Regulation of mTOR Complexes in Long-Lived Growth Hormone Receptor Knockout and Snell Dwarf Mice

by

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Table of Contents

List of Tables

List of Figures

List of Abbreviations

αKG: α-ketoglutarate

4EBP1: eukaryotic translation initiation factor 4E-binding Protein 1

AMPK: AMP-activated protein kinase

Arf1: ADP ribosylation factor 1

ATF4: activating transcription factor 4

ATG101: autophagy related 101

ATG13: Autophagy Related 13

CAD: carbamoyl-phosphate synthetase 2, aspartate transcarbamylase, and dihydroorotase

CDK1: cyclin-dependent kinase 1

CIT: cap-independent translation

CMA: chaperone-mediated autophagy

DEPTOR: DEP-domain-containing mTOR-interacting protein

EGF: epidermal growth factor

eIF3: eukaryotic initiation factor 3

ERK: extracellular signal-regulated kinase

FIP200: 200 KDa FAK family kinase-interacting protein

FIRKO: fat-specific insulin receptor knockout

FKBP12: FK506-binding protein 12

FKBP38: FK506-binding protein 38

FOXO: forkhead box protein O

GAP: GTPase-activating protein

GATOR2: GAP activity towards the Rags 2

GEF: guanine nucleotide exchange factor

GH: growth hormone

GHR: growth hormone receptor

GHRH-KO: growth hormone releasing hormone knockout

GHRH: growth hormone releasing hormone

GHRKO: growth hormone receptor knockout

GSK3: glycogen synthase kinase 3

HIF-1α: hypoxia-inducible factor 1-alpha

HSP: heat shock protein

IGF-1: insulin-like growth factor 1

IGF-1R: insulin-like growth factor 1 receptor

IGFBP: IGF-binding protein

IP: immunoprecipitation

IR: insulin receptor

IRS: insulin receptor substrate

LKO: liver-specific growth hormone receptor knockout

m6A: N6-methyladenosine

MKO: muscle-specific growth hormone receptor knockout

mLST8: mammalian lethal with SEC13 protein 8

mSIN1: mammalian stress-activated protein kinase-interacting protein

MTHFD2: methylenetetrahydrofolate dehydrogenase 2

mTOR: mechanistic target of rapamycin

mTORC1: mTOR complex 1

mTORC2: mTOR complex 2

PAPPA: pregnancy-associated plasma protein A

Pdcd4: programmed cell death 4

PDK1: pyruvate dehydrogenase kinase 1

PI3K: phosphoinositide 3-kinase

PIKK: phosphatidylinositol 3-kinase-related kinase

PIP3: phosphatidylinositol (3,4,5)-trisphosphate (PtdIns(3,4,5)P3)

Pit1: pituitary transcription factor 1

PKC: protein kinase C

PRAS40: proline-rich AKT substrate of 40 kDa

Prop1: PROP paired-like homeobox 1

PROTOR1: protein observed with rictor-1

Rags: RAS- related GTP-binding proteins

RAPTOR: regulatory associated protein of mTOR complex 1

REDD1: regulated in development and DNA damage responses 1

RICTOR: rapamycin-insensitive companion of mTOR

RSK: ribosomal S6 kinase

RUBICON: Rubicon autophagy regulator

S6: ribosomal protein S6

S6K: P70-S6 kinase

SAM: S-adenosyl methionine

Ser: serine

SGK1: serum- and glucocorticoid-induced protein kinase 1

SLC38A9: solute carrier family 38 member 9

SREBP: sterol regulatory element binding transcription factor

TBC1D7: Tre2-Bub2-Cdc16 1 domain family, member 7

Tel2: telomere maintenance 2

TFEB: transcription factor EB

Thr: threonine

TSC: tuberous sclerosis complex

TSC2: tuberous sclerosis complex 2

TSH: thyroid stimulating hormone

Tti1: Tel2 interacting protein 1

UCP1: uncoupling protein 1

ULK1: unc-51 like autophagy activating kinase 1

UTR: untranslated region

UVRAG: UV irradiation resistance-associated gene

v-ATPase: vacuolar H+-adenosine triphosphatase

Abstract

mTOR is a serine/threonine kinase that binds with multiple proteins to form two complexes, mTORC1 and mTORC2. mTORC1 receives upstream signals including amino acids, growth factors, energy and oxygen levels to regulate cell growth and metabolism. mTORC2 is regulated by insulin/IGF-1 signaling to modulate cell survival and proliferation.

mTOR is well known to play a critical role in aging. A reduction in mTORC1 activity extends lifespan in multiple species, including yeast, worms, flies and mice. Growth hormone receptor knockout (GHRKO) mice and Snell dwarf mice are two long-lived mouse models with lower body weight and significantly extended lifespan. Tissues of these mice have lower mTORC1 activity, but the mechanisms are not clear. Here, two ideas were tested about the regulation of mTOR in GHRKO and Snell mice, one involving alteration in the components of the mTOR complexes, and the other focused on the upstream regulation of mTORC1 function.

GHRKO and Snell dwarf liver, kidney and skeletal muscle were used to study the protein expression of mTORC components. Only two proteins, DEPTOR and PRAS40 which inhibit mTOR activity, were reduced in the liver, opposite in the direction expected to cause lower mTORC1 function. In addition, DEPTOR or PRAS40 knockdown in GHRKO fibroblasts did not alter mTORC substrate phosphorylation, indicating that the deficit in mTORC1 activity in GHRKO or Snell dwarf mice is not likely to reflect differences in levels of DEPTOR or PRAS40.

TSC is a master regulator of mTORC1 activity that integrates upstream signals from growth factors, energy and oxygen levels. TSC components TSC1 and TSC2 were tested in GHRKO and Snell dwarf liver, kidney and muscle. The results showed increased TSC1 and TSC2 protein expression in all tissues tested. Relative phosphorylation of TSC2 at Ser 939 and Thr 1462, which inhibits activity of TSC2, were decreased in these tissues as well, suggesting increased TSC inhibition of mTORC1. TSC2 knockdown in GHRKO fibroblasts augmented the phosphorylation of mTORC1 substrates, consistent with the model in which mTORC1 activity was regulated by TSC.

In summary, the results showed that the lower mTORC1 activity in GHRKO and Snell dwarf mice is not regulated by the levels of mTORC components. Instead, the increased amounts of unphosphorylated, active, inhibitory TSC might contribute to lower mTORC1 function in these long-lived mice. This study highlights the potential role of TSC in regulating mTORC1 activity in long-lived mutant mice. Future studies on enhancing TSC function by overexpressing TSC2 or using TSC2 activating molecules may generate additional mouse models that can be used as comparisons with GHRKO and Snell dwarf mice, which would provide more evidence about the role of mTOR in regulating the pace of aging.

Chapter 1 Introduction

1.1 Aging

Aging is a gradual process featuring accumulated physiological damage and functional decline, eventually leading to death. Understanding the aging mechanisms and slowing the aging process are among the most interesting topics in biological research. There are two main categories of aging theories, programmed and error theories. Programmed aging is controlled by genetics that determines the progress of aging. Error theories involves the accumulated cellular and molecular damage in the organism [1]. There have been a variety of approaches developed to extend lifespan, such as caloric restriction, inhibiting the growth hormone or the insulin/IGF-1 signaling pathway, diminishing mTOR activity, combating cellular senescence, targeting mitochondria and oxidative stress [2].

The first genetic model of extended longevity was established in *C. elegans* in 1988 [3]. A single gene mutation in *age-1*, which encodes a subunit of the phosphoinositide 3-kinase (PI3K) complex, could significantly increase the worm lifespan without affecting their food intake, movement or behavior. Later in 1993, Kenyon *et al.* found that worms with *daf-2* gene mutations had doubled lifespan. However, this effect was eliminated when *daf-16* was also mutated, suggesting that *daf-2* extends lifespan through the downstream gene *daf-16* [4]. *daf-2* is the *C. elegans* homolog of the insulin-like growth factor 1 receptor (IGF-1R) and *daf-16* corresponds to FOXO in mammals. Both are critical players from the insulin/IGF-1 signaling pathway.

1.1.1 The insulin/IGF-1 signaling pathway

In mammals, insulin is secreted from β-cells in the pancreas in response to glucose [5]. Insulin circulates to tissues across the body and binds with insulin receptor (IR) present in most cells [6,7]. Insulin-like growth factor 1 (IGF-1) is primarily secreted by hepatocytes in the liver under the stimulation of growth hormone [8]. Circulating IGF-1 is transported by IGF-binding proteins (IGFBPs) and binds with IGF-1 receptor expressed in many tissues and cell types [9]. Due to the highly homologous structure of IR and IGF-1R, they can form heterodimers that bind at high affinity with IGF-1 but not insulin [10]. The binding of insulin or IGF-1 with their receptors triggers the autophosphorylation of the receptors at tyrosine residues to be activated, which then bind and phosphorylate IRS (insulin receptor substrate). Phosphorylated IRS binds PI3K, and the phospholipid product phosphatidylinositol (3,4,5)-trisphosphate (PIP3) generated by PI3K can then recruit AKT and PDK1 to the plasma membrane causing AKT to be phosphorylated and activated by PDK1 [11].

Later studies of this pathway in other species found similar lifespan extension effects. For example, *insulin-like receptor* mutant flies lived 85 % longer in females and had lower mortality in old males [12]. In mice, insulin receptor knockout led to neonatal death [13,14]. However, fat-specific insulin receptor knockout (FIRKO) mice were viable and showed 18% increased lifespan [15]. Mice with heterozygous IGF-1 receptor knockout ($IGF1R^{+/-}$) had 30 % lifespan extension in females, whereas the effect in males was not significant [16]. IRS has two isoforms, IRS-1 and IRS-2, and both function as adaptors between IR/IGF-1R and PI3K. IRS-1 knockout in mice resulted in 32% lifespan extension in females and 16% in males [17,18]. These mice in old

age also showed better rotarod performance and delayed age-associated immune system biomarkers. Interestingly, IRS-2 knockout ($IRS2^{-/-}$) mice were short-lived but IRS-2 heterozygous mice ($IRS2^{+/-}$) had lifespan extended for 17% [17,19]. Findings from these studies support the idea that disruption of the insulin/IGF-1 signaling pathway would benefit longevity.

1.1.2 The growth hormone signaling pathway

Growth hormone (GH) is secreted from somatotroph cells in the anterior pituitary gland under the stimulation of growth hormone releasing hormone (GHRH) from the hypothalamus. Growth hormone release is inhibited by another hypothalamic hormone, somatostatin. Growth hormone circulates to multiple tissues such as liver, kidney, muscle and heart, to bind with growth hormone receptor (GHR) [20]. GHR binds and activates Janus kinase 2 (JAK2) which then phosphorylates a group of signal transducer and activator of transcription (STAT) proteins including STAT1, STAT3 and STAT5. These STAT proteins enter the nucleus and bind to DNA to activate the transcription of IGF-1 and other target genes involved in cell proliferation and survival [21].

Several mouse models with defects in growth hormone signaling are long-lived, such as Ames dwarf mice, Snell dwarf mice, Little (GHRHR-deficient) mice, growth hormone releasing hormone knockout mice (GHRH-KO) and growth hormone receptor knockout (GHRKO) mice. Ames dwarf mice carry a missense mutation in the gene PROP paired-like homeobox 1 (Prop1), which is a transcription factor in pituitary gland. This mutation causes abnormal pituitary development lacking three cell types, somatotrophs, lactotrophs, and thyrotrophs, and leads to reduced production of growth hormone, prolactin and thyroid stimulating hormone (TSH) [22].

Ames dwarf mice have significantly reduced body size but their lifespan is dramatically increased, with 68% longer lifespan in females and 49% in males [23]. Little mice are also small in size [24], carrying a missense mutation in GHRH receptor which cannot bind GHRH, leading to significantly reduced growth hormone production [25,26]. A longevity study showed 23% lifespan extension in males and 25% in females [27]. GHRH knockout mice resemble Little mice. GHRH-KO mice have about two thirds the body weight of controls and 51% and 43% extended lifespan in males and females, respectively [28]. Snell dwarf mice and GHRKO mice are also long-lived, and details are introduced below. In brief, these mice with disrupted growth hormone signals provide valuable models for studying slow-aging mechanisms.

1.1.3 Mouse models with defective GH/IGF-1 signaling

1.1.3.1 Snell dwarf mice

Snell dwarf mice are a strain of house mouse first described by George D. Snell in 1929 [29]. He found that homozygous Snell mice were about one-fourth the size of their wildtype siblings but were still healthy. These dwarf mice were sterile, and crossing the heterozygotes generated a mendelian ratio, indicating a recessive phenotype. Early studies found these mice had reduced production of thyroid stimulating hormone, growth hormone and prolactin [30–32]. These phenotypes resulted from a loss-of-function mutation in pituitary transcription factor 1 (Pit1), which, similar to the Prop1 mutation, leads to defective pituitary glands lacking somatotrophs, lactotrophs, and thyrotrophs [33,34]. Pit1 binds to the promotor regions of these hormone genes and activates their transcription [35]. The significant decline of growth hormone secretion also results in reduced downstream IGF-1 production by the liver.

In 2001, these mice were shown to live over 40% longer than littermate controls with approximately equal increases in both sexes. They also showed delayed age-associated effects on immune system markers and collagen crossing-linking [27]. Fibroblasts isolated from the tail tips of these mice were resistant to multiple stresses, including H_2O_2, UV light, heat, paraquat, and cadmium [36]. After UV-induced damage, Snell fibroblasts showed higher levels of DNA-repair enzymes and faster recovery of mRNA synthesis than control cells [37]. mRNA levels of multiple heat shock proteins (HSPs) were tested in tissues of the Snell dwarf mice and they presented complicated effects in different tissues, with some decreased in certain tissues and some increased in other tissues [38]. A more comprehensive analysis of the protein levels of these HSPs would help to reveal the mechanism of heat shock stress resistance in these fibroblasts.

1.1.3.2 GHRKO mice

A mouse model with direct disruption of growth hormone signaling was established in 1997 [39]. These mice had a whole-body knockout of growth hormone receptor by deleting exon 4 region of the gene. They had only 10% IGF-1 but several folds of growth hormone in the serum, and they showed significantly retarded growth, ending up with a body size less than half of the controls. Unlike Snell dwarf mice, these GHRKO mice were fertile. However, they had compromised reproductive function with reduced litter size and lower body weight of the offspring [40]. Moreover, they exhibited insulin sensitivity with lower insulin and glucose concentrations in the blood [41]. The lifespan study of these mice were done in 2000, and dramatically, GHRKO male mice lived 55% longer and GHRKO females lived 38% longer than wildtype controls [42].

1.1.3.3 Liver-specific GHRKO mice

To understand further the hormone signaling that leads to the extended lifespan, tissue-specific growth hormone receptor knockout mice were developed. When GHR was only deleted in the liver (LKO), the mice had three times higher levels of growth hormone and 10% of IGF-1 in the serum [43]. IGF-1 mRNA levels were very low in liver tissue but were higher in other tissues such as skeletal muscle and brown adipose tissues due to the increased circulating growth hormone levels. Adult mice showed reduced body weight and fat mass. More importantly, these mice had no extended lifespan compared with controls, suggesting that GHR deficiency only in the liver is not enough to benefit longevity despite depression of circulating IGF-1 levels [44].

1.1.3.4 Muscle-specific GHRKO mice

Muscle-specific GHRKO (MKO) mice were also developed to study whether deficient growth hormone signaling in muscle reproduces the longevity phenotypes of whole body GHR knockout mice [45]. Serum GH and IGF-1 levels were not different between MKO and wildtype mice. Male MKO mice showed enhanced glucose tolerance with lower glucose and insulin levels in the blood. However, these effects were not observed in the females. Interestingly, body weight, fat and lean mass were lower in males but higher in females. Furthermore, the lifespan study showed that male MKO mice had slightly extended lifespan (9% median lifespan extension, p-value 0.024; 6% maximum lifespan extension, p-value 0.03) at one testing site but not the other, and female mice had no lifespan extension at either site. These findings suggest that growth hormone signaling interruption in muscle have some health beneficial effects in a sex-specific way, but more studies still need to be done to evaluate effects on other age-sensitive traits.

Multiple other tissue-specific GHRKO mouse models have also been generated such as GHR deletion in adipose tissue, bone, brain, or cardiac myocytes [46]. These mouse models exhibit diverse phenotypes and provide insights into the GH signaling regulation in individual tissues. However, the fundamental question about the lifespan extension mechanism of whole-body GHRKO mice still awaits to be answered with more integrated and thorough studies.

1.1.3.5 PAPPA-KO mice

When IGF-1 circulates throughout the body, it is usually bound by IGF binding proteins. Binding with one of the six types of IGFBPs prolongs the half-life of IGF-1 and prevents its interaction with the insulin receptor which has a similar structure with IGF-1 receptor [47]. There are proteases that cleave IGF-1 from IGFBPs, releasing IGF-1 to bind with IGF-1R at the cell membrane to activate downstream intracellular signaling. One of these proteases, pregnancy-associated plasma protein A (PAPPA), cleaves IGFBP4, releasing IGF-1, suggesting that deletion of PAPPA would cause inhibited IGF-1 signaling [48]. PAPPA knockout mice were viable and had 40% lower body weight [49]. There was no alteration of glucose, insulin, GH or IGF-1 levels in the serum, but the mice had lower rates of tumors. A lifespan study showed a 33% increase of male lifespan and a 41% increase of female lifespan [50]. PAPPA-KO mice fed with a high fat diet started at 12 months of age still had about 27% increase of their lifespan compared with littermates despite similar weight gains [51]. These PAPPA mutants had lower inflammation and degeneration in multiple tissues including kidney, heart and testes, suggesting extended health span as well.

1.1.4 Common mechanisms of these mouse models

Multiple signaling pathways have been studied to search for common longevity mechanisms of these mouse models. In response to amino acid withdrawal, skin-derived fibroblasts from Snell dwarf and GHRKO mice showed increased levels of autophagy markers, indicating subtle regulation of autophagy in these two long-lived mouse models [52]. Lysosomes isolated from the liver tissue of these mice had higher uptake of the substrates of a specific type of autophagy, chaperone-mediated autophagy (CMA), suggesting higher CMA activity in these mice. Meanwhile, lysosomes from LKO mice did not show similar CMA substrate uptake, indicating that GH deficiency only in the liver is not enough to regulate hepatic CMA activity [53].

Examination of adipose tissue from Snell and GHRKO mice found increased expression of uncoupling protein 1 (UCP1), a mitochondrial protein that decouples the proton gradient in oxidative phosphorylation to generate heat. Interestingly, similar effects were not observed in liver-specific or fat-specific GHRKO mice, but were present in muscle-specific GHRKO mice, revealing regulation of adipose tissue by signals from muscle [54].

In addition, Snell dwarf, GHRKO and PAPPA-KO mice had elevated levels of methyltransferases METTL3 and METTL14, enzymes that add N^6-methyladenosine (m6A) marks to mRNA. Reader proteins that bind with these m6A modifications, YTHDF1 and YTHDF2, were also increased in multiple tissues [55]. m6A can facilitate cap-independent translation (CIT), an alternative mechanism to cap-dependent translation, to enable translation of selected mRNAs under stress conditions [56,57]. Further analysis found increased expression of m6A-mediated CIT targets in fibroblasts isolated from Snell dwarf and GHRKO mice [55]. These results suggest that elevated m6A-mediated CIT is a novel pathway to regulate longevity.

Mechanistic target of rapamycin (mTOR) activity has also been studied in the Snell dwarf and GHRKO mice. There was lower mTOR complex 1 (mTORC1) activity in multiple tissues including liver, kidney, muscle and heart when the mice were refed after fasting. Interestingly, three of the four mTOR complex 2 (mTORC2) substrates tested showed higher phosphorylation in the long-lived mice compared with fasted controls, but one substrate had lower phosphorylation after refeeding [58]. These results show lower mTORC1 but partially higher mTORC2 activities in tissues of Snell dwarf and GHRKO mice, and these activities are also dependent on fasting and feeding conditions. Interestingly, higher mTORC2 activity was also observed in male mice fed with two drugs, acarbose and 17-α-estradial, which mainly extends male mice lifespan, indicating that higher mTORC2 activity may be beneficial for longevity [59].

1.2 mTORC1 and mTORC2

mTOR is a serine, threonine kinase that belongs to the phosphatidylinositol 3-kinase-related kinase (PIKK) family with members involved in DNA repair, cell growth, RNA decay and oncogene transcriptional activation [60]. mTOR can bind with other proteins to form two complexes, mTORC1 and mTORC2, which are regulated in different ways and have different cellular functions.

1.2.1 mTORC1 and mTORC2 composition

The composition of mTORC1 and mTORC2 are shown in Figure 1A. Both complexes share three common components, including mTOR, mammalian lethal with SEC13 protein 8 (mLST8)

and DEP domain-containing mTOR-interacting protein (DEPTOR). As an essential subunit of the complexes, mLST8 binds to the kinase domain of mTOR and stimulates its activity, and it also stabilizes the interaction between mTOR and RAPTOR [61]. DEPTOR interacts with mTOR and inhibits the kinase activity of both mTORC1 and mTORC2. Intriguingly, the expression of DEPTOR itself can be suppressed at the transcriptional and translational levels by mTORC1 and mTORC2 [62].

In addition to these common components, mTORC1 also has two specific components, regulatory associated protein of mTOR complex 1 (RAPTOR) and proline-rich AKT substrate of 40 kDa (PRAS40). RAPTOR serves as a scaffold for the complex by binding with mTORC1 substrates to facilitate their phosphorylation by mTOR [63,64]. PRAS40 interacts with RAPTOR and inhibits mTORC1 activity by preventing substrate binding with the complex [65,66].

mTORC2 contains three specific subunits not present in mTORC1: rapamycin-insensitive companion of mTOR (RICTOR), mammalian stress-activated protein kinase-interacting protein (mSIN1) and protein observed with RICTOR-1 (PROTOR1). Similar to RAPTOR, RICTOR functions as a scaffold by binding directly with the other components of the complex [62,67–69]. mSIN1 is a critical component mediating the reciprocal regulation between the complex and its substrate AKT. There are multiple isoforms of mSIN1, three of which can be incorporated into mTORC2 [68,70,71]. More work is needed to explore the differential functions of these isoforms and the complexes containing them. PROTOR1 interacts directly with RICTOR, and PROTOR1 knockdown does not affect the complex assembly, but inhibits AKT phosphorylation at Ser 473 and decreases cell proliferation [69,72].

There are some other proteins that can interact with mTOR complexes and affect their functions. For example, telomere maintenance 2 (Tel2) and its binding partner Tel2 interacting protein 1 (Tti1) can interact and stabilize the PIKK family of kinases including mTOR. Tel2 or Tti1 knockdown inhibits the phosphorylation of mTORC1 and mTORC2 substrates, suggesting a positive role in regulating mTOR function [73].

Despite the many studies of these mTORC components, their detailed roles in specific conditions are still not well investigated. Higher mTORC1 and mTORC2 activities are frequently observed in cancer, but the roles of these mTOR components are not well examined. For instance, some cases of multiple myeloma have overexpressed DEPTOR, which inhibits mTORC1 and leads to activated PI3K/AKT signaling [62]. Thus, targeting specific mTORC components in individual diseases would be an alternative strategy to regulate mTOR activity. In addition, mTOR is widely studied in aging but how these particular components are involved is mostly unknown. More research about the mTORC components would shed light on mTOR regulation and function in aging.

1.2.2 mTORC1 functions

1.2.2.1 mTORC1 positively regulates protein synthesis

mTORC1 phosphorylates multiple substrates to regulate various aspects of cellular growth and metabolism. mTORC1 controls RNA translation and this is mainly executed through phosphorylating substrates eukaryotic translation initiation factor 4E-binding protein 1 (4EBP1) and p70-S6 kinase (S6K) [74,75]. mRNA translation is initiated by the binding of the m7G cap at

the 5' end of mRNA with the translation initiation complex, termed cap-dependent translation. eIF4E first binds with m7G, which then recruits eIF4F and other components to form a complex [76]. This process can be regulated by 4EBP1 which binds with eIF4E to interrupt the interaction between eIF4E and eIF4F, thus inhibiting cap-dependent translation. The activity of 4EBP1 can be suppressed by phosphorylation at multiple sites by mTORC1. 4EBP1 is first phosphorylated by mTORC1 at Threonine (Thr) 37 and Thr 46, which are required for the subsequent phosphorylation at Thr 70 and Serine (Ser) 65 by mTORC1 to be fully suppressed [77,78]. Thus, Thr 37/46 phosphorylation by mTORC1 is a priming event for 4EBP1 regulation, which is often used as a marker for mTORC1 activity.

S6K is a serine/threonine kinase that also plays an important role in protein synthesis. S6K can be phosphorylated by mTORC1 at Thr 389 within the hydrophobic motif. Together with the T-loop site Thr 229 phosphorylated by pyruvate dehydrogenase kinase 1 (PDK1), S6K is then activated to phosphorylate its downstream targets [79]. Ribosomal protein S6 (S6) is one of the S6K targets, and is an indispensable component of the ribosomal 40S subunit. S6 can be phosphorylated by S6K at multiple sites, including Ser 235, 236, 240, 244, and 247. S6 is involved in the regulation of cell size, cell proliferation, apoptosis and glucose homeostasis, but its exact role in protein synthesis is still ambiguous [80]. Inactive S6K binds with eukaryotic initiation factor 3 (eIF3) and this interaction is disrupted upon the activation of S6K by mTORC1. S6K then phosphorylates eIF4B at Ser 422 allowing it to bind with the translation initiation complex [81,82]. Another substrate of S6K is programmed cell death 4 (Pdcd4), which binds to and inhibits the helicase activity of eIF4A to unwind the secondary structure of mRNA at their 5'-untranslated region (UTR) [83].

1.2.2.2 mTORC1 mediates nucleotide and lipid synthesis

Apart from protein synthesis, mTORC1 also regulates the synthesis of certain nucleotides and lipids. mTORC1 stimulates the activating transcription factor (ATF4) through some unknown mechanism that is independent of the canonical integrated stress response. ATF4 then further induces the expression of methylenetetrahydrofolate dehydrogenase 2 (MTHFD2) to stimulate de novo purine synthesis [84]. The mTORC1 target S6K phosphorylates carbamoyl-phosphate synthetase 2, aspartate transcarbamylase, and dihydroorotase (CAD), an enzyme functioning in the early steps of pyrimidine synthesis. This phosphorylation stimulates CAD enzymatic activity and therefore promotes de novo pyrimidine synthesis [85,86]. mTORC1 can also phosphorylate Lipin1 and limits its localization to the nucleus, which can no longer inhibits sterol regulatory element binding transcription factor (SREBP) transcription [87]. SREBP is a transcription factor that controls the expression of sterol biogenesis genes [88]. Thus activated mTORC1 enhances SREBP functions in lipid synthesis. These events reveal a multifunctional role of mTORC1 in promoting cell growth by facilitating nucleotide and lipid synthesis.

1.2.2.3 mTORC1 negatively regulates autophagy

Autophagy is a cellular process to degrade and recycle cellular components in the lysosome under limited energy and nutrient status. It may also play an active role in molding the proteome through post-translational effects. The unc-51 like autophagy activating kinase 1 (ULK1) complex is an early mediator that receives upstream energy and nutrient signals to induce the formation of autophagosome which engulfs cytoplasmic components and delivers them to the lysosome. The ULK1 complex is composed of ULK1, autophagy related 13 (ATG13), 200 KDa

FAK family kinase-interacting protein (FIP200) and autophagy related 101 (ATG101) [89]. Two of these components, ULK1 and ATG13, can be phosphorylated by mTORC1, which results in suppressed function of ULK1 complex [90–92]. mTORC1 also phosphorylates UV irradiation resistance-associated gene (UVRAG), which facilitates autophagosome formation and maturation [93]. When UVRAG is phosphorylated by mTORC1, it is recruited away from autophagosome maturation machinery by its binding partner Rubicon autophagy regulator (RUBICON) [94]. Another critical autophagy gene phosphorylated by mTORC1 is transcription factor EB (TFEB), a master transcription factor in lysosomal biogenesis. Phosphorylation of TFEB by mTORC1 inhibits its activity and prevents its translocalization from the cytosol to the nucleus, suppressing the transcription of lysosomal hydrolases, lysosomal membrane proteins and lysosomal acidification complex components [95,96]. These observations show a negative role of mTORC1 in autophagy, making sure the major activity happening in the cell is anabolism instead of catabolism.

In summary (Figure 1B), mTORC1 promotes protein, nucleotide and lipid synthesis to provide building blocks for cell growth and maintain balanced cell metabolism. mTORC1 inhibits autophagy to prevent premature degradation of newly synthesized cellular components and directs energy and resources for anabolism over catabolism. mTORC1 downstream targets are also involved in multiple other biological pathways that are important for cell homeostasis, and investigating their specific roles in certain physiological and pathological conditions would expand our understanding of the broad biological functions of mTOR.

1.2.3 mTORC2 functions

1.2.3.1 mTORC2 regulates cell survival and proliferation

One of the well-characterized mTORC2 substrates is AKT, a serine/threonine kinase regulating cell survival and proliferation. AKT is activated downstream of the insulin/IGF-1 PI3K pathway. When insulin/IGF-1 receptors get activated after binding insulin/IGF-1, they phosphorylate IRS which, as an adapter, interacts with PI3K. PI3K phosphorylates PIP2 to PIP3, which recruits AKT and PDK1 to the plasma membrane. PDK1 phosphorylates AKT at Thr 308 within the AKT kinase domain, resulting in partial activation of AKT. AKT then phosphorylates mTORC2 subunit mSIN1 at Thr 86 to activate mTORC2. mTORC2 in turn phosphorylates AKT at Ser 473 in the C-terminal regulatory domain, leading to full AKT activation [70,97]. mTORC2 also phosphorylates AKT at other sites within the regulatory domain, including Thr 450, Ser 477 and Ser 479. Thr 450 helps with C-terminal folding and maintains the stability of AKT [98]. Ser 477 and Ser 479 promote AKT activation possibly by enhancing Ser 473 phosphorylation [99].

As a kinase, AKT phosphorylates a variety of targets, such as glycogen synthase kinase 3 (GSK3), forkhead box protein O (FOXO) and tuberous sclerosis complex 2 (TSC2), to regulate cell growth, metabolism, survival and proliferation. GSK3 is a serine/threonine kinase that phosphorylates and inhibits the expression of genes involved in glycogen metabolism, cell survival and proliferation. AKT phosphorylates and inhibits the substrate recruitment of GSK3, thus enhancing the expression of cell metabolism, survival and proliferation genes [97]. FOXO is a family of transcription factors that induce the expression of genes involved in apoptosis, cell-cycle arrest and growth inhibition. Phosphorylation by AKT leads to binding of FOXO with its partner 14-3-3 proteins, which keeps FOXO from entering the nucleus to activate the target gene expression. Thus, by inhibiting the FOXO target gene expression, AKT promotes cell survival

and cell cycle progression [97]. TSC2, an upstream negative regulator of mTORC1, can be phosphorylated by AKT at Ser 939 and Thr 1462 to have its activity inhibited, leading to elevated mTORC1 activity [100,101]. Together, AKT regulates a series of downstream effectors to promote cell metabolism, survival and proliferation.

1.2.3.2 mTORC2 regulates ion transport and cytoskeleton morphology

Serum- and glucocorticoid-induced protein kinase 1 (SGK1) is also a serine/threonine kinase phosphorylated by mTORC2. SGK1 controls multiple ion channels and pumps to regulate sodium, potassium and calcium transport. mTORC2 phosphorylates SGK1 at Ser 422 within the hydrophobic motif to activate its function in ion transport, affecting cell volume, hormone release and neuroexcitability [102,103].

Another mTORC2 substrate is protein kinase C (PKC), a group of kinases which, similarly with AKT and SGK1, also belongs to the AGC kinase family that share a conserved catalytic kinase domain [104]. PKC transduces a variety of signals to regulate cell cycle, tumorigenesis and cell migration [105,106]. mTORC2 phosphorylates PKC to alter the morphology of the actin cytoskeleton [67].

In summary (Figure 1C), mTORC2 phosphorylates several AGC family of kinases, including AKT, SGK1 and PKC, to regulate cell survival, proliferation, ion transport, cytoskeleton and cell migration. Each of these substrates has plenty of downstream targets that are involved in numerous biological processes. Except for the few well-studied target genes, how mTORC2

affects the other targets is mostly unclear. Therefore, more specific mTORC2 functions still need to be characterized to give a more comprehensive understanding of the mTORC2 network.

1.2.4 mTORC1 regulation

mTORC1 is regulated mainly through two pathways (Figure 2). On one hand, amino acid levels induce the localization of mTORC1 at the lysosome. On the other hand, upstream signals like growth factors, energy or oxygen sensor control the activation of mTORC1 at the lysosomal surface. Signals from both pathways are required for mTORC1 to be fully activated [107].

1.2.4.1 mTORC1 lysosomal localization regulated by amino acids

Leucine, arginine, methionine and glutamine are the primary amino acids stimulating mTORC1 activity. Cytoplasmic leucine level is detected through binding with its sensor protein Sestrin2. This conformational change dissociates Sestrin2 from interacting and inhibiting the target protein complex GAP activity towards the Rags 2 (GATOR2) [108]. GATOR2 further inhibits the activity of another protein complex GATOR1, which localizes at the lysosome surface tethered by the complex KICSTOR [109–111]. GATOR1 is a GTPase-activating protein complex that inhibits mTORC1 activity through a group of four RAS-related GTP-binding proteins (Rags) [109]. Two Rags from each subgroup form heterodimers so that either RagA or RagB interacts with RagC or RagD. When RagA/B binds with GTP and RagC/D binds with GDP, this dictates the active state of the complex, which then recruits mTORC1 to the lysosome through binding with RAPTOR [112]. GATOR1 exerts GTPase-activating protein (GAP) function and turns GTP-binding RagA/B to be GDP-binding, thus switching off the activity of the Rags and inhibiting mTORC1 lysosomal localization [109]. The Rag complex is tethered to the lysosome by the complex

Regulator which also functions as a guanine nucleotide exchange factor (GEF) to switch the GDP-binding RagA/B to the GTP-binding active status, allowing mTORC1 lysosomal translocalization [113].

Cytoplasmic arginine is sensed by the protein CASTOR1, and arginine bound CASTOR1 can no longer bind and inhibit GATOR2, leading to mTORC1 activation through GATOR1 and the Rag GTPases [114]. Arginine inside the lysosome is sensed by another protein solute carrier family 38 member 9 (SLC38A9) that stays at the lysosome membrane and functions through the Regulator and Rag proteins to activate mTORC1 [115,116]. Methionine is sensed in an indirect way through the methionine metabolite S-adenosyl methionine (SAM). SAMTOR is the sensor of SAM and acts as an inhibitor of GATOR1-Rag signaling. The binding of SAM with SAMTOR disrupts the interaction between SAMTOR and GATOR1, leading to mTORC1 activation [117]. Glutamine is metabolized through a process termed glutaminolysis to α- ketoglutarate (αKG), which enhances the GTP loading of RagB, contributing to mTORC1 activation [118]. In addition, glutamine acts through ADP ribosylation factor 1 (Arf1) to activate mTORC1 independent of the Rag proteins. This process requires the vacuolar H^+-adenosine triphosphatase (v-ATPase) at the lysosomal membrane [119]. v-ATPase is a proton pump that acidify the lysosome using energy from ATP hydrolysis. Amino acids in the lysosome lumen stimulate v-ATPase to interact with Regulator, resulting in mTORC1 activation [120].

In summary, mTORC1 localization to the lysosome membrane is regulated by multiple amino acids and their sensing pathways that converge on the Rag complex. Despite these detailed mechanisms, whether these signals are regulated in the aging context remains unknown. It would

be interesting to investigate the amino acid sensing pathways and the regulation of mTORC1 localization in slow-aging mice.

1.2.4.2 mTORC1 activation regulated by upstream signals through TSC-Rheb

mTORC1 activation can be regulated by multiple upstream signals like growth factors, energy and oxygen levels, and these signaling pathways converge on the master regulator tuberous sclerosis complex (TSC). TSC is composed of three components: TSC1 (also called hamartin), TSC2 (also called tuberin) and TBC1D7 (Tre2-Bub2-Cdc16 1 domain family, member 7). TSC1 binds and stabilizes TSC2 by blocking the ubiquitination and degradation of TSC2 [121]. TBC1D7 promotes the interaction between TSC1 and TSC2 [122]. TSC2 is the main functional subunit of the complex and acts as a GTPase-activating protein towards the downstream GTPase Rheb [123]. GTP-binding Rheb interacts with FK506-binding protein 38 (FKBP38) to prevent FKBP38 from binding and inhibiting the kinase activity of mTOR, leading to mTORC1 activation [124]. TSC2 activates GTP hydrolysis and switches the GTP-binding active state of Rheb to the GDP-binding inactive state, thus inhibiting mTORC1 activation [123].

There are several growth factors signaling through TSC to regulate mTORC1 activity, including insulin, IGF-1 and epidermal growth factor (EGF). Insulin and IGF-1 activate the PI3K-AKT signaling pathway, after which AKT phosphorylates TSC2 at Ser 939 and Thr 1462, which inhibits TSC2 to stimulate mTORC1 activity [100,101]. The binding of EGF with EGF receptor triggers the activation of multiple downstream targets including extracellular signal-regulated kinase (ERK) and ribosomal S6 kinase (RSK) [125]. ERK phosphorylates TSC2 at Ser 664 to dissociate TSC1 and TSC2, resulting in elevated mTORC1 function [126]. RSK, downstream of

ERK, also phosphorylates TSC2, and Ser 1798 phosphorylation by RSK inhibits TSC activity to enhance mTORC1 functions [127].

In addition to growth factors, energy level sensed by AMP-activated protein kinase (AMPK) regulates TSC activity as well. Low ATP levels activate AMPK to promote catabolic and inhibit anabolic cellular processes to maintain energy homeostasis [128]. AMPK phosphorylates TSC2 at Thr 1227 and Ser 1345 to augment TSC2 activity, inhibiting mTORC1 mediated cell growth and metabolism [129]. Moreover, AMPK can inhibit mTORC1 independent of TSC2 through phosphorylating RAPTOR at Ser 722 and Ser 792, leading to the sequestration of RAPTOR by its binding partner protein 14-3-3 [130]. In response to low oxygen levels, the hypoxia master regulator hypoxia-inducible factor 1-alpha (HIF-1α) induces the expression of regulated in development and DNA damage responses 1 (REDD1) [131], which disrupts the interaction between TSC2 and the binding protein 14-3-3, and releases TSC2 to inhibit mTORC1 signaling [132,133].

In brief, TSC functions as a central hub to relay upstream extracellular and intracellular signals to regulate mTORC1 activity. Together with the translocalization to the lysosome, mTORC1 receives diverse signals through TSC, and further controls multiple targets to modulate cell growth and metabolism.

1.2.5 mTORC2 regulation

mTORC2 is mainly regulated by the PI3K-AKT signaling pathway. Insulin or IGF-1 signals activate the insulin or IGF-1 receptors, which phosphorylate IRS to form a platform for PI3K binding. Then PI3K phosphorylates a group of phosphatidylinositols to produce PIP3, which can

recruit both AKT and PDK1 to the plasma membrane. PDK1 phosphorylates and partially activates AKT function [11]. AKT then phosphorylates mSIN1 to activate mTORC2 [70,97].

1.2.6 Crosstalk between mTORC1 and mTORC2

mTORC1 and mTORC2 can regulate each other through phosphorylating their components. mTORC1 substrate S6K can phosphorylate mTORC2 component RICTOR at Thr 1135 [134]. This modification does not affect the complex integrity or the kinase activity but inhibits mTORC2 substrate phosphorylation of AKT at Ser 473. However, phosphorylation of two other substrates, SGK1 and PKC, is not affected. These results indicate that mTORC1 can regulate mTORC2 activity on certain targets. Furthermore, both mTORC1 and S6K can directly phosphorylate and inhibit IRS-1, providing negative feedback for the PI3K-AKT-mTORC2 signaling [135–137]. In contrast, mTORC2 target AKT phosphorylates and suppresses TSC2 function to relieve inhibition on mTORC1 activity, displaying positive signals from mTORC2 to mTORC1 [100,101].

1.2.7 mTOR and aging

mTOR deficiency is associated with longer lifespan in multiple species. Yeast has two orthologs of mammalian mTOR, TOR1 and TOR2 [138]. Although both can be incorporated with other components to form TOR complex 1, only TOR2 is an essential gene. Single gene deletion of TOR1 increased yeast mean and maximum lifespan by about 20% [139]. RNAi of *C. elegans* TOR gene *let-363* doubles the lifespan of these worms. *let-363* null mutants showed even more dramatic effects by tripling the lifespan [140]. Overexpression of a dominant-negative form of the

Drosophila mTOR gene dTOR also extended mean lifespan by 24% compared with control constructs [141].

In mice, complete mTOR deletion is embryonically lethal. However, a hypomorphic mTOR model was established by inserting a cassette between exons 12 and 13 to disrupt mTOR transcription, leading to 25% of mTOR expression compared with wildtype mice. These mutant mice had slightly lower body weight, and similar food intake and glucose levels, but exhibited 22% median lifespan extension in males, and 19% in females. Moreover, these mice showed improved health in old age by behavioral measurements such as maze, rotarod or grip strength tests [142].

In addition to the genetic approach, the mTORC1 inhibitor rapamycin is a widely used drug to study the effects of mTOR on aging. Rapamycin was originally a natural bacteria metabolite with anti-fungal effects and was later used as an immunosuppressant in human organ transplantation. Rapamycin binds with FK506-binding protein 12 (FKBP12) to interact with mTOR and inhibit substrate recruitment of mTORC1. mTORC2 component RICTOR blocks this interaction site and leaves mTORC2 insensitive to acute rapamycin treatment. Prolonged rapamycin treatment keeps mTOR from forming new complexes, therefore inhibiting mTORC2 as well [143,144]. Similar to the effects of mTOR mutants, rapamycin treatment extends lifespan in diverse species, including yeast, worms, flies and mice. *S. cerevisiae* exposed to rapamycin showed lifespan extension in a dose-dependent way [145]. Rapamycin treated *C. elegans* had strengthened oxidative stress resistance and increased lifespan [146]. *D. melanogaster* fed with

rapamycin prolonged lifespan and increased resistance to starvation and the oxidative stressor paraquat [147].

A number of studies have shown the lifespan-extending effects of rapamycin in mice. Mice were given food containing rapamycin started at 9 months and showed increased lifespan in a dose-dependent manner. The highest dose led to 23% longer lifespan in males and 26% in females [148]. Strikingly, even when rapamycin was given late in life, starting at around 20-month old, it still extended male lifespan by 9% of male and female lifespan by 14% [149]. Rapamycin treatment not only extended lifespan but also prolonged health span by reducing pathological conditions in multiple tissues, including liver, heart, kidney and tendon [150]. However, these mice had impaired glucose tolerance and more severe cataract and testicular degeneration under rapamycin treatment [149,150]. Side effects in human may involve anemia, diabetes, and impaired wound healing [151]. These side effects as well as the low solubility and stability in vivo limit its potential as an anti-aging drug in clinical use [151,152]. Other mTOR inhibitors are being developed or tested that may provide more promising effects in the future [153].

Other mTORC1 components in addition to the mTOR kinase itself have also been tested for their effects in longevity. There was no lifespan extension in mice with one allele deleted of mTOR ($mTOR^{+/-}$), RAPTOR ($RAPTOR^{+/-}$) or mLST8 ($mLST8^{+/-}$), or double mutants $mTOR^{+/-}$ $RAPTOR^{+/-}$. However, female but not male $mTOR^{+/-}$ $mLST8^{+/-}$ mice had 14% increased lifespan [154]. These studies are consistent with the idea that mTOR deficiency extends lifespan but also suggest that the extent of compromise in mTOR function is an important factor in lifespan regulation.

Manipulation of mTORC1 targets is another direction to test the role of mTOR in longevity. Flies overexpressing a dominant-negative form of S6K increased lifespan by 22% [141]. Mice with S6K1 deletion showed 19% of lifespan extension in females but not in males [155]. These findings suggest that targeting mTORC1 downstream genes is an alternative approach to targeting mTOR directly and may provide useful insights in aging and longevity research.

As a critical upstream regulator of mTORC1, TSC has also been investigated in lifespan studies. Overexpression of TSC1 or TSC2 in *Drosophila* increased lifespan by 14% and 12%, respectively [141]. A TSC1 overexpression mouse model has also been generated [156]. These mice carried a TSC1 transgene which produced 30% higher TSC1 protein levels, leading to reduced mTORC1 signaling but enhanced mTORC2 signaling. This moderate TSC1 overexpression did not change body weight, food consumption, glucose tolerance or insulin sensitivity. However, when these mice were exposed to intense treadmill exercise, TSC1 overexpressing mice had higher running endurance and reached exhaustion later than wildtype mice. Moreover, when isoproterenol was injected to induce cardiac hypertrophy, TSC1 overexpressing mice retained better heart function and were more resistant to this treatment than control mice. Interestingly, a lifespan study found a 12% increase in female median lifespan but not in male mice. These results suggest that TSC1 overexpression exhibits similar lifespan extension phenotypes as mTOR deficiency, and future studies about the TSC2 overexpression would be informative.

1.3 Hypothesis and aims

Given the important role of mTOR in aging, efforts have been made to study mTOR signaling in long-lived GHRKO and Snell dwarf mice to see whether there is lower mTORC1 activity [58]. In the fasted condition, the mTORC1 targets pS6K and p4EBP1 were expressed at low levels. Feeding increased the phosphorylation of these proteins, but compared with wildtype mice, GHRKO and Snell mice ended up with lower pS6K and p4EBP1 in multiple tissues including liver, kidney, skeletal muscle and heart. Intriguingly, GHRKO and Snell mice showed higher phosphorylation of mTORC2 targets AKT at Thr 450 and SGK under fasting condition. AKT phosphorylation at Ser 473 was not different between these mice and the controls. Upon refeeding, the difference of pAKT Thr 450 and pSGK disappeared and instead, GHRKO and Snell mice showed lower levels of pAKT Ser 473 than controls. These results suggest that these two long-lived GHRKO and Snell mouse models have lower mTORC1 activity and higher mTORC2 activity towards some mTORC2 targets. It is still unknown what is regulating the mTORC activity or substrate specificity in these long-lived mice.

As mTOR complexes are each composed of several components, and the levels of these components could affect the kinase activity, complex integrity and substrate recruitment, the regulation of the mTORC targets could be from alteration of the mTORC components. Therefore, the hypothesis was that **the activity of the mTOR complexes in long-lived GHRKO and Snell dwarf mice could be regulated by alteration in their components**. Since upstream signaling of mTORC1 converge on TSC to control mTORC1 activity, it was further hypothesized that **the lower mTORC1 activity in these mice might be regulated by alternation in TSC activity or levels of TSC components**.

To test these hypotheses, two aims were developed.

Aim 1 was to study **whether there are alterations of mTORC components in tissues of GHRKO and Snell dwarf mice, and whether these changes affect mTORC activity**. To test this idea, the protein levels of mTORC1 and mTORC2 components were examined in three tissues of GHRKO and Snell dwarf mice, including liver, kidney and skeletal muscle. There were reductions in DEPTOR and PRAS40, two negative regulators of mTOR activity, in GHRKO and Snell liver. However, DEPTOR or PRAS40 knockdown in skin-derived GHRKO fibroblasts did not change the phosphorylation of mTORC1 or mTORC2 substrates, indicating that phosphorylation of mTORC1 or mTORC2 targets are not regulated by alteration of the mTORC components.

Aim 2 was to study **whether there are changes in the TSC components in GHRKO and Snell dwarf mice and whether they regulate mTORC1 activity**. Protein levels of TSC1 and TSC2 were tested in GHRKO and Snell liver, kidney and skeletal muscle, and both showed increased expression. Further examination of the phosphorylation of TSC2 found reduced levels of Ser 939 and Thr 1462 , which are AKT targeted sites that inhibit TSCs, indicating more active TSC that could inhibit mTORC1. TSC2 knockdown in GHRKO fibroblasts reversed the effects on the phosphorylation of mTORC1 substrates, suggesting regulated mTORC1 activity by TSC. These data showed that the levels of TSC components and the activity of the complex contributed to lower activity of mTORC1 in long-lived GHRKO and Snell dwarf mice.

Together, the findings provide no support for the hypothesis that alterations in mTORC components regulate mTORC activity or substrate specificity in GHRKO or Snell dwarf mice, but support the hypothesis that lower mTORC1 activity is regulated by inhibitory signaling from TSC.

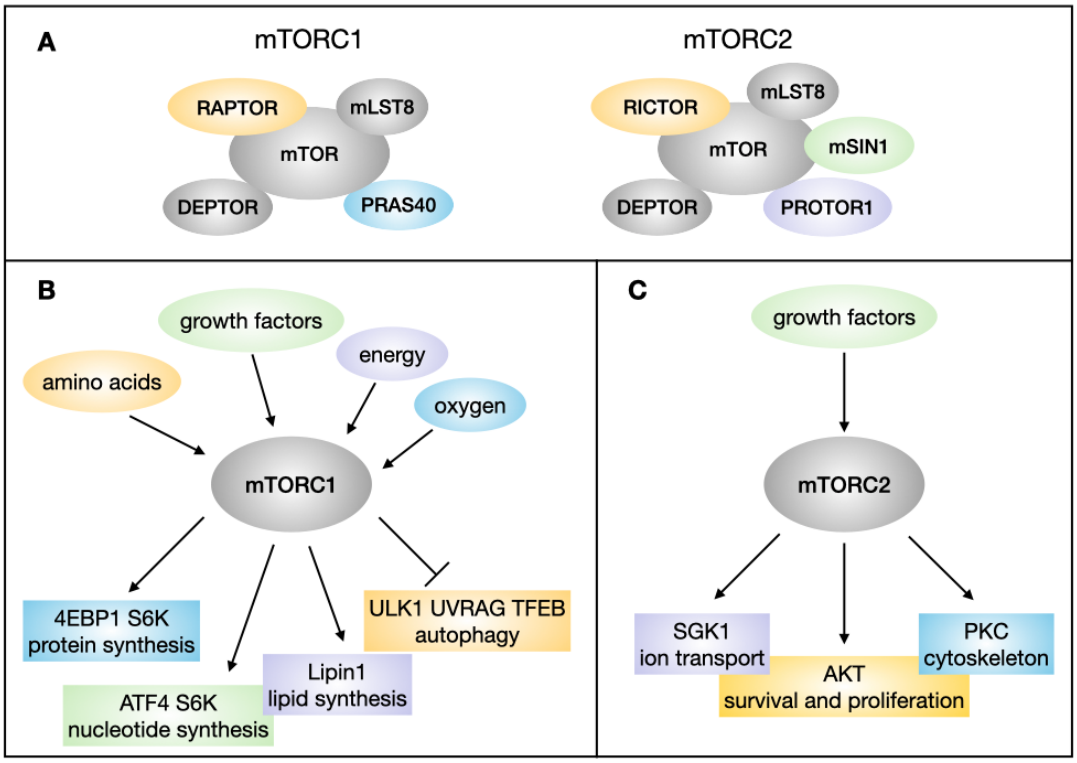

Figure 1 mTORC1 and mTORC2 composition, regulation and function
(A) mTORC1 and mTORC2 structure. mTORC1 and mTORC2 share common components mTOR, mLST8 and DEPTOR. mTORC1 has specific components RAPTOR and PRAS40. mTORC2 has specific components RICTOR, mSIN1 and PROTOR1. (B) mTORC1 regulation and function. mTORC1 receives signals from amino acids, growth factors, ATP and oxygen levels. mTORC1 phosphorylates and regulates 4EBP1 and S6K in protein synthesis, ATF4 and S6K in nucleotide synthesis, Lipin1 in lipid synthesis, ULK1, UVRAG and TFEB in autophagy. (C) mTORC2 regulation and function. mTORC2 is regulated by growth factors to affect SGK1 in ion transport, AKT in cell survival and proliferation, and PKC in cytoskeleton arrangement.

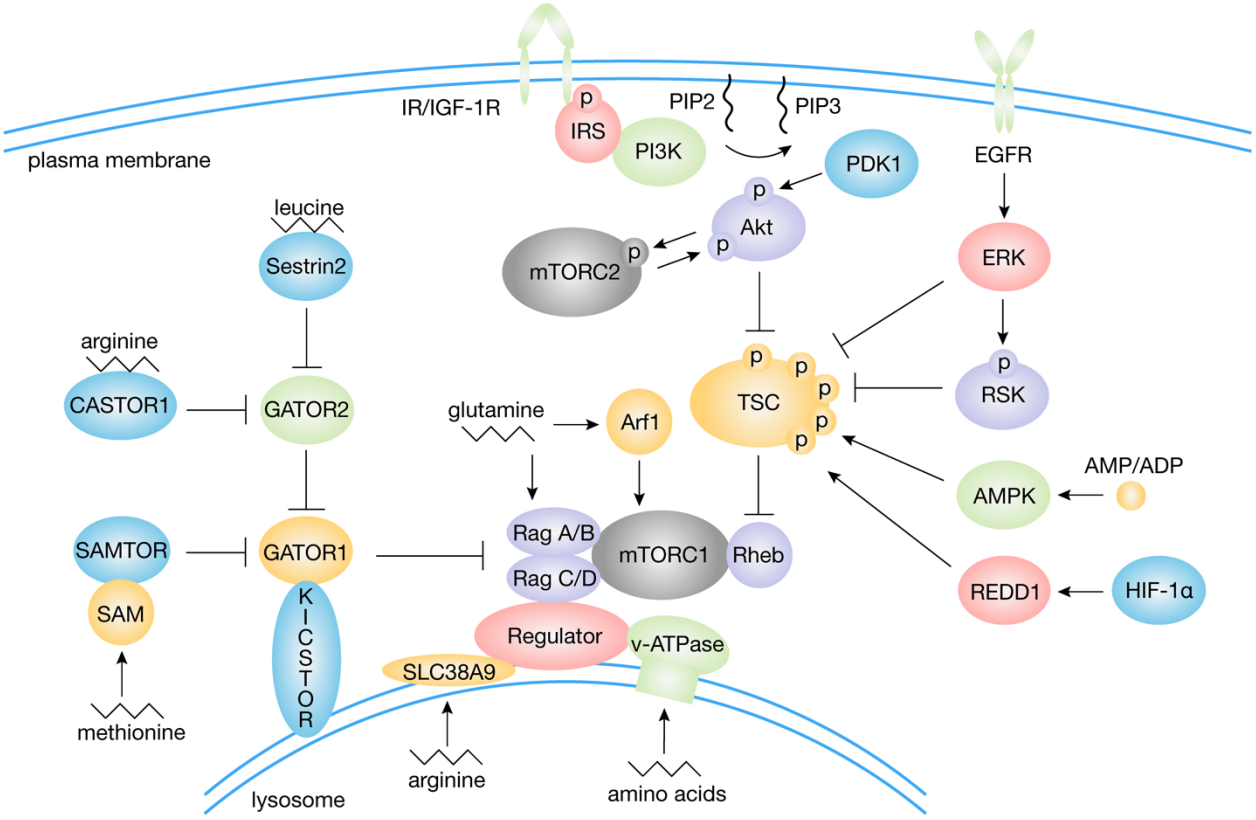

Figure 2 Regulation of mTORC1 and mTORC2

mTORC1 is regulated at two aspects, localization and activation. mTORC1 localization to the lysosome is regulated by amino acid levels. Leucine, arginine, and the methionine metabolite SAM are bound with their sensors, and inhibit GATOR1 which is tethered to the lysosome membrane by the KICSTOR complex. GATOR1 inhibits the Rag proteins that are bound by the Regulator complex to the lysosome, blocking the recruitment of mTORC1. Glutamine functions by the glutamolysis pathway to enhance RagB activity. Glutamine also acts through Arf1 to activate mTORC1. Arginine levels in the lysosome are sensed by SLC38A9, which stimulates the Regulator and Rag proteins. Lysosomal amino acid levels are also sensed by v-ATPase, which then interacts with Regulator to enable mTORC1 translocalization. Insulin or IGF-1 signals through IRS to bind PI3K which phosphorylates PIP2 to PIP3. PIP3 recruits PDK1 and AKT for AKT to be phosphorylated and partially activated by PDK1. AKT can both phosphorylate mTORC2 and be phosphorylated by mTORC2, leading to full AKT activation. AKT then phosphorylates and inhibits TSC, which further inhibits the mTORC1 activator Rheb. EGF acts through downstream ERK and RSK to phosphorylate and inhibit TSC. Low ATP levels activates AMPK to phosphorylate and stimulate TSC. Hypoxia induces HIF-1α and REDD1 to phosphorylate and augment TSC activity.

Chapter 2 The mTORC1 and mTORC2 Substrates Are Not Regulated by mTORC Components in GHRKO and Snell Dwarf Mice

2.1 Abstract

Downregulation of mTOR can extend lifespan in multiple species, including mice. GHRKO and Snell dwarf mice have 40% or greater lifespan increase, and have lower mTORC1 function, which might reflect alteration in mTORC1 components. Here, protein expression of mTORC1 and mTORC2 components were examined in GHRKO and Snell dwarf liver, kidney and skeletal muscle. Two inhibitory proteins, DEPTOR and PRAS40, were decreased in liver, which is opposite in direction to changes that would be expected to lead to lower mTORC1 function. DEPTOR or PRAS40 knockdown by RNAi in GHRKO fibroblasts did not lead to higher phosphorylation of mTORC1 or mTORC2 substrates, suggesting that DEPTOR and PRAS40 do not inhibit mTORC1 or mTORC2 activity in these cells. Overall, these findings indicate that mTORC1 and mTORC2 are not regulated by the level of their components in GHRKO and Snell dwarf mice.

2.2 Introduction

GHRKO and Snell dwarf mice are two long-lived mutant mouse models with deficient GH/IGF-1 signaling. Previous study found these mice have lower mTORC1 targets pS6K and p4EBP1, higher mTORC2 targets pAKT Thr 450 and pSGK, and lower pAKT Ser 473 under the refeeding condition [58]. However, it is unclear what signals are causing these effects.

mTORC1 is composed of the core kinase mTOR, the scaffold protein RAPTOR, the stabilizer mLST8, and internal inhibitors DEPTOR and PRAS40 [61–66]. mTORC2 consists of the mTOR kinase, the scaffold protein RICTOR, the stabilizer mLST8, the internal inhibitor DEPTOR, the kinase mSIN1, and the positive regulator PROTOR1 [67–70].

Changes of mTORC targets could be regulated at the level of the mTORC components. To test this idea, protein expression of these mTORC components were tested in tissues of GHRKO and Snell mice. Immunoprecipitation (IP) experiments were done to study the complex composition and followed up with knockdown experiments to study the effects of certain components. To investigate the hormonal signaling, additional mouse models were used to study their mTORC components, including PAPPA-KO, LKO and MKO mice.

2.3 Results

2.3.1 The protein levels of DEPTOR and PRAS40 but no other mTORC components are reduced in GHRKO liver

Immunoblotting with specific antibodies was carried out to test the protein levels of mTORC1 and mTORC2 components in GHRKO liver, kidney and skeletal muscle. Figure 3 shows the results of GHKRO liver with 6 pairs of male and 6 pairs of female mice at six-month old. Since two-way ANOVA found no effects of sex and no (sex × genotype) interactions for any of these proteins, data were pooled for males and females for the analyses.

Interestingly, DEPTOR, an inhibitor of both mTORC1 and mTORC2, had decreased protein expression in GHRKO liver compared with wildtype mice (Figures 3A and 3D). This finding is in opposite direction expected for mTORC1 regulation, indicating that DEPTOR is not the reason for lower mTORC1 activity in these mice. However, since DEPTOR is also a component of mTORC2, a reduction in DEPTOR may be upregulating mTOR2 activity towards certain targets. PRAS40 also inhibits mTORC1 activity and similarly, there was decreased protein expression of PRAS40 in GHRKO liver (Figures 3A and 3F). Again, this finding does not explain the lower mTORC1 activity observed in GHRKO mice. There was no difference in the protein levels of other components, including common components mTOR and mLST8, mTORC1 specific component RAPTOR, and mTORC2 specific components RICTOR and mSIN1 (Figures 3A, 3B, 3C, 3E, 3G and 3H). PROTOR1 was not tested by immunoblot because there were no specific antibodies identified for this purpose. The alternative method immunoprecipitation was used and results are shown in Figure 8. Together, GHRKO liver showed no alternation in most mTORC components, and reductions in two inhibitory components DEPTOR and PRAS40.

2.3.2 The protein changes of DEPTOR and PRAS40 are not seen in GHRKO kidney and skeletal muscle

Next, two other tissues, kidney and skeletal muscle, were tested to see whether the results in liver are also true for other tissues. In kidney, neither DEPTOR or PRAS40 was different between GHRKO and wildtype mice (Figure 4A). In muscle, DEPTOR protein level was higher in GHRKO than control mice. PRAS40 protein levels were similar in these two groups (Figure 4B). These results showed that the reduction of DEPTOR and PRAS40 observed in GHRKO liver

does not present in kidney or muscle, suggesting tissue-specific regulation of these two proteins. These findings also argue against critical roles of DEPTOR and PRAS40 in mTOR regulation because the alteration of mTORC targets is universal in these tissues but the expression of DEPTOR and PRAS40 is not.

2.3.3 DEPTOR and PRAS40 but no other mTORC components are reduced in Snell dwarf liver

To further confirm the findings, a very similar mouse model, Snell dwarf mice, was used to test the mTORC proteins in liver, kidney and skeletal muscle. As expected, Snell liver showed decreased expression of DEPTOR and PRAS40, as GHRKO liver (Figures 5A, 5D and 5F). The other components were not differentially regulated in Snell liver, including mTOR, mLST8, RAPTOR, RICTOR and mSIN1 (Figures 5A, 5B, 5C, 5E, 5G and 5H). Together, most mTORC components were not altered in Snell dwarf liver. Only two proteins, DEPTOR and PRAS40, were decreased in this tissue. These results confirm those from GHRKO liver, suggesting similar regulation of mTORC composition in these two mouse models.

2.3.4 DEPTOR and PRAS40 changes are not present in Snell dwarf kidney and skeletal muscle

Two other Snell tissues were also tested to see whether DEPTOR and PRAS40 are regulated in not just liver. Snell kidney showed similar levels of DEPTOR and PRAS40 with wildtype controls (Figure 6A). Snell muscle had lower levels of PRAS40 but not DEPTOR (Figure 6B). These results are not consistent with Snell liver, suggesting tissue-specific regulation of these

two proteins. GHKRO kidney and Snell kidney gave similar results but muscles from these two models were slightly different. GHRKO muscle had higher DEPTOR and unaltered PRAS40. In contrast, Snell muscle had unaltered DEPTOR but lower PRAS40. These subtle differences suggest distinct signaling between GHRKO and Snell dwarf mice which are not well-understood. Together with the results from GHRKO tissues, proteins of mTOR complexes are largely not altered in liver except for DEPTOR and PRAS40, which are in the opposite direction expected for mTORC1 regulation. In addition, the effects of DEPTOR and PRAS40 are not consistent in kidney or skeletal muscle, opposing a causal relationship between these proteins and altered mTOR activity.

2.3.5 DEPTOR and PRAS40 are decreased in immunoprecipitated mTOR complexes

DEPTOR and PRAS40 were reduced in whole liver lysates but whether there is less DEPTOR and PRAS40 in the mTOR complexes was unknown. Thus, immunoprecipitation experiment was done to isolate mTORC1 and mTORC2 from wildtype or GHRKO liver using antibodies against RAPTOR or RICTOR, respectively. The input was shown as Figure 7C. Then the protein expression of the other mTORC1 components were tested from the RAPTOR pull down samples. mTOR and mLST8 were not different between wildtype and GHRKO, but there was a decrease of DEPTOR and PRAS40 in GHRKO compared with control (Figures 7A and 7D). This indicates that there is less DEPTOR and PRAS40 incorporated into mTOR complex 1, which is consistent with reduced levels of these proteins in whole tissue lysates. mTORC2 components in the RICTOR pull down samples were also tested and there were similar levels of mTOR, mLST8 and mSIN1 in wildtype and GHRKO. However, the level of DEPTOR was decreased in GHRKO sample, suggesting less DEPTOR incorporated into mTOR complex 2

(Figures 7B and 7E). These results confirmed that there is not only less DEPTOR and PRAS40 in GHRKO whole liver lysates but also inside mTOR complexes.

2.3.6 Unaltered PROTOR1 level in mTOR complex 2 in GHRKO liver

Since immunoblotting did not work for PROTOR1 because of non-specific antibodies, an alternative strategy was used which is to pull down mTOR complex 2 with a specific PROTOR1 antibody for immunoprecipitation purpose. Then immunoblot was done for the other mTORC2 proteins to see whether their levels are different using PROTOR1 as a reference. Figure 8 showed that the levels of RICTOR, mTOR, mLST8 and mSIN1 were not different between wildtype and GHRKO liver, indicating that the amount of PROTOR1 inside mTORC2 is also similar in these mice. As expected, DEPTOR was decreased in the GHRKO IP samples, confirming less DEPTOR in mTORC2 in GHRKO liver.

2.3.7 DEPTOR or PRAS40 knockdown did not regulate mTORC1 or mTORC2 substrates

To further test whether DEPTOR or PRAS40 is regulating mTORC activity, knockdown experiments were performed in wildtype and GHRKO cells. These cells were isolated from the mouse tail tips and cultured in vitro. Immunoblot was first done to test the protein levels of the mTORC substrates. As expected, GHRKO cells had significantly reduced expression of GHR (Figure 9A). DEPTOR was also decreased in these cells as in liver lysates. However, PRAS40 was not decreased, again showing tissue-specific expression of this protein (Figure 9). Previous study using GHRKO and Snell dwarf mice found reduced phosphorylation of mTORC1 substrates S6K and 4EBP1, and lower mTORC2 substrates pAKT Ser 473, but unchanged

mTORC2 substrate pAKT Thr 450 under feeding condition [58]. These observation were also true for the GHRKO fibroblasts, suggesting that these cells are representative of GHRKO mice.

Next, cells were treated with either control siRNA or siRNA against DEPTOR, and DEPTOR was successfully knocked down (Figure 10). Then targets of both mTORC1 and mTORC2 were tested in these knockdown samples. pS6K, p4EBP1 and pAKT S473 were lower in GHRKO cells, and pAKT T450 was not different, which are all as expected and indicate no effects from control siRNAs. However, there was no increase or decrease in DEPTOR knockdown groups compared with controls, suggesting negligible regulation of DEPTOR on mTOR complexes in wildtype or GHRKO cells. These results further confirm the former findings that DEPTOR is not responsible for the lower mTORC1 activity in GHRKO or Snell dwarf mice. In addition, these results suggest that the reduced DEPTOR levels does not upregulate mTORC2 activity or modulate mTORC2 substrate specificity in these mice.

PRAS40 was also knocked down using siRNA in wildtype or GHRKO fibroblasts, and PRAS40 protein expression was significantly blocked (Figure 11). mTORC1 targets pS6K and p4EBP1 were tested in these cells but there were no effects of PRAS40 knockdown on any of the mTORC1 substrate phosphorylation. This data suggests that PRAS40 does not regulate mTORC1 activity in GHRKO cells and strengthens the former results that the lower mTORC1 activity in GHRKO or Snell dwarf mice is not from decreased PRAS40 expression.

2.3.8 DEPTOR and PRAS40 protein expression in PAPPA-KO mice

To understand the hormonal regulation of DEPTOR and PRAS40, three other mouse models, PAPPA-KO, LKO and MKO mice, were used. GHRKO mice have disrupted growth hormone signaling pathway and lower IGF-1 [42]. Snell dwarf mice have both lower growth hormone and IGF-1 [27]. It is unknown which hormone is leading to the decreased DEPTOR and PRAS40 expression. PAPPA-KO mice have defective IGF-1 signaling and are long-lived [50]. If IGF-1 plays an important role in regulating DEPTOR and PRAS40 levels, PAPPA mice should also exhibit decreased DEPTOR and PRAS40 expression. This idea was tested using three tissues of PAPPA mice, liver, kidney and skeletal muscle. However, none of these tissues showed any alteration of DEPTOR and PRAS40 protein levels (Figure 12), indicating that IGF-1 signal is not critical in DEPTOR and PRAS40 regulation.

2.3.9 DEPTOR and PRAS40 protein expression in liver-specific GHRKO mice

Growth hormone receptor knockout in specific tissues are also alternative models to study the hormones that regulate mTORC proteins. Liver-specific growth hormone receptor knockout (LKO) mice have disrupted growth hormone signaling in the liver, higher circulating growth hormone but lower circulating IGF-1 [43]. These mice are not long-lived, but they provide a model for the hormonal regulation of mTORC components in comparison to whole body GHRKO mice. DEPTOR and PRAS40 protein levels were tested in liver, kidney and muscle, and the only alteration is decreased DEPTOR expression in LKO liver (Figure 13A). DEPTOR was not decreased in kidney or muscle, and PRAS40 was not decreased in any of the three tissues (Figure 13). The common decrease of DEPTOR in GHRKO liver and LKO liver indicates that the expression of DEPTOR in liver appeared to be regulated by growth hormone. In contrast, PRAS40 expression is more complicated and seems to involve combined signaling pathways.

2.3.10 DEPTOR and PRAS40 protein expression in muscle-specific GHRKO mice

The intriguing results from LKO mice prompted the study of another similar mouse model, muscle-specific growth hormone receptor knockout (MKO) mice. These mice have unaltered circulating growth hormone and IGF-1 levels. The lifespan study did not give convincing results because one site showed extended lifespan of male but not female mice, and the other site showed no lifespan extension for both sexes [45]. Results from liver, kidney and muscle found no alteration of DEPTOR or PRAS40 protein expression (Figure 14). The DEPTOR result does not resemble the increased expression of DEPTOR in whole-body GHRKO, again suggesting tissue-specific regulation of DEPTOR. The lack of change in PRAS40 levels opposes the idea that PRAS40 protein expression is directly regulated by growth hormone signaling pathway.

To summarize, comprehensive data were collected from three tissues, liver, kidney and skeletal muscle, in five mouse models, GHRKO, Snell dwarf, PAPPA-KO, liver-specific GHRKO and muscle-specific GHRKO mice (Table 1). Only DEPTOR and PRAS40 were reduced in GHRKO and Snell dwarf liver but none of the other mTORC components were altered in these tissues. In addition, there were also less DEPTOR and PRAS40 incorporated into the mTOR complexes. Examination of their expression in kidney and muscle of GHRKO and Snell mice showed inconsistent results, suggesting tissue-specific regulation of these two proteins. Moreover, tests of three other mouse models, PAPPA, LKO and MKO mice, with different growth hormone or IGF-1 signaling, did not reveal the hormonal regulation of DEPTOR and PRAS40, and cannot support any definite conclusion about whether growth hormone or IGF-1 is leading to the

alteration of DEPTOR and PRAS40 protein expression. However, further molecular studies that manipulate growth hormone or IGF-1 directly at the cellular level would be more helpful.

2.4 Discussion

This project began with the hypothesis that the activity of the mTOR complexes in long-lived GHRKO and Snell dwarf mice could be regulated by alteration in mTORC components. The protein expression of mTORC components was tested in GHRKO and Snell dwarf liver, kidney and muscle lysates. Two proteins that inhibit mTOR activity, DEPTOR and PRAS40, were decreased in GHRKO and Snell liver, but results from other tissues were inconsistent. DEPTOR and PRAS40 were unaltered in GHRKO or Snell kidney. DEPTOR was increased in GHRKO muscle while PRAS40 was unaltered. In Snell muscle, only PRAS40 but not DEPTOR was decreased. Since lower mTORC1 activity was observed in all these tissues in GHRKO and Snell dwarf mice [58], these inconsistent changes of DEPTOR and PRAS40 are not very likely to be responsible for the lower mTORC1 activity in these mice. Immunoprecipitation experiments using GHRKO liver showed less DEPTOR and PRAS40 incorporated into mTOR complexes, disapproving the possibility that these two proteins could be enriched in the complexes despite lower levels in whole liver lysates. Consistently with these results, DEPTOR and PRAS40 knockdown in GHRKO fibroblasts did not alter mTORC1 or mTORC2 substrate phosphorylation, showing that the changes of DEPTOR and PRAS40 do not regulate mTORC activity or substrate specificity. This indicates that players other than DEPTOR and PRAS40 are controlling mTORC1 and mTORC2 signaling in these cells. Thus the conclusion is that alterations in composition of mTORC1 and mTORC2, though present in the mutant mice, could

not explain changes in mTORC activity previously documented [58] in GHRKO and Snell dwarf mice.

Three other mouse models, liver-specific, and muscle-specific GHRKO, and PAPPA-KO mice, were used to study the hormonal regulation of DEPTOR and PRAS40. If DEPTOR and PRAS40 are directly regulated by growth hormone signaling, lower DEPTOR and PRAS40 would be observed in liver tissue of liver-specific GHRKO mice and muscle tissue of muscle-specific GHRKO mice. However, DEPTOR showed reduction only in liver of liver-specific GHRKO mice but not in muscle of muscle-specific GHRKO mice, suggesting tissue-specific regulation of DEPTOR. In addition, DEPTOR was not differentially regulated in any of the PAPPA knockout mouse tissues, arguing against the direct involvement of IGF-1 signaling. These surprising results suggest tissue-specific regulation of DEPTOR controlled by some unknown signaling pathway(s). PRAS40 was not different in any of these mouse models, suggesting that PRAS40 is not directly regulated by growth hormone or IGF-1. Combined with results from GHRKO and Snell dwarf mice, PRAS40 is regulated by some unknown mechanism in a tissue-specific way.

2.5 Materials and methods

2.5.1 Mice

GHRKO mice were originally generated by deleting part of the growth hormone receptor gene around the fourth exon and fourth intron as described in [39]. GHRKO (GHR-/-) and littermate control (GHR+/+) breeding stocks were maintained with a mix of C57BL/6J and BALB/cByJ background. Offspring of these two genotypes are heterozygous GHR+/- mice. These mice were crossed to breed GHR+/+, GHR+/- and GHR-/- mice. GHR+/+ mice were used as controls. Snell

dwarf mice carry the Pit1 gene mutation and were bred as described in [27]. C3H/HeJ-Pit1$^{dwJ/+}$ females and DW/J-Pit1$^{dw/+}$ males were crossed to generate C3H/HeJ × DW/J F1, including dwarf mice (dwJ/dw) and controls which lack the dwarf phenotypes (+/+, dwJ/+ and dw/+). The generation of conditional GHR knockout mice (GHR$^{flox/flox}$) used methods described in [157]. Crossing of conditional GHR$^{flox/flox}$ mice with B6.Cg-Tg(albcre) 21Mgn/J mice carrying liver-specific Cre-recombinase expression produced liver-specific GHRKO and littermate controls [43]. Muscle specific GHRKO mice were bred by crossing conditional GHR$^{flox/flox}$ mice with B6.FVB(129S4)-Tg(Ckmm-cre)5Khn/J mice that have muscle-specific Cre-recombinase [45]. PAPPA-KO mice were initially generated by deleting exon 4 of the PAPPA gene as described in [49]. Crossing C57BL/6 heterozygous mice with homozygous knockout mice produced PAPPA +/- and -/- mice. PAPPA +/- mice were used as controls.

The protocols for mouse experiments were approved by the Institutional Animal Care & Use Committee at University of Michigan. All mice were kept in cages with free access to food and water. Mutant mice and controls were housed in the same cage. Mice were euthanized at six-months of age without fasting. Tissues were isolated and immediately frozen in liquid nitrogen, then transferred to and stored in a -80 ℃ freezer.

2.5.2 Cell culture

Fibroblasts were isolated from mouse tail tips. 1 cm long tail tips were cut from mice and temporarily stored in 50 mL centrifuge tubes with 5 mL complete medium, DMEM (Gibco, 11965092) with 10% fetal bovine serum (Corning, 35011CV) and 1% antibiotic-antimycotic (Gibco, 15240062). Tail tips were washed with 5 mL PBS (Thermofisher, SH3025601) first,

then soaked in 5 mL 70% ethanol for 5 min. The ethanol wash was repeated a second time to minimize bacterial and fungal contamination. Tail tips were rinsed again with 5 mL PBS and transferred to petri dishes with a 6 cm diameter. Collagenase (Gibco, 17101-015) was dissolved in complete DMEM to make a 400 U/mL solution and 0.5 mL was added to each tail tip. Tail tips were diced with a sterile blade to around 1 mm pieces. Another 3.5 mL collagenase was added to the dish and the dish was transferred to a 37 ℃ incubator with 10% CO_2. 24 hr later, the cells and cell clumps were pipetted for five to ten times and filtered through a 40 μm nylon cell strainer into 50 mL tubes. The mixture was centrifuged at 200 g for 5 min then resuspended with 5 mL complete DMEM. Cells were counted and 5×10^5 cells were seeded into 25 cm^2 flasks with 5 mL complete DMEM.

Medium was changed every three days, and cells were passaged about every six days. For passaging, medium was first removed and PBS was added to rinse the surface. 1 mL 0.05% trypsin (Thermofisher, 25300054) was added and incubated for 3 min to dislodge the cells. 2 mL complete DMEM was added to stop the process and the mixture was transferred to tubes and centrifuged at 200 g for 5 min. Supernatant was discarded and cells were resuspended with 3 mL complete DMEM. Cells were then counted and seeded to 75 cm^2 or 175 cm^2 flasks. Cells were used for experiments at passage 3 or passage 4. Cells were seeded into six-well plates at 0.5×10^6 per well (3.5 cm diameter) and harvested 24 hr later to make lysates for immunoblot analysis of the protein expression of mTORC components, substrates, and TSC components.

2.5.3 RNAi

Fibroblasts were seeded into six-well plates at 0.2×10^6 per well to reach 60-80% confluence on the second day. Lipofectamine (Invitrogen, 13778150) was used for RNA interference following the manufacturer's instructions. For a single well, 7.5 µL lipofectamine was added to 125 µL Opti-MEM (Invitrogen, 31985062), and 2.5 µL 10 µM siRNA was added to 125 µL Opti-MEM. The latter solution was added to the former one and incubated for 5 min at room temperature. Then 250 µL was added to each well. Two days later, cells were harvested for immunoblot analysis. The following siRNAs were used, control (Invitrogen, 4390844), DEPTOR (Invitrogen, n424206), PRAS40 (Invitrogen, s85345) and TSC2 (Invitrogen, s75509).

2.5.4 Immunoblot

To make protein lysates, lysis buffer was prepared to contain 33 mM Tris-HCl pH 6.8, 5% glycerol and 1% SDS and stored at 4 ℃. One tablet of protease inhibitor (Sigma, 11836170001) and 1 tablet of Phosstop (Sigma, 4906845001) were added to every 10 mL lysis buffer right before using. 50 µL lysis buffer was added to each well of six-well plates and cells were scraped to be collected, then transferred to 1.5 mL centrifuge tubes. Frozen tissues were ground with liquid nitrogen using pestle and mortar. 20 mg tissue powder was added with 600 µL lysis buffer. Samples were sonicated till fully homogenized and centrifuged at 17,000 g for 20 min. 360 µL supernatant was taken to a new tube. Protein concentration was measured using the BCA assay (Thermofisher, 23227) and diluted to 6.7 µg/ µL. One third volume of 4X Laemmli sample buffer (Biorad, 1610747) was added to the protein and heated at 95 ℃ for 10 min. The final concentration of protein was 5 µg/ µL for tissue lysates and 2 µg/ µL for cell lysates.

For immunoblotting, 50 µg tissue protein or 20 µg cell protein were loaded into SDS-PAGE gels and run at 100 V for about 1.5 hr in electrophoresis buffer (0.3% tris base, 1.44% glycine and 0.1% SDS dissolved in H_2O). Protein standards (Biorad, 1610374) were used as a molecular weight comparison. Samples were transferred onto PVDF membrane (Biorad, 1620177) at 90V for 1.5 hr using transfer buffer (0.3% tris base and 0.1% SDS dissolved in H_2O). Blocking buffer was made using TBS buffer (diluted from Biorad, 1706435) added with 0.1% Tween-20 and 5% BSA (MP Biomedicals, 160069). Membranes were blocked at room temperature for 1 hr. Antibodies were diluted according to the manufacturer's instructions. The specific antibodies used are listed in Table 2. Membranes were incubated with primary antibodies at 4 ℃ overnight, then washed with TBST (TBS+0.1% Tween-20) for 20-30 min before incubating with secondary antibodies at room temperature for 1 hr. Membranes were washed again then developed using ECL prime reagents (Cytiva, RPN2232). Immunoblots were analyzed and quantified by ImageJ. Quantification of the bands were normalized to GAPDH, and the average of controls was set as 1. Phosphorylation levels were compared directly to total levels of the same protein then normalized to controls.

2.5.5 Immunoprecipitation

Immunoprecipitation lysis buffer contains HEPES 40 mM pH 7.5, NaCl 120 mM, EDTA 1 mM, Na-pyrophosphate 10 mM, glycerophosphate 10 mM, NaF 50 mM and 0.3 % CHAPS. This was made one day ahead of time and kept at 4 ℃. One protease inhibitor tablet was added to 10 mL IP lysis buffer before using. The manufacturer's instructions were followed to prepare protein A magnetic beads (Biorad, 1614013) by washing the beads three times with wash buffer (PBS+0.1% Tween-20). 2 µg antibody was added to the beads and incubated on a rotating mixer

at 4 ℃ for 2 hr. 600 µL lysis buffer was added to 20 mg tissue powder and homogenized with a Douncer. Then samples were incubated on ice for 30 min and mixed in every 10 min. Samples were centrifuged at 12,000 g, 4 ℃ for 20 min. 90 µL supernatant was taken to make input samples by adding 30 µL 4X Laemmli buffer and heating at 95 ℃ for 5 min. After beads were bound with antibodies, they were washed three times and added to 300 µL tissue lysates. The mixer was incubated at 4 ℃ overnight. On the second day, beads were washed three times, then added to 40 µL 1X Laemmli buffer and incubated at 70 ℃ for 10 min to elute protein. Samples were heated at 95 ℃ for 5 min and were used for immunoblotting.

2.5.6 Statistics

Quantification of immunoblots were analyzed by two-way ANOVA (Sex × Genotype, with Interaction), to see if the genotype effects were sex specific. Since none of the interaction terms were statistically significant, data were then combined from both males and females and significance assessed using the Student's t-test. Figures were made using Graphpad Prism and Adobe Illustrator. Mean and standard error of the mean (SEM) were shown in dot plots.

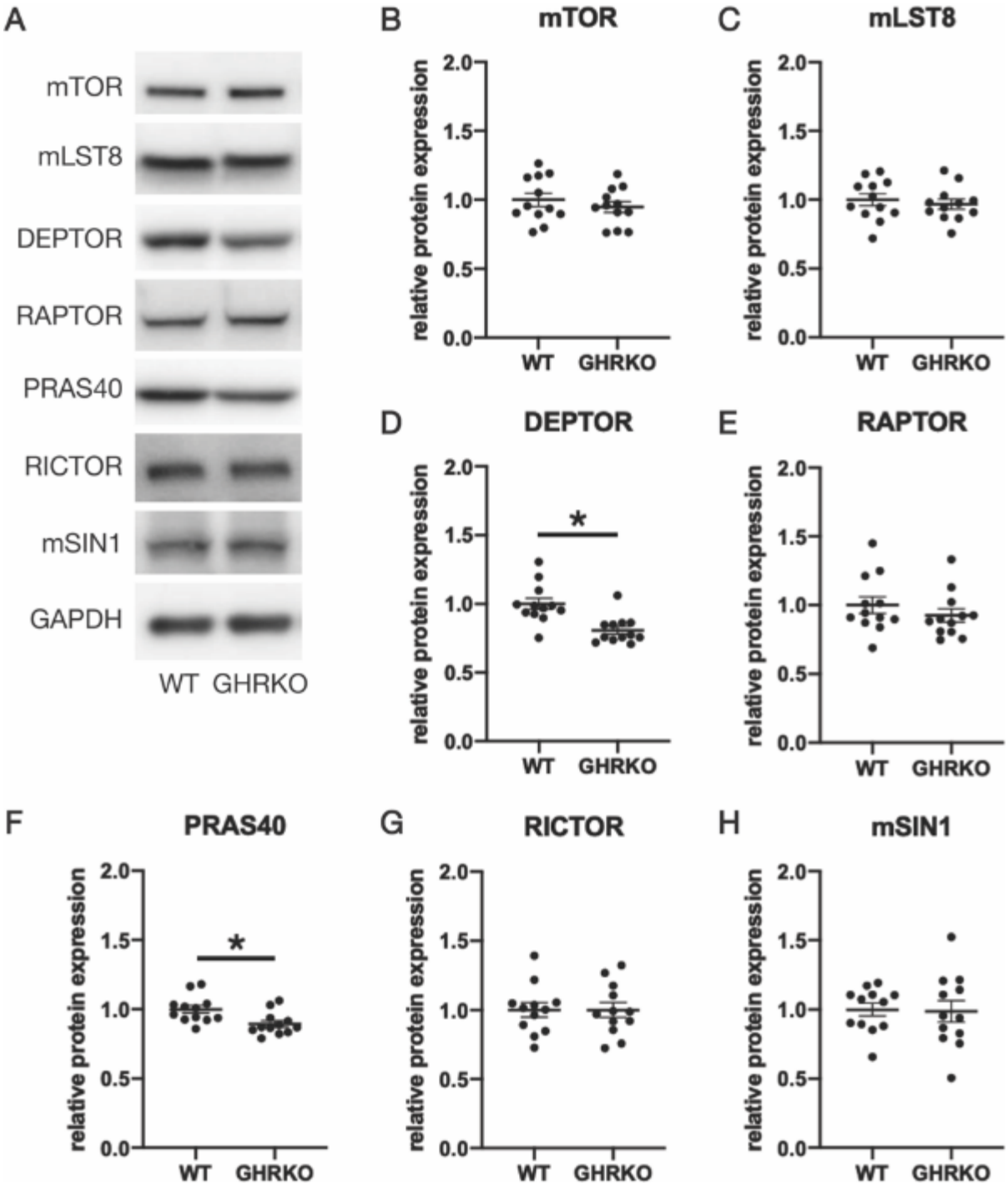

Figure 3 Reduced DEPTOR and PRAS40 protein expression in GHRKO liver
(A) Representative immunoblots of protein expression for mTORC1 and mTORC2 components. (B-H) Quantification of protein expression, for N=6 male and N = 6 female mice, with mean and SEM. (*) for t-test p-value < 0.05

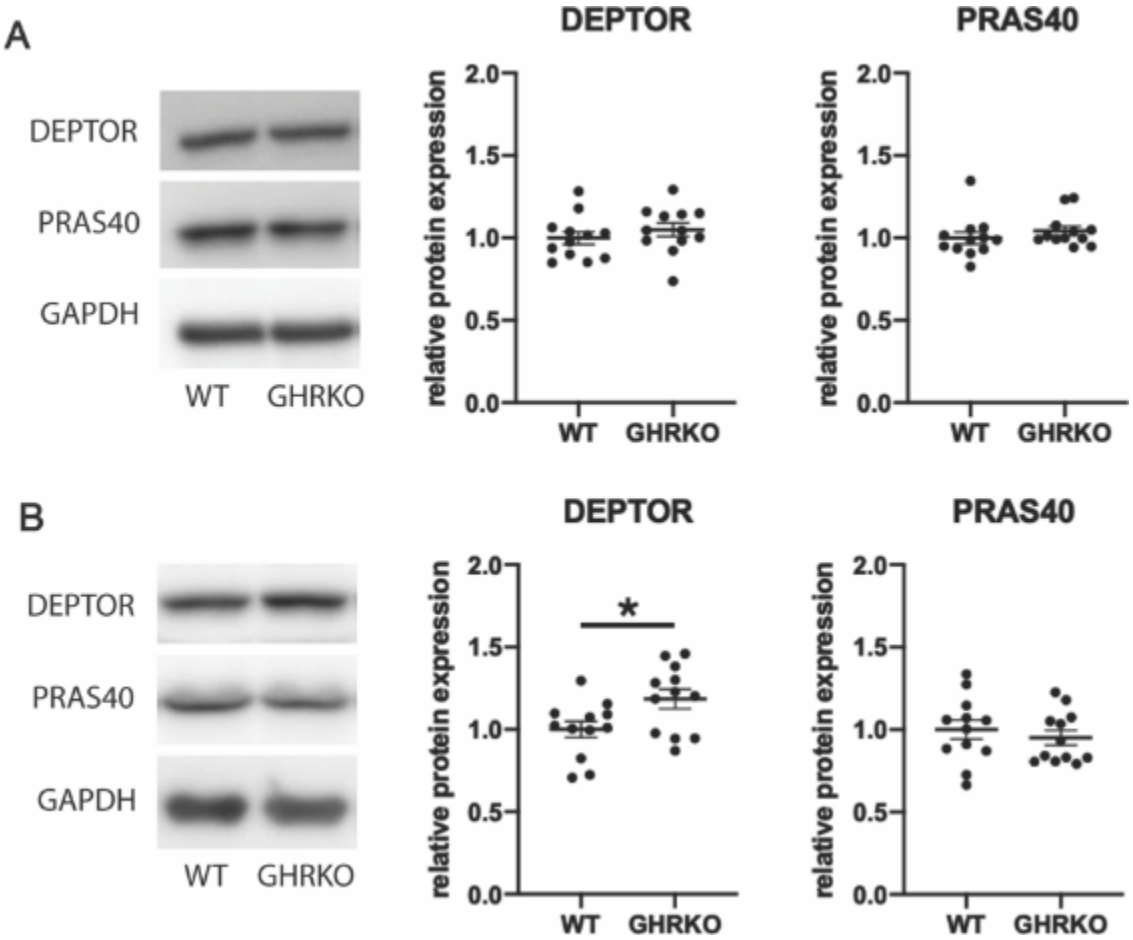

Figure 4 DEPTOR and PRAS40 are not reduced in GHRKO kidney and muscle
(A) Protein expression of DEPTOR and PRAS40 in GHRKO kidney. (B) Protein expression of DEPTOR and PRAS40 in GHRKO muscle. N=6 male and N=6 female mice. (*) for t-test $p < 0.05$

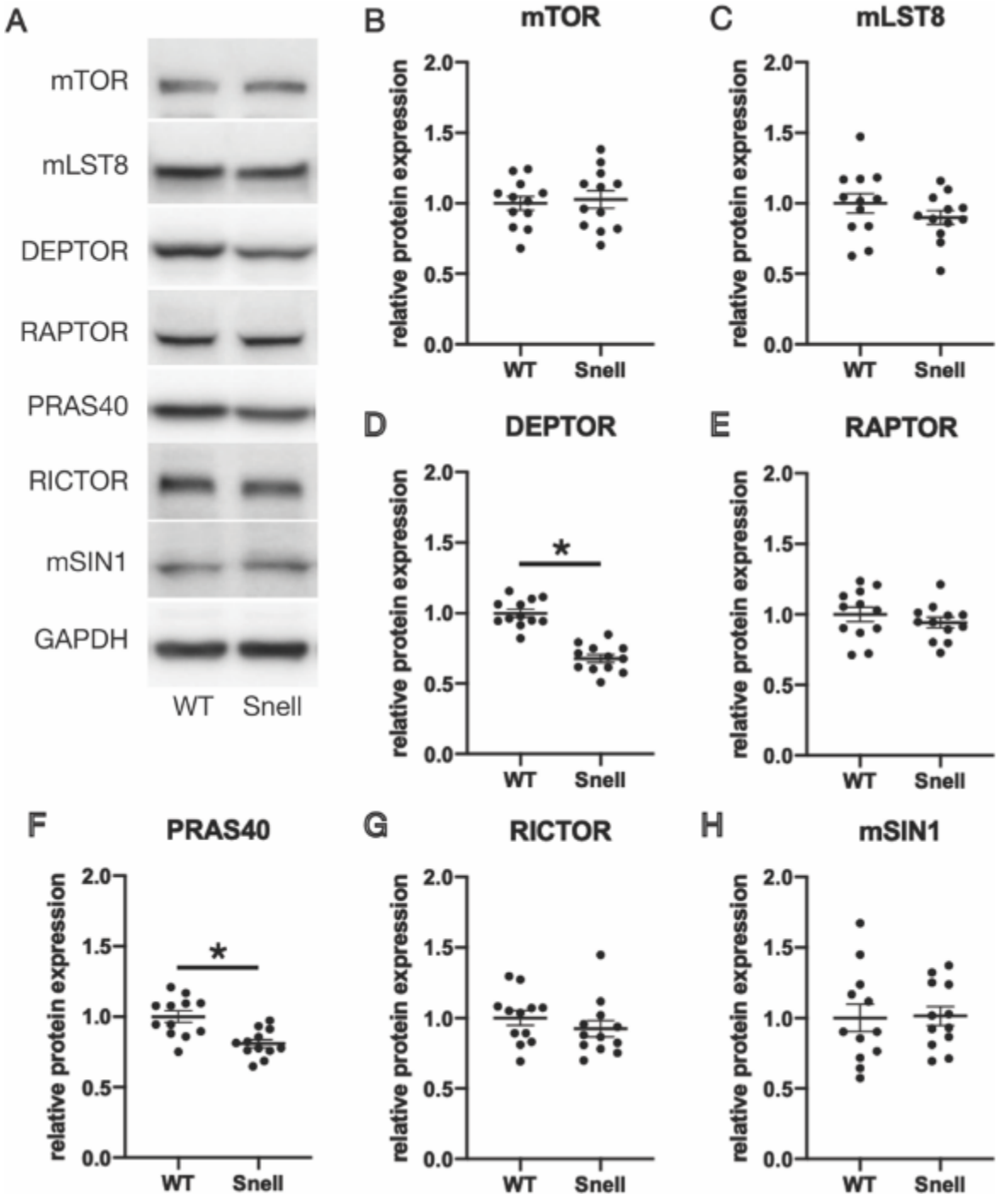

Figure 5 Reduced DEPTOR and PRAS40 protein expression in Snell dwarf liver
(A) Representative immunoblots of protein expression for mTORC1 and mTORC2 components. (B-H) Quantification of protein expression, for N=6 male and N = 6 female mice, with mean and SEM. (*) for t-test $p < 0.05$

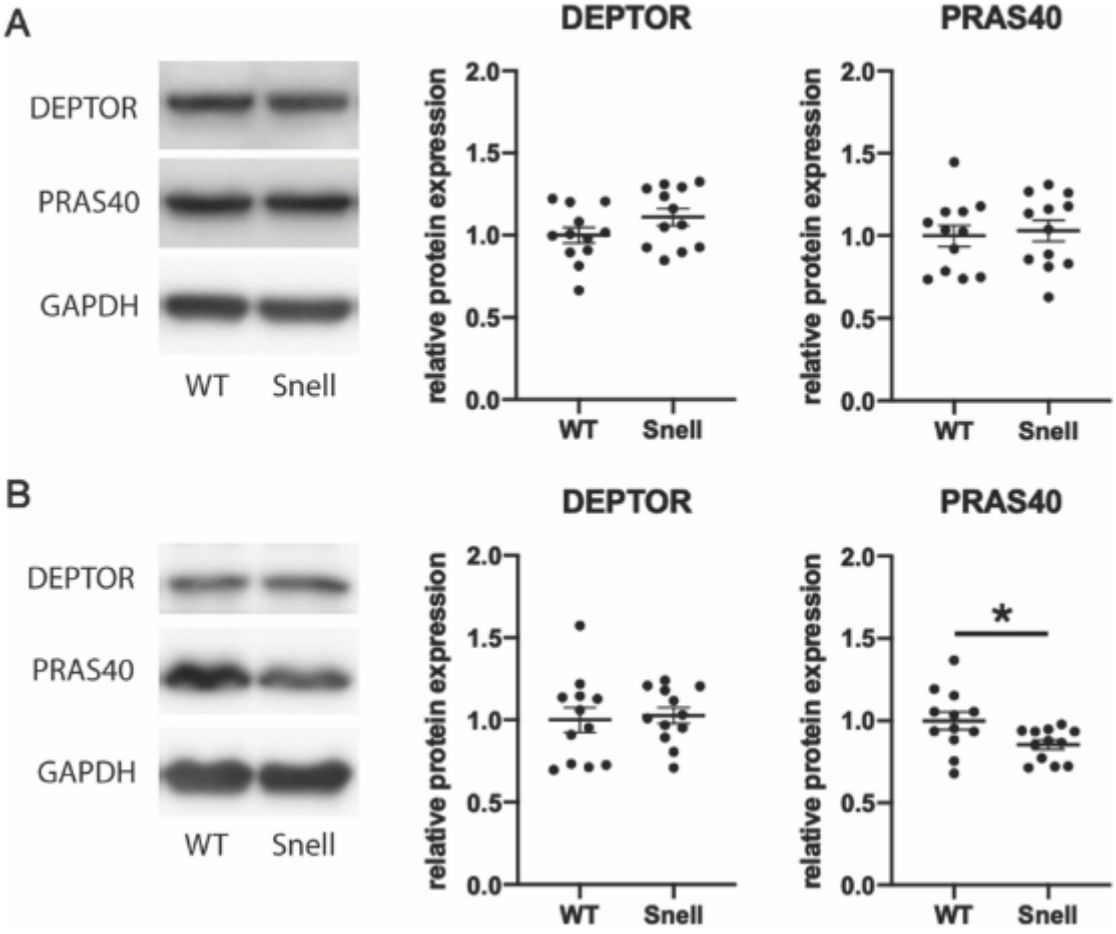

Figure 6 DEPTOR and PRAS40 protein expression in Snell dwarf kidney and skeletal muscle

(A) Protein expression of DEPTOR and PRAS40 in Snell dwarf kidney. (B) Protein expression of DEPTOR and PRAS40 in Snell dwarf muscle. N=6 male and N=6 female mice. (*) for t-test $p < 0.05$

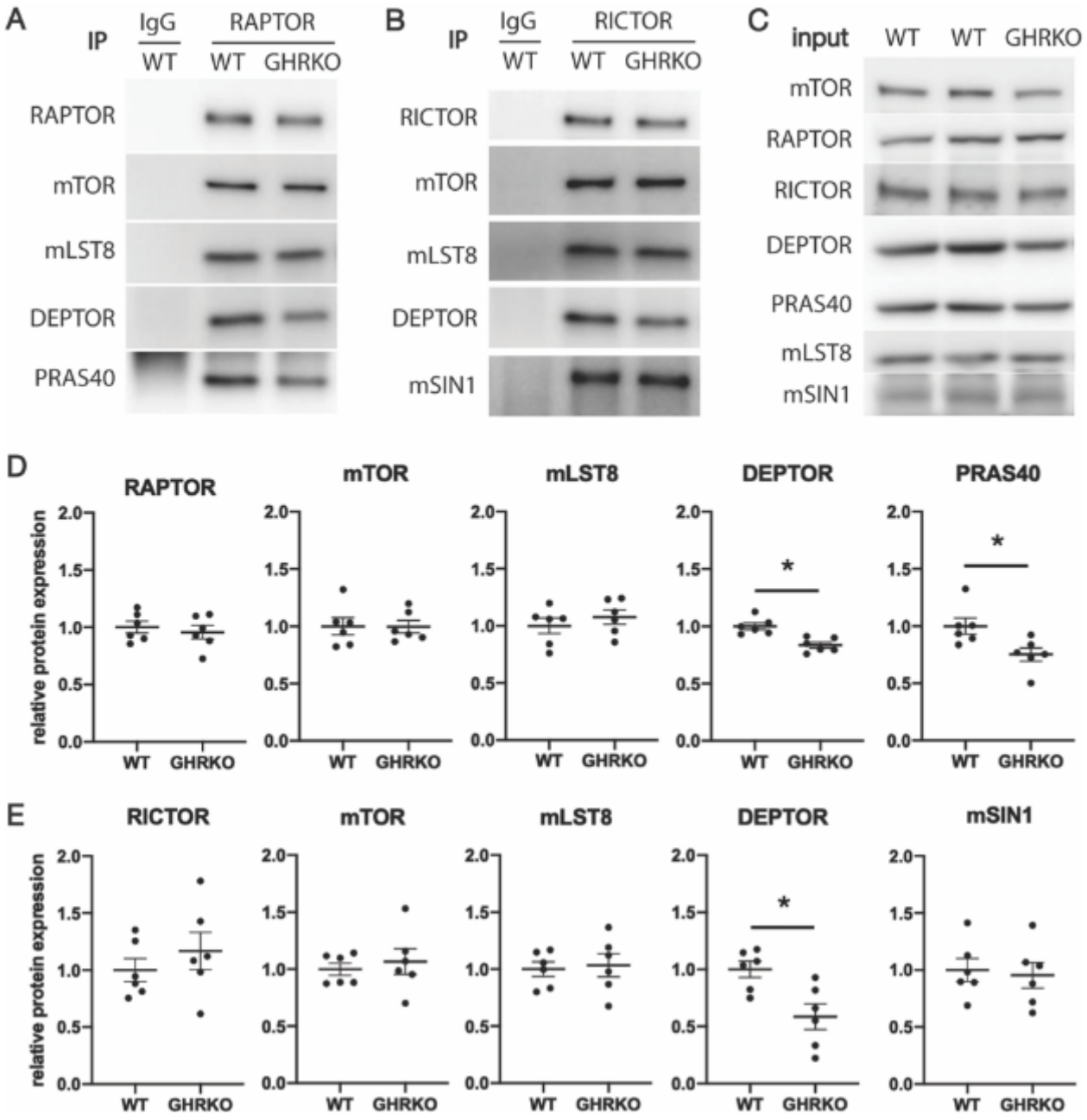

Figure 7 Decreased DEPTOR and PRAS40 in immunoprecipitated mTOR complexes
(A) Representative immunoblots of the mTORC1 components from samples immunoprecipitated with anti-RAPTOR antibody. (B) Representative immunoblots of the mTORC2 components immunoprecipitated with anti-RICTOR antibody. (C) Representative immunoblots of the input samples. (D) Quantification of the protein levels in RAPTOR-IP samples, as in (A). (E) Quantification of the protein levels in RICTOR-IP samples, as in (B), for N=6 male mice, with mean and SEM. (*) for t-test $p < 0.05$

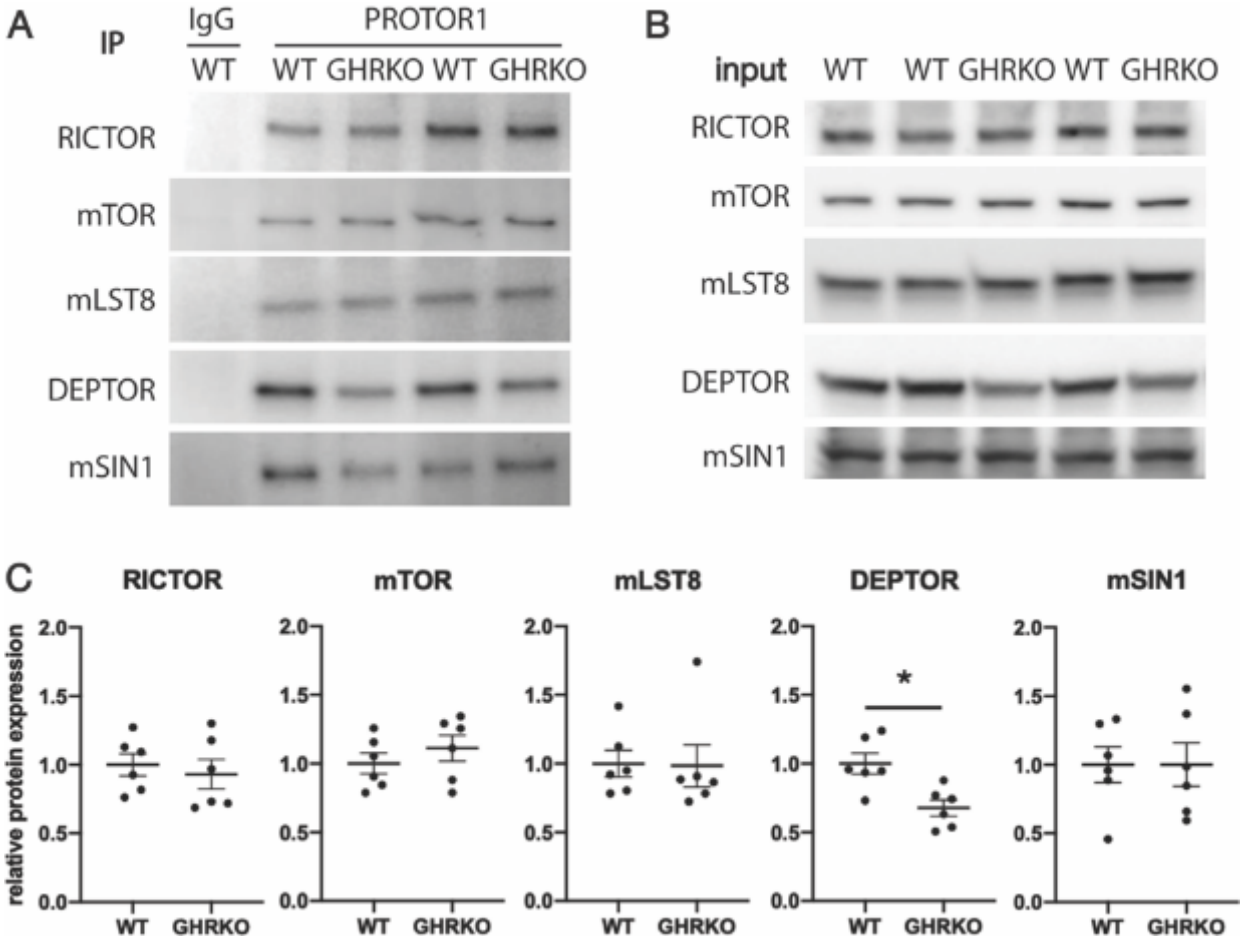

Figure 8 Unaltered PROTOR1 level in mTORC2
(A) Representative immunoblots of mTORC2 components in PROTOR1 immunoprecipitation samples. (B) Input samples for immunoprecipitation. (C) Quantification of mTORC2 components in (A). N=6 male mice, with mean and SEM. (*) for t-test $p < 0.05$

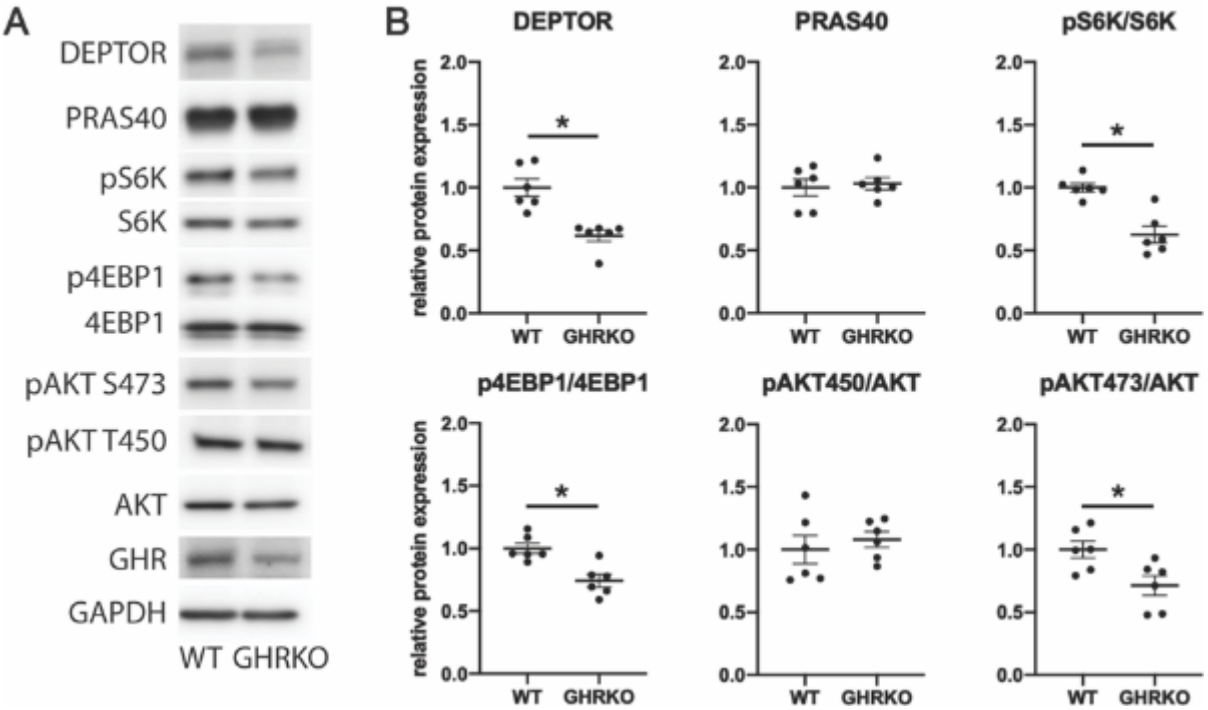

Figure 9 Protein levels of mTORC1 and mTORC2 substrates in GHRKO cells
(A) Representative immunoblots of the protein expression in wildtype and GHRKO fibroblasts. (B) Quantification of the protein levels in (A). N=6. (*) for t-test $p < 0.05$

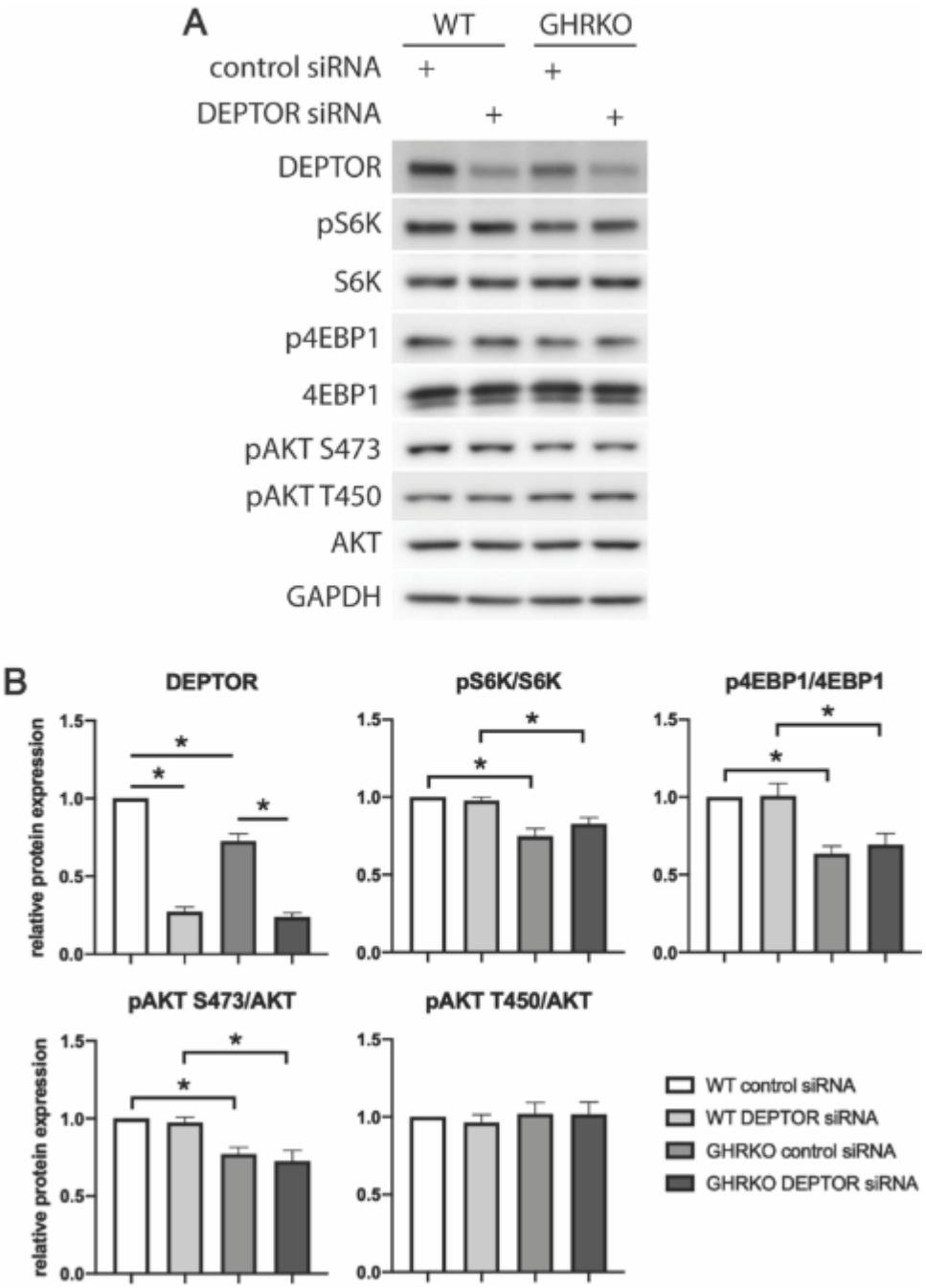

Figure 10 mTORC1 or mTORC2 substrates were not regulated by DEPTOR knockdown in GHRKO cells

(A) Representative images of the protein expression of mTORC1 and mTORC2 substrates in DEPTOR knockdown cells. (B) Quantification of the protein levels in (A). N=6. (*) for t-test $p < 0.05$

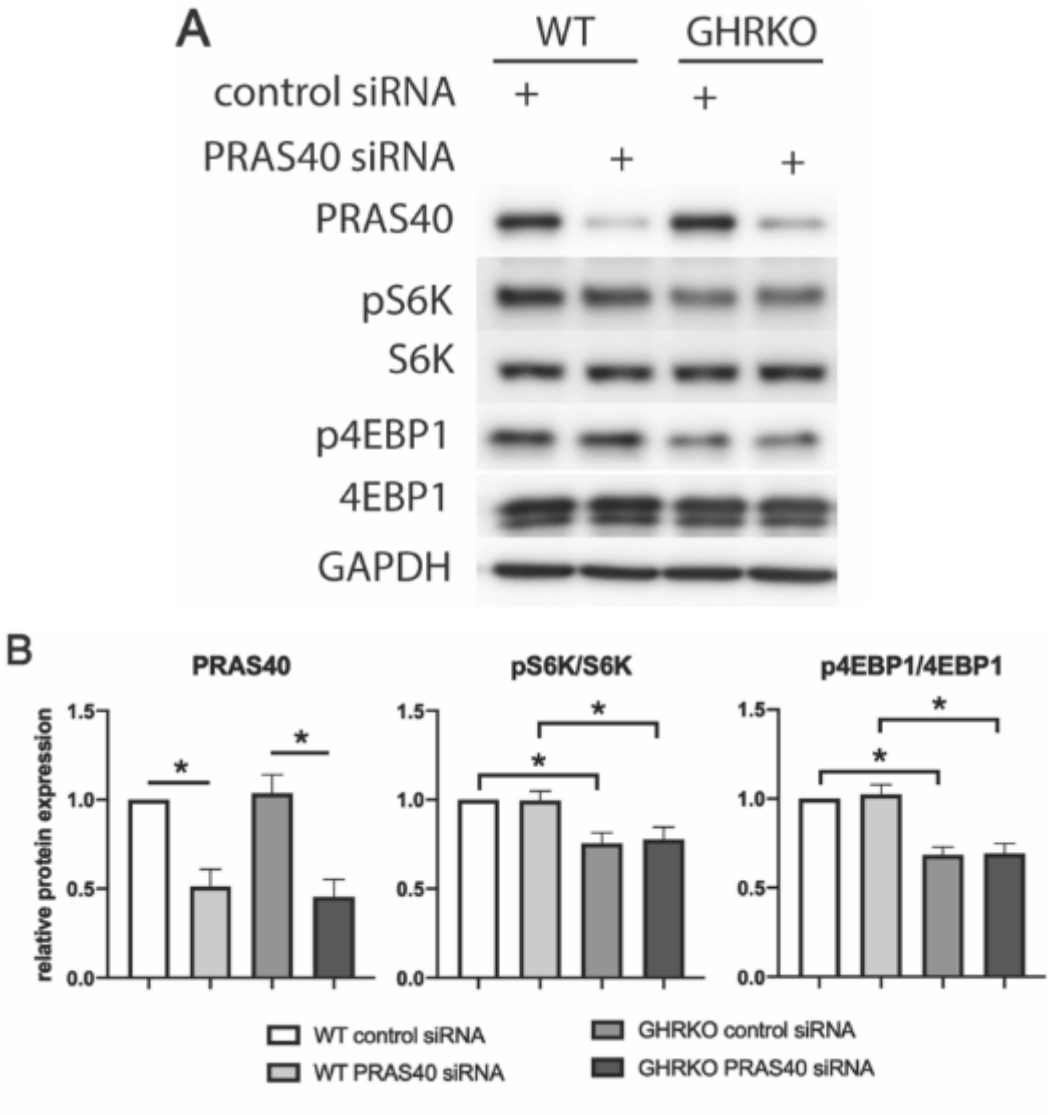

Figure 11 mTORC1 substrates were not regulated by PRAS40 knockdown in GHRKO cells
(A) Representative immunoblots of the mTORC1 target protein expression in PRAS40 knockdown cells. (B) Quantification of the protein levels in (A). N=6. (*) for t-test $p < 0.05$

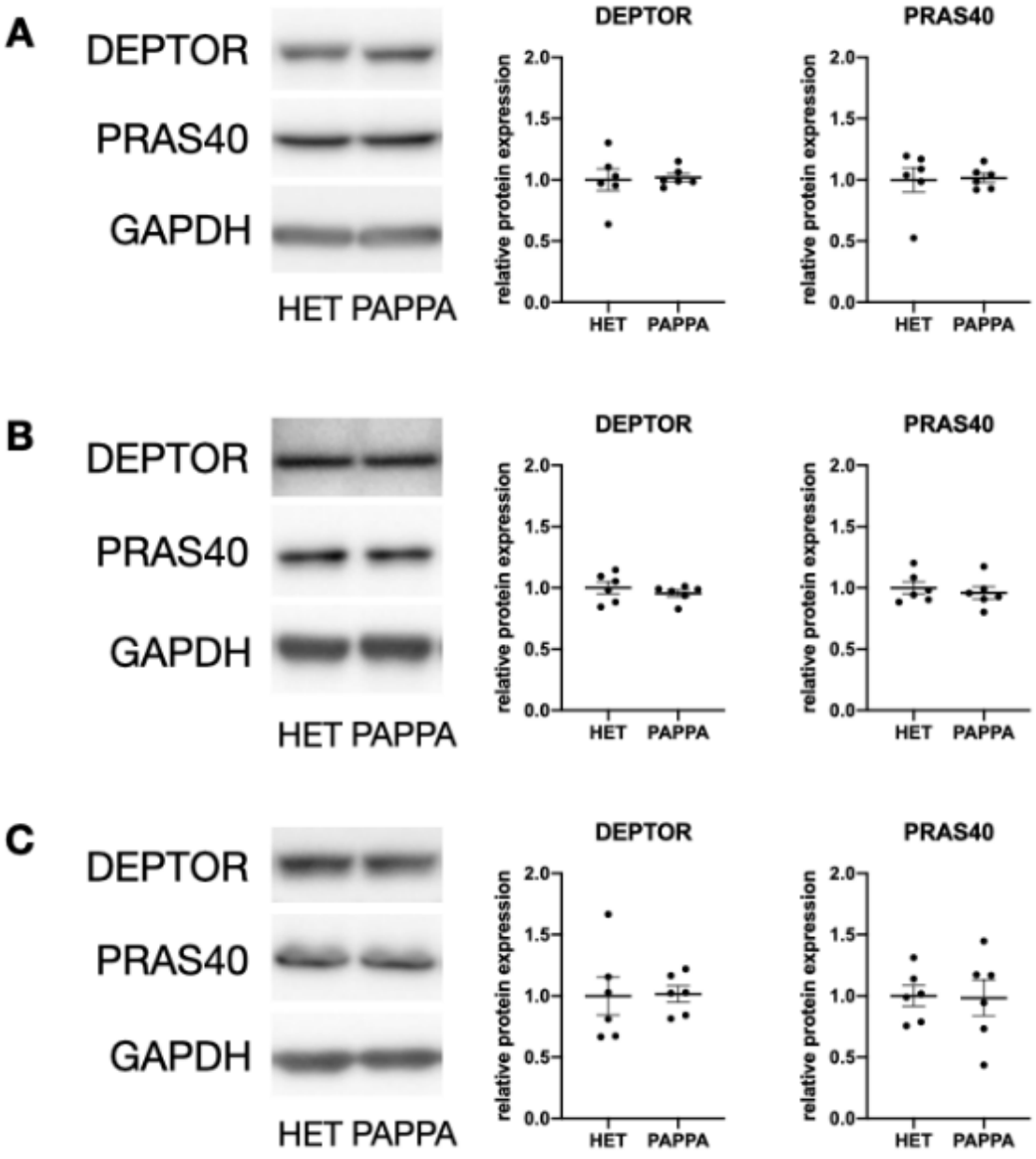

Figure 12 DEPTOR and PRAS40 protein expression in PAPPA-KO mice
Representative immunoblots and quantification of DEPTOR and PRAS40 protein levels in PAPPA-KO liver (A), kidney (B) and muscle (C). N=6 male mice. (*) for t-test $p < 0.05$

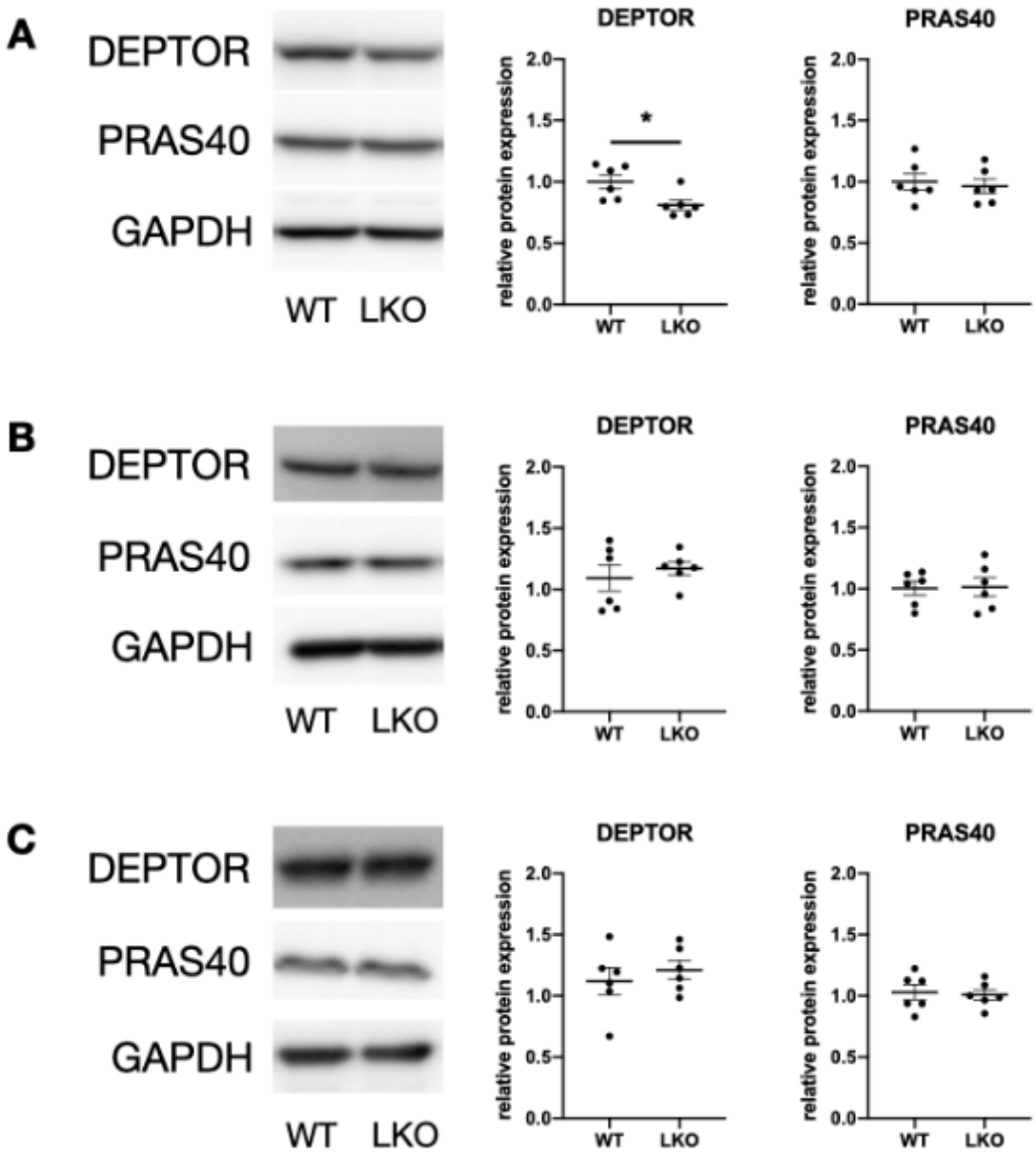

Figure 13 DEPTOR and PRAS40 protein expression in LKO mice
Representative immunoblots and quantification of DEPTOR and PRAS40 protein levels in LKO liver (A), kidney (B) and muscle (C). N=6 male mice. (*) for t-test $p < 0.05$

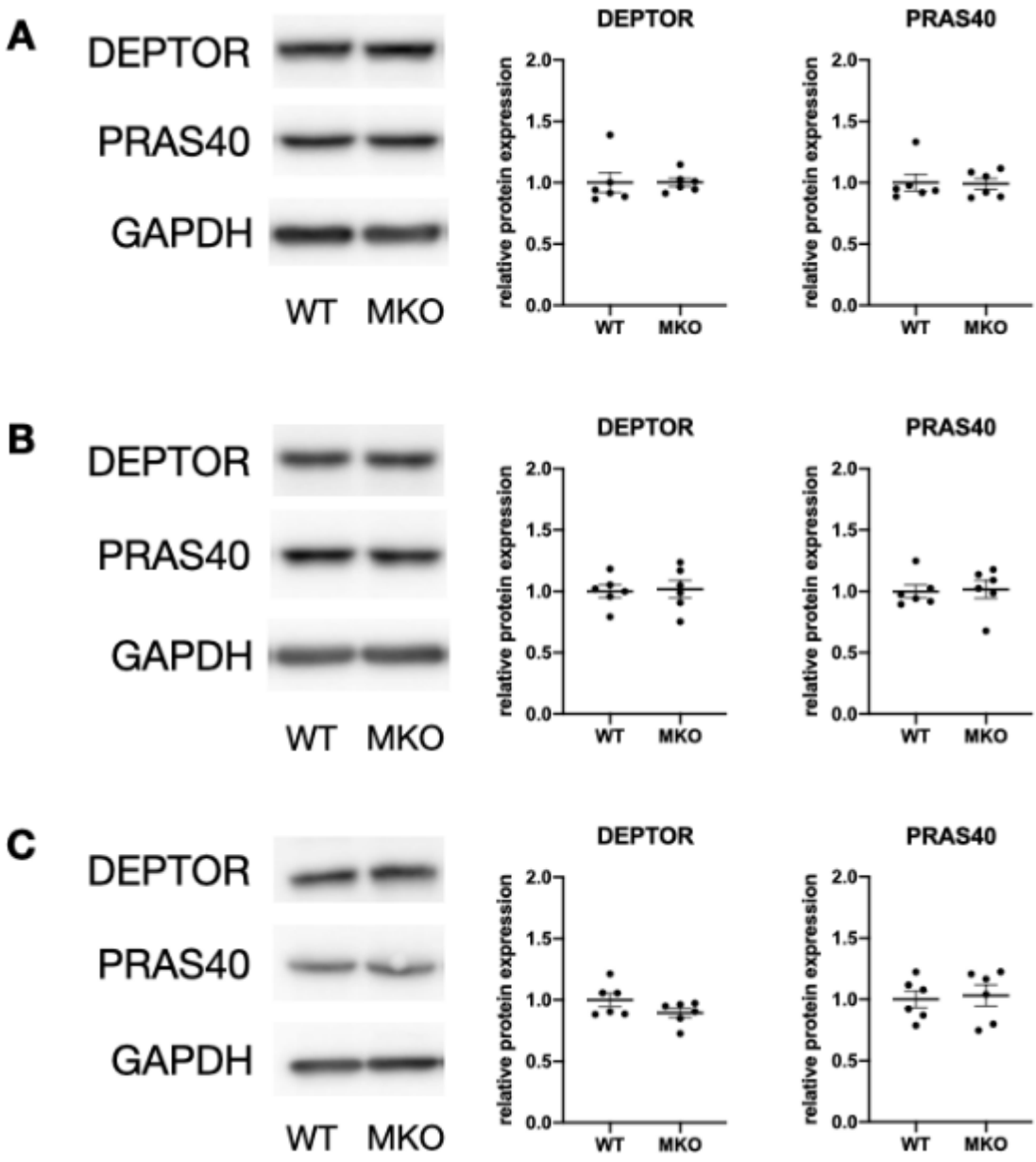

Figure 14 DEPTOR and PRAS40 protein expression in MKO mice
Representative immunoblots and quantification of DEPTOR and PRAS40 protein levels in MKO liver (A), kidney (B) and muscle (C). N=6 male mice. (*) for t-test $p < 0.05$

Mouse model	Tissue	DEPTOR	PRAS40
GHRKO	Liver	↓	↓
	Kidney	-	-
	Muscle	↑	-
Snell	Liver	↓	↓
	Kidney	-	-
	Muscle	-	↓
PAPPA-KO	Liver	-	-
	Kidney	-	-
	Muscle	-	-
LKO	Liver	↓	-
	Kidney	-	-
	Muscle	-	-
MKO	Liver	-	-
	Kidney	-	-
	Muscle	-	-

Table 1 Summary of DEPTOR and PRAS40 protein expression in five mouse models
DEPTOR and PRAS40 protein expression in liver, kidney and muscle of GHRKO, Snell dwarf, PAPPA-KO, LKO and MKO mice. ↑ increased, ↓ decreased, - unaltered

Name	Source	Catalog
GAPDH	Cell Signal	2118S
mTOR	Cell Signal	2972S
mLST8	Cell Signal	3274S
DEPTOR	Novus Bio	NBP1-49674
RAPTOR	Thermofisher	42-4000
PRAS40	Cell Signal	2691S
RICTOR	Abcam	ab70374
mSIN1	Abcam	ab64188
PROTOR1	Bethyl lab	A304-187A
TSC1	Cell Signal	6935S
TSC2	Cell Signal	4308S
pTSC2 S939	Cell Signal	3615S
pTSC2 T1462	Cell Signal	3617S
p4EBP1	Cell Signal	2855S
4EBP1	Cell Signal	9644S
pS6K	Cell Signal	9234S
S6K	Cell Signal	9202S
pAKT S473	Cell Signal	4060S
pAKT T450	Cell Signal	9267S
AKT	Cell Signal	9272S
GHR	R&D Systems	AF1360
rabbit IgG	Cell Signal	2729S
anti-rabbit	Abcam	ab205718
anti-goat	R&D Systems	HAF017

Table 2 List of antibodies

Chapter 3 TSC Regulates mTORC1 Activity in GHRKO and Snell Dwarf Mice

3.1 Abstract

Long-lived GHRKO and Snell dwarf mice have lower mTORC1 activity, but it remains unknown what specific factors cause this phenotype. Results from Chapter 2 showed that mTORC1 or mTORC2 components are not responsible for this phenomenon. Upstream regulators that transmit signals to control mTORC1 activity would be good candidates to study. TSC receives signals from insulin/IGF-1 signaling, ATP and oxygen level to inhibit mTORC1 activation. Here, higher protein expression of TSC1 and TSC2 were found in liver, kidney and muscle of GHRKO and Snell dwarf mice. A lower ratio of phosphorylated TSC2 to total TSC2, which indicates inhibited TSC2, was seen in these tissues as well, suggesting higher TSC activity. TSC2 knockdown through siRNA in GHRKO fibroblasts increased phosphorylation of mTORC1 targets, suggesting inhibited mTORC1 activity by TSC in these cells. Together, higher TSC level and activity may contribute to inhibited mTORC1 functions in GHRKO and Snell dwarf mice.

3.2 Introduction

The results on mTORC components in long-lived mutant mice suggested that the proteins of mTOR complexes do not explain the effects of these mutations on mTORC activity. For this reason we extended this study to evaluate the upstream signaling network. mTORC1 is regulated by amino acid levels, growth factors, energy and oxygen levels 144. Amino acid levels are

detected by their sensors and regulate the localization of mTORC1 to the lysosome. The other signals converge on an upstream master regulator of mTORC1, TSC. This complex is composed of TSC1, TSC2 and TBC1D7, among which TSC2 is the catalytic subunit. TSC1 stabilizes TSC2, whereas TBC1D7 promotes the interaction between TSC1 and TSC2 [121,122]. Growth factors, including insulin and IGF-1, signal through the PI3K-AKT pathway to regulate TSC2 phosphorylation. Specially, AKT phosphorylates TSC2 at Ser 939 and Thr 1462 to inhibit TSC activity and therefore elevate mTORC1 functions [100,101]. Both GHRKO and Snell dwarf mice have lower circulating IGF-1 levels [27,42], and AKT activity marker pAKT Ser 473 is lower in GHRKO and Snell dwarf mice, suggesting inhibited insulin/IGF-1-AKT pathway. As a result, TSC activity may be higher in these mice, which could inhibit mTORC1 activation. These observations make TSC an interesting target to study the upstream regulation of mTORC1.

For this purpose, the protein levels of two TSC components, TSC1 and TSC2, were tested in tissues of GHRKO and Snell dwarf mice. Phosphorylation of TSC2 was also tested to study the activity of the complex. To identify a causal link between TSC and mTORC1, TSC2 was knocked down in GHRKO fibroblasts, and mTORC1 targets were tested. In addition to GHRKO and Snell dwarf mice, three other mouse models, PAPPA, LKO and MKO mice, were also used to study the hormonal regulation of TSC.

3.3 Results

3.3.1 Increased TSC1 and TSC2 expression in GHRKO mice

To study whether the TSC complex regulates mTORC1 activity in the long-lived mice, the protein expression of TSC1 and TSC2 was measured in three tissues of GHRKO mice, including

liver, kidney and skeletal muscle. The results showed that both TSC1 and TSC2 protein levels were increased in all three tissues of GHRKO mice compared with wildtype mice (Figure 15). Upregulated TSC levels should lead to inhibition towards mTORC1 activity, which might contribute to the reduced mTORC1 function in these mice.

3.3.2 Increased TSC1 and TSC2 expression in Snell dwarf mice

To find out whether the above phenotype is also seen in another long-lived mouse model, Snell dwarf mice were used to test TSC1 and TSC2 protein expression in liver, kidney and muscle. Like the GHRKO mice, Snell dwarf mice also had elevated TSC1 and TSC2 protein levels in all three tissues (Figure 16), suggesting common TSC regulation in these two models. These data support the idea that TSC upregulation might explain the lower mTORC1 activity in GHRKO and Snell dwarf mice.

3.3.3 TSC2 phosphorylation is downregulated in GHRKO mice

TSC2 can be phosphorylated by AKT at Ser 939 and Thr 1462; phosphorylation at these sites inhibits TSC function, leading to mTORC1 activation [100,101]. It has been reported that non-fasting GHRKO and Snell dwarf mice had reduced pAKT Ser 473 levels, which is a marker for AKT activation [58]. Thus TSC2 phosphorylation might be decreased due to inactive AKT in GHRKO mice. To test this idea, immunoblotting was carried out using two antibodies targeting these two TSC2 phosphorylation sites, respectively. However, neither pTSC2 S939 nor pTSC2 T1462 showed different levels between wildtype and GHRKO liver, kidney and muscle (Figure 17). Since total TSC2 was increased in all tissues, the ratio of pTSC2 to total TSC2 was thus

decreased (Figure 17). Reduced TSC2 phosphorylation reflects less inhibition from AKT, resulting in elevated amount of unphosphorylated, active, inhibitory TSC2. This additional regulation of TSC2 activity is also in the direction expected to lead to lower mTORC1 signaling in these mice.

3.3.4 TSC2 knockdown upregulates mTORC1 substrates

These data showed both increased TSC levels and elevated TSC activity, but whether this leads to lower mTORC1 activity in Snell and GHRKO mice still needs to be tested. Therefore, the TSC level was manipulated in cultured GHRKO cells to see whether this would alter mTORC1 substrate phosphorylation. First, the protein levels of TSC1 and TSC2 were tested in wildtype and GHRKO fibroblasts. As shown in Figure 18A, both TSC1 and TSC2 were increased in these cells as they are in tissues. Next, TSC2 was knocked down using siRNA in wildtype and GHRKO fibroblasts. Interestingly, the level of TSC1 was also decreased by TSC2 siRNA, suggesting coordinate regulation of these two TSC components (Figure 18B). The phosphorylation status of mTORC1 substrates pS6K and p4EBP1 were tested, and both were upregulated under TSC2 knockdown in wildtype and GHRKO fibroblasts (Figure 18B). These results showed that TSC can indeed regulate mTORC1 activity in GHRKO cells and support the idea that the higher TSC activity in GHRKO mice might contribute to inhibition of mTORC1 function.

3.3.5 TSC1 and TSC2 expression in PAPPA-KO mice

Since both TSC1 and TSC2 were increased in GHRKO liver, kidney, muscle and skin-derived fibroblasts, it would be interesting to know which hormone is upregulating TSC expression across the tissues. To test this idea, PAPPA-KO mice with disrupted IGF-1 signaling were used. The data showed that TSC1 and TSC2 were not differentially expressed in liver, kidney and muscle of heterozygous or homozygous PAPPA knockout mice (Figure 19), suggesting that these two genes might not be regulated by IGF-1. These findings are consistent with the previous results that mTORC1 components were not altered in these mice, either, suggesting that both TSC and mTOR complexes in long-lived GHRKO and Snell dwarf mice are probably not directly regulated by IGF-1 levels.

3.3.6 TSC1 and TSC2 expression in liver-specific GHRKO mice

To find out whether TSC levels are regulated by growth hormone directly, liver-specific GHRKO mice serve as a good model for this purpose. If this hypothesis is true, higher TSC1 and TSC2 levels would be seen in the liver but in no other tissues of these mice. Indeed, LKO kidney or muscle showed similar levels of TSC1 and TSC2 as wildtype mice (Figures 20B and 20C). In contrast, liver of LKO mice had increased TSC1 protein expression but not TSC2 (Figure 20A). It seems that growth hormone receptor knockout only in the liver is sufficient to alter TSC1, but not TSC2. The implication is that these two components of the TSC complex may be under separate, or overlapping, controls in kidney.

3.3.7 TSC1 and TSC2 expression in muscle-specific GHRKO mice

To further explore questions of direct versus indirect GH action, TSC1 and TSC2 protein levels were also tested in muscle-specific GHRKO mice. No alteration was seen in liver or kidney (Figures 21A and 21B). Interestingly, TSC2 but not TSC1 was increased in MKO muscle compared with control mice (Figure 21C), suggesting that growth hormone receptor knockout limited to muscle is sufficient to upregulate TSC2 expression. In conjunction with the liver data, the implication is that these two proteins may be under separate control pathways in the two tissues. It is also possible that signals from one or more other tissues could modulate TSC1 or TSC2 in a tissue-specific fashion not yet apparent from our data.

In summary (Table 3), GHRKO and Snell dwarf mice had elevated TSC1 and TSC2 protein levels in liver, kidney and muscle. The phosphorylation status of TSC2, which indicates inhibitory TSC2 activity, was downregulated in GHRKO tissues. In addition, TSC2 knockdown in GHRKO fibroblasts upregulated mTORC1 substrate phosphorylation. These results showed that more active TSC signals could inhibit mTORC1 activity in GHRKO and Snell dwarf mice. Moreover, studies using mouse models with different hormone signaling suggest that TSC1 and TSC2 might be regulated by growth hormone but not IGF-1 in a tissue-coordinated manner. Further experiments with growth hormone receptor or IGF-1 receptor knockout in cells would answer this question more explicitly.

3.4 Discussion

The second hypothesis of the study was that the lower mTORC1 activity in GHRKO and Snell dwarf mice might be regulated by alternation in the TSC components. The protein expression of two TSC components, TSC1 and TSC2, was examined in GHRKO and Snell dwarf liver, kidney

and muscle. Both TSC1 and TSC2 protein levels were increased in all these tissues. Two TSC2 phosphorylation sites which are under AKT signaling, were unaltered in GHRKO tissues. Since the total levels of TSC2 were increased, the relative phosphorylation of TSC2 is then decreased, suggesting less TSC2 inhibition from AKT signaling. Moreover, TSC2 knockdown in GHRKO fibroblasts upregulated phosphorylation of mTORC1 substrates, reversing the effects of GHRKO mutation on mTORC1 activity. These results provide evidence to support the hypothesis that GHRKO and Snell dwarf mice have higher TSC activity which contributes to inhibited mTORC1 functions.

Further analysis of tissues from liver-specific GHRKO, muscle-specific GHRKO, and PAPPA-KO mice, showed mixed results. There was higher TSC1 but not TSC2 protein expression in liver of liver-specific GHRKO mice. Kidney and muscle showed no effects in these liver-specific KO mice. Higher TSC2 but not TSC1 was observed in muscle of muscle-specific GHRKO mice. Liver and kidney showed no effects. It seemed that TSC1 or TSC2 can be upregulated in tissues without growth hormone receptor, but not in the other tissues of these mice, where GHR remains intact. This phenomenon resembles an AND switch that signals from different tissues are required to turn on overall TSC expression. It is possible that some signal under growth hormone regulation is involved in this process to communicate across tissues to enable whole body TSC upregulation. Further analysis of PAPPA knockout mice found no alternation of TSC1 or TSC2 in liver, kidney or muscle. Since these mice have inhibited IGF-1 signaling, these results indicate that TSC1 and TSC2 regulation is probably not a direct effect of IGF-1. Taken together, results from these mouse models suggest that growth hormone but not IGF-1 might regulate TSC proteins in a tissue-coordinated way.

3.5 Materials and methods

Materials and methods for mice, cell culture, RNAi, immunoblot and statistics are the same as in chapter 2.

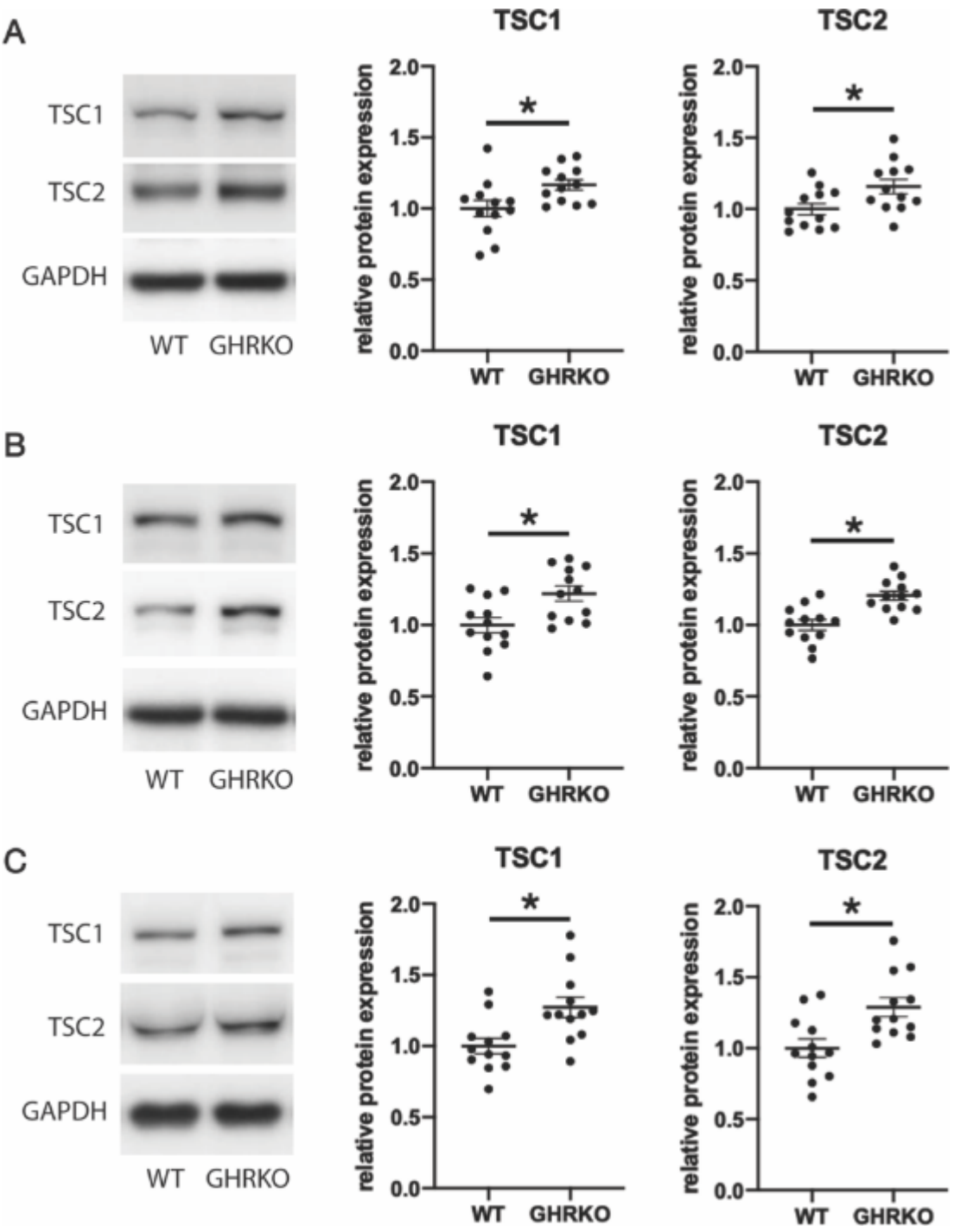

Figure 15 TSC1 and TSC2 are increased in GHRKO tissues
TSC1 and TSC2 protein expression in GHRKO liver (A), kidney (B), and muscle (C). N=6 male and N=6 female mice. (*) for t-test $p < 0.05$

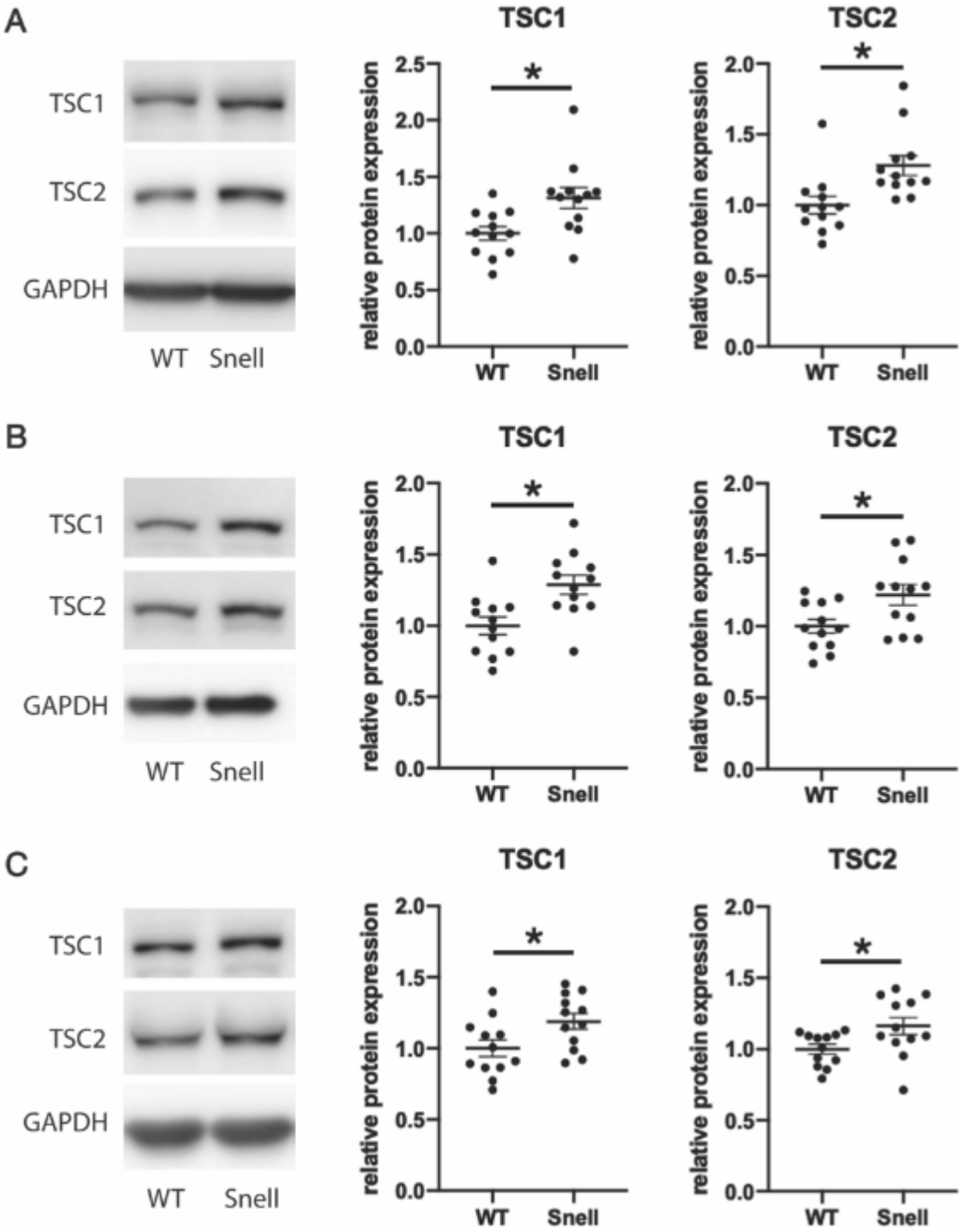

Figure 16 TSC1 and TSC2 are increased in Snell dwarf tissues
TSC1 and TSC2 protein expression in Snell dwarf liver (A), kidney (B), and muscle (C). N=6 male and N=6 female mice. (*) for t-test $p < 0.05$

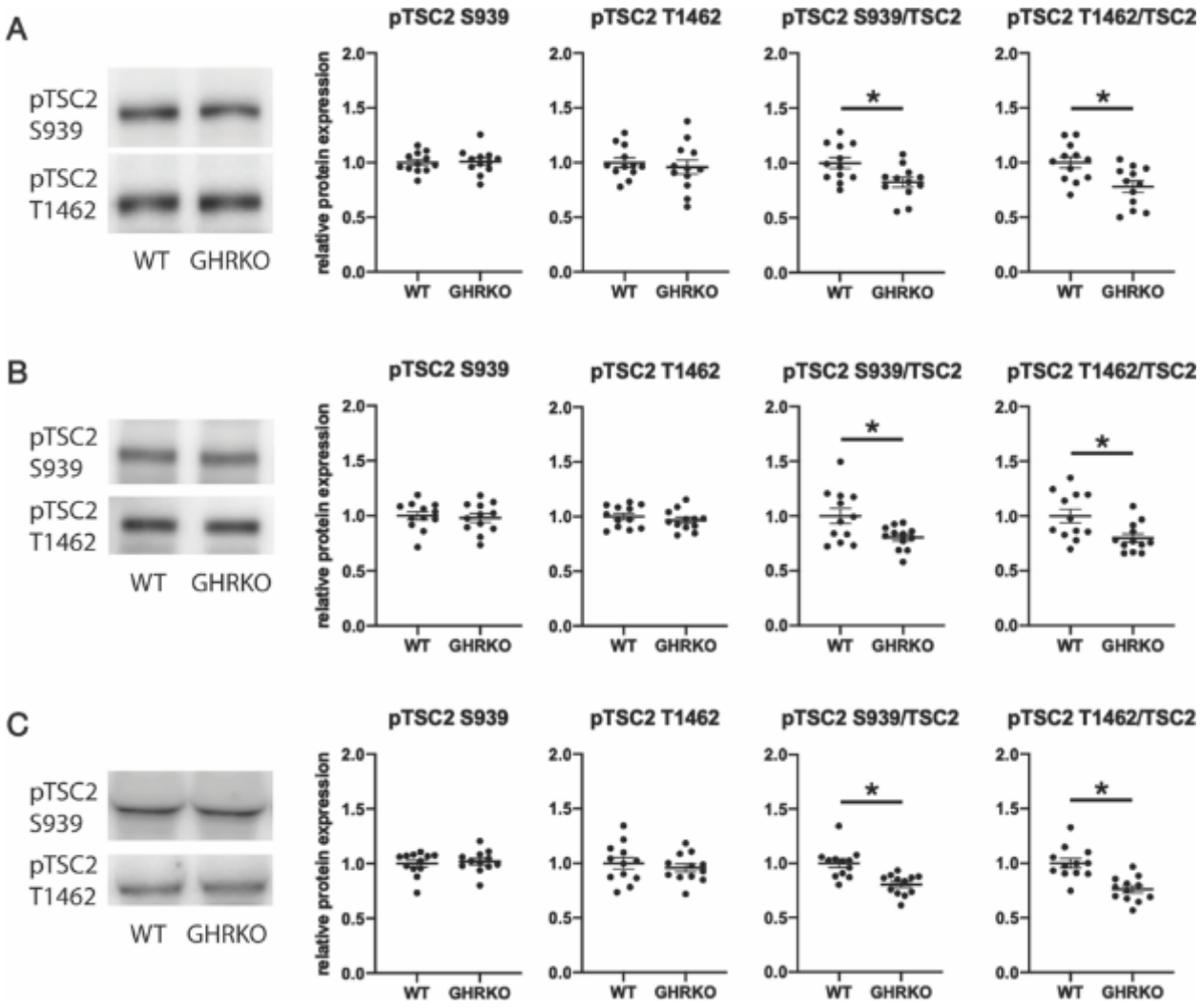

Figure 17 Relative phosphorylation of TSC2 is decreased in GHRKO tissues
The relative TSC2 phosphorylation at two sites, S939 and T1462, were decreased in GHRKO liver (A), kidney (B), and muscle (C). N=6 male and N=6 female mice. (*) for t-test $p < 0.05$

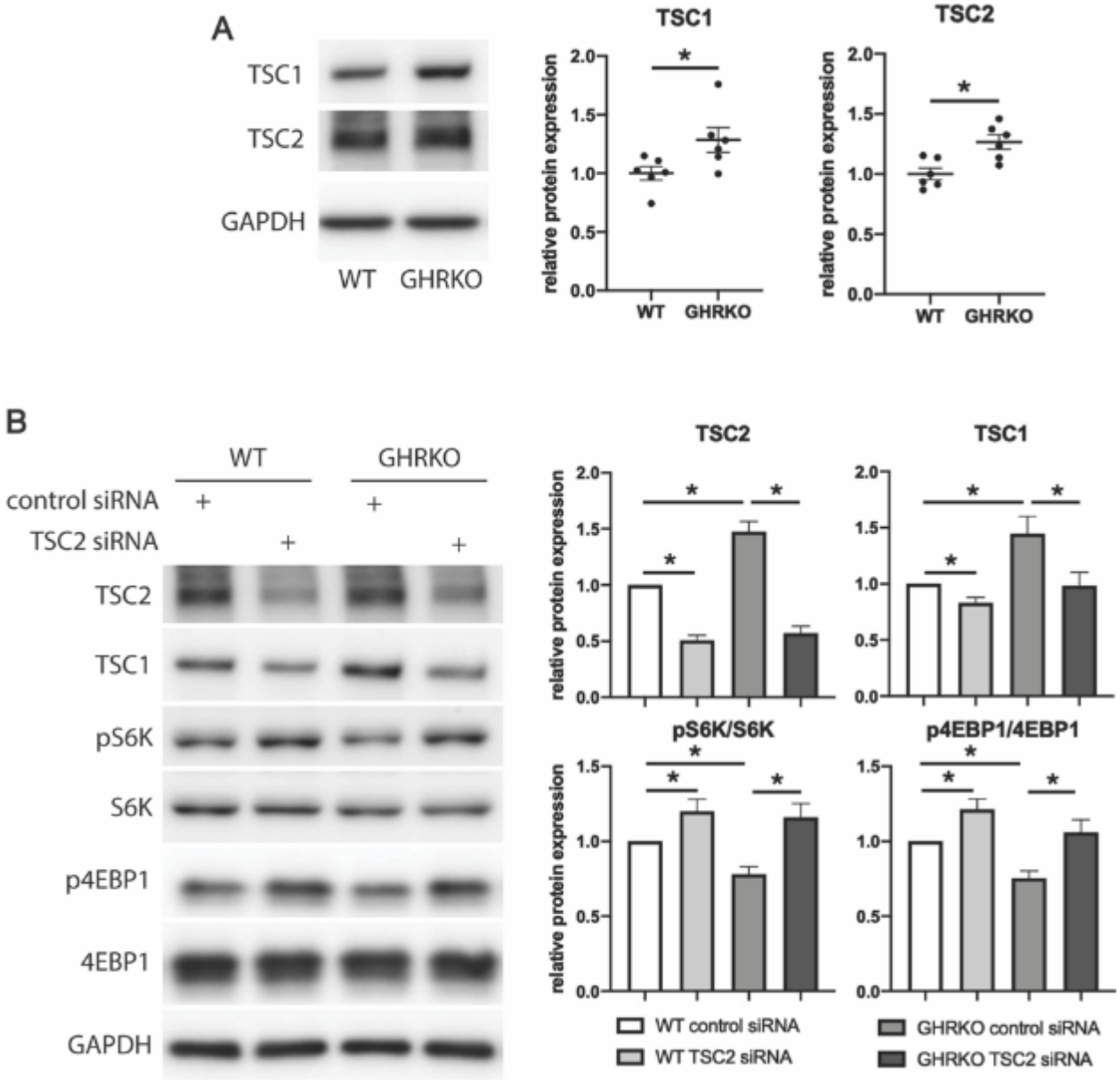

Figure 18 Upregulation of mTORC1 substrates by TSC2 knockdown in GHRKO cells
(A) TSC1 and TSC2 protein expression in GHRKO cells. (B) Protein expression for mTORC1 substrates in TSC2 knockdown cells. N=6. (*) for t-test $p < 0.05$

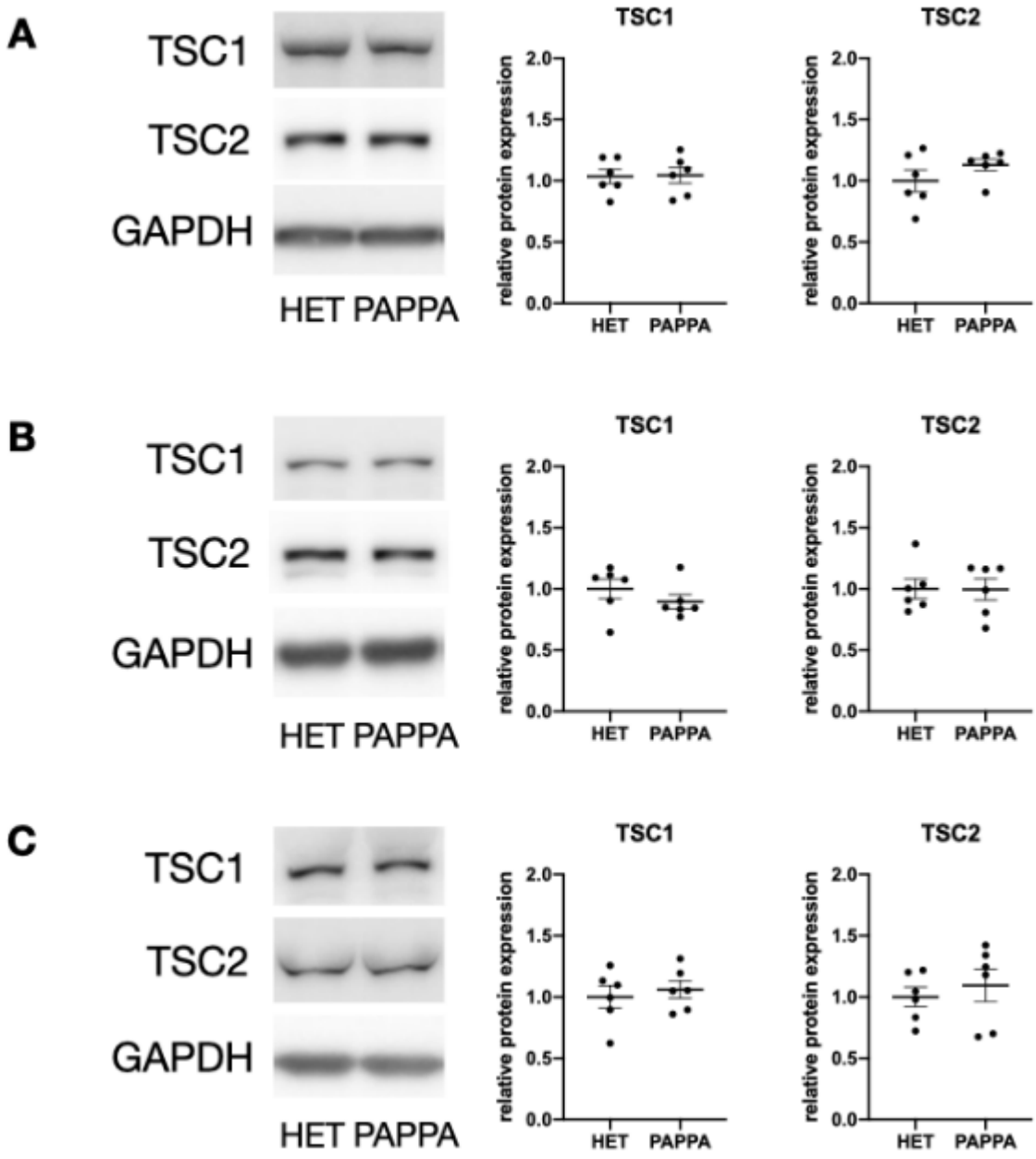

Figure 19 TSC1 and TSC2 protein expression in PAPPA-KO mice
Representative immunoblots and quantification of TSC1 and TSC2 protein levels in PAPPA-KO liver (A), kidney (B) and muscle (C). N=6 male mice. (*) for t-test $p < 0.05$

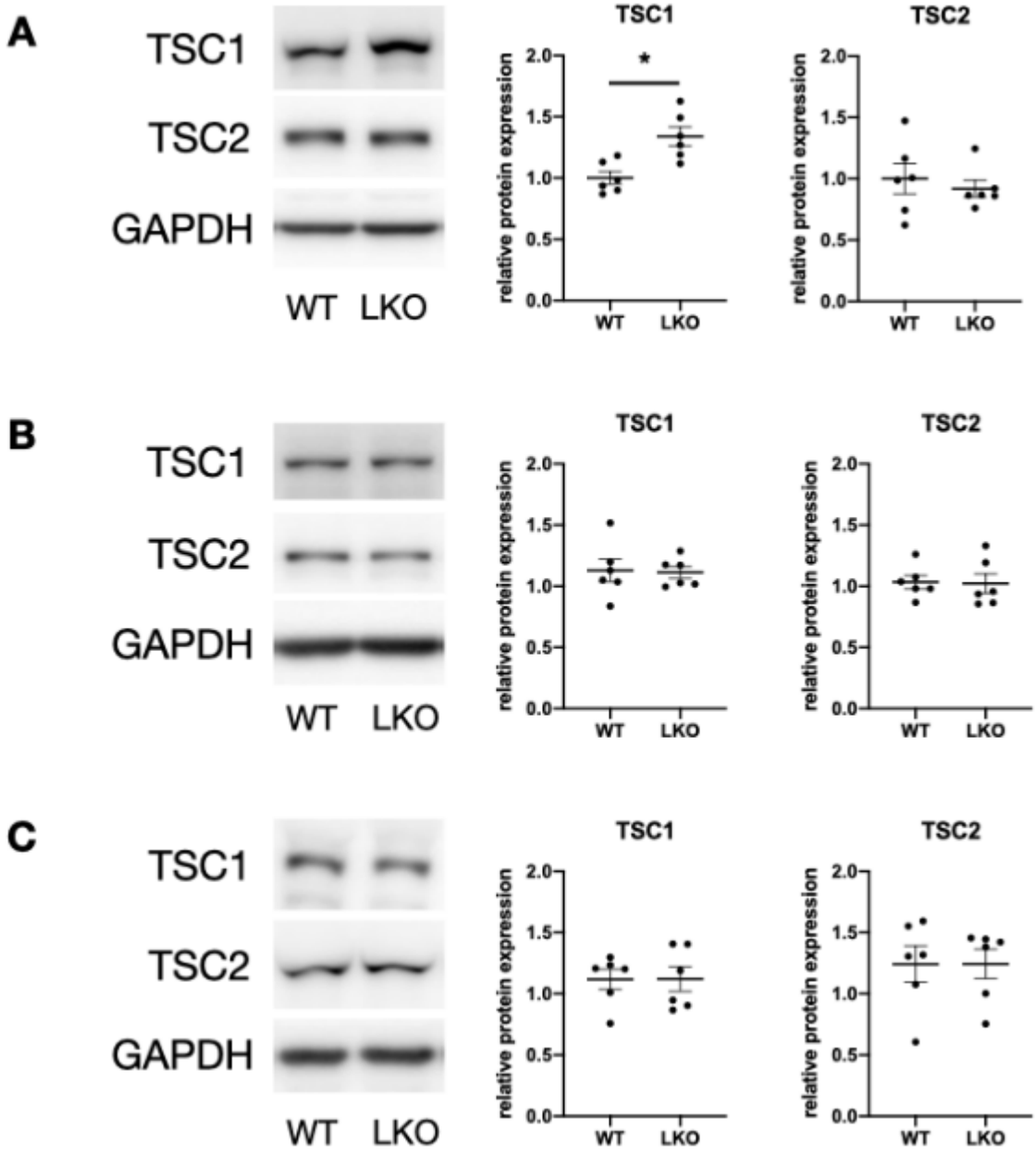

Figure 20 TSC1 and TSC2 protein expression in LKO mice
Representative immunoblots and quantification of TSC1 and TSC2 protein levels in LKO liver (A), kidney (B) and muscle (C). N=6 male mice. (*) for t-test $p < 0.05$

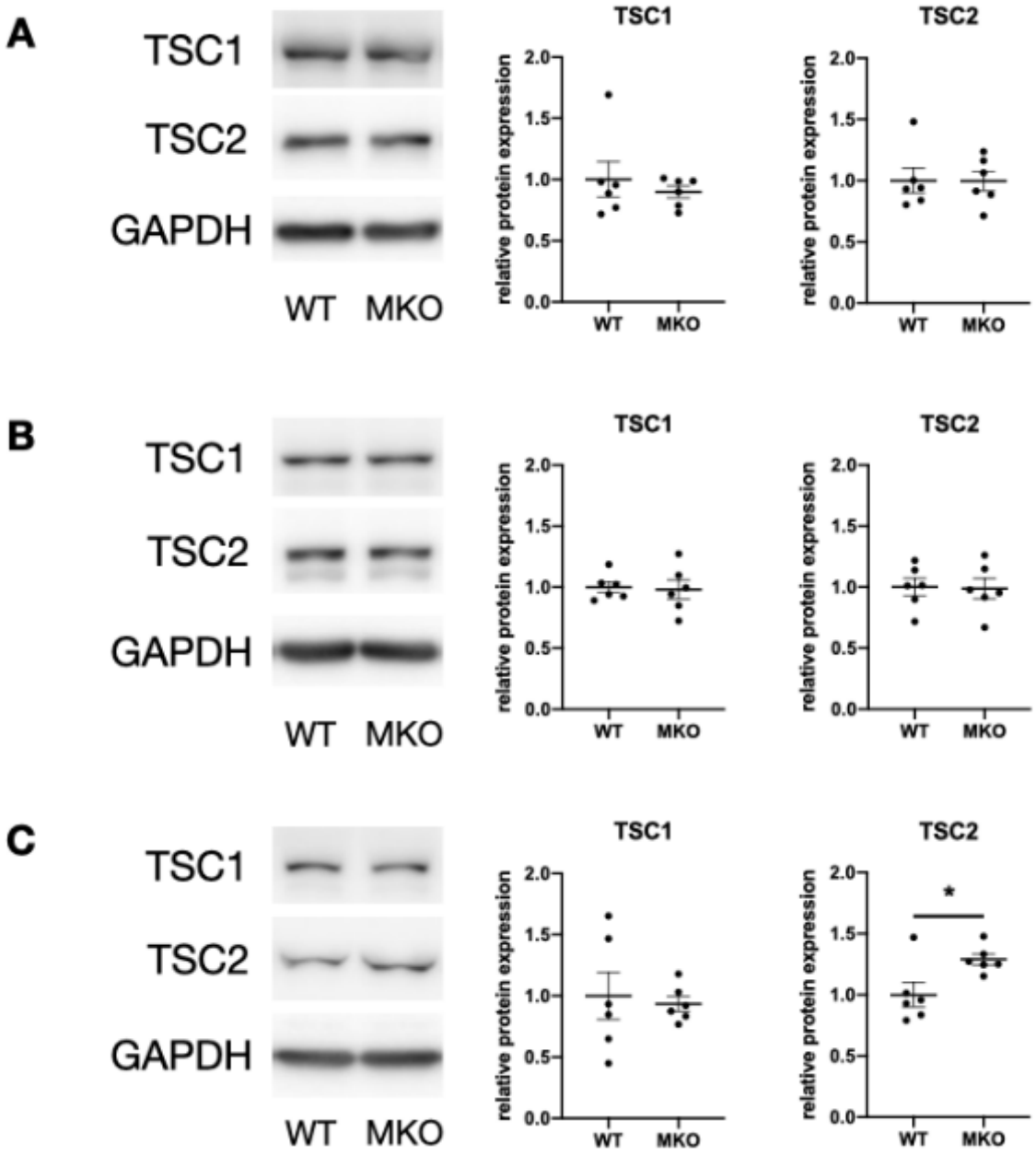

Figure 21 TSC1 and TSC2 protein expression in MKO mice
Representative immunoblots and quantification of TSC1 and TSC2 protein levels in MKO liver (A), kidney (B) and muscle (C). N=6 male mice. (*) for t-test $p < 0.05$

Mouse model	Tissue	TSC1	TSC2
GHRKO	Liver	↑	↑
	Kidney	↑	↑
	Muscle	↑	↑
Snell	Liver	↑	↑
	Kidney	↑	↑
	Muscle	↑	↑
PAPPA-KO	Liver	-	-
	Kidney	-	-
	Muscle	-	-
LKO	Liver	↑	-
	Kidney	-	-
	Muscle	-	-
MKO	Liver	-	-
	Kidney	-	-
	Muscle	-	↑

Table 3 Summary of TSC1 and TSC2 protein expression in five mouse models
TSC1 and TSC2 protein expression in liver, kidney and muscle of GHRKO, Snell dwarf, PAPPA-KO, LKO and MKO mice. ↑ increased, ↓ decreased, - unaltered

Chapter 4 Concluding Remarks and Future Directions

4.1 Summary of the study

It is well known that mTOR plays a crucial role in aging. Studies have shown that diminished mTOR function can extend lifespan in multiple species [138-142]. There are also studies characterizing mTOR regulation in certain longevity or disease models. For example, lower mTORC1 activity was observed in long-lived GHRKO and Snell dwarf mice, but the underlying mechanism by which mTORC1 function is diminished is unclear [58]. This study tried to answer this question by testing the mTORC components and the upstream TSC regulator in these mouse models. The findings shown here suggest that TSC levels and activity, but not the levels of mTORC components, contribute to inhibited mTORC1 functions in GHRKO and Snell dwarf mice (Figure 22). However, there are still limitations of this study and future work will be necessary to further elucidate the mechanisms. For instance, direct manipulation of TSC in vivo instead of in cells would provide more convincing evidence about the importance of TSC in mTOR regulation in the long-lived mice. Other aspects of mTOR complexes, such as phosphorylation and localization, would be helpful to determine whether mTOR is regulated by multiple signaling pathways or mainly by TSC. Overall, these results emphasize the critical roles of TSC in mTOR regulation as well as the importance of mTOR in aging. The future directions mentioned in this chapter would help to understand more about the long-lived mouse models and may aid in developing more approaches to extend mammalian lifespan.

4.2 Mouse models and tissues in this study

There are five mouse models used in this study. GHRKO and Snell dwarf mice are two similar dwarf mouse models, and using both in this study help limit the strain-specific phenotypes and generalize the conclusions. Snell dwarf mice have defective pituitary structure, and produce lower levels of three hormones, GH, prolactin and TSH [27], whereas GHRKO mice only have defective growth hormone signaling. The results presented here are similar between these two models, indicating that GH but not the two other hormones affected in the Snell mice, is probably the factor leading to mTOR protein changes. Both GHRKO and Snell dwarf have significantly lower circulating IGF-1 resulted from defective GH signaling. This raises the question whether GH or IGF-1 signaling causes the alteration in TSC and mTOR proteins. To answer this question, three other mouse models were used including PAPPA-KO, LKO and MKO mice. PAPPA-KO have inhibited IGF-1 signaling [48] ,and if IGF-1 is the critical factor in mTOR regulation, the changes seen in GHRKO and Snell dwarf mice would also be observed in PAPPA-KO mice. However, none of those changes are present in PAPPA-KO mice, suggesting that mTOR is probably not directly regulated by IGF-1 signaling, although regulation of IGF-1 action in local tissues may depend on a complex balance of PAPPA function, IGF-1 and IGF-1R levels, and levels of the various IGFBPs, some of which are not subject to cleavage by PAPPA. Next, to test whether GH actions represent direct or indirect effects, two tissue-specific GHRKO mouse models were used. LKO mice have GHR deletion only in the liver while MKO mice have GHR deletion only in the muscle. If GH signals are entirely tissue-specific, alteration of TSC and mTOR proteins would be evident in liver but not in other tissues of LKO mice, and in muscle but not in other tissues of MKO mice. DEPTOR reduction was observed in LKO liver, consistent with direct effects of GH on liver, but, more surprisingly, was not seen in muscle of MKO,

suggesting that effects on muscle of GHRKO mice may reflect indirect effects of GH action on some unknown other tissue. Moreover, TSC1 was only increased in LKO liver but not MKO muscle, and TSC2 was increased in MKO muscle but not LKO liver. This phenomenon suggests some fascinating tissue-specific regulation of these two proteins as well as potential tissue-tissue communication mechanism. In whole body GHR deletion mice, some signal downstream of GH may be secreted from liver and travel to muscle, and at the same time, another signal could be secreted from muscle and travel to liver, which enables upregulation of both TSC1 and TSC2 in liver and muscle. This would not happen in tissue-specific GHR deletion mice. For example, in LKO mice, a signal in liver would increase TSC1 expression, but there is no signal coming from muscle to increase TSC2 expression in liver. Additional mouse models with GHR knockout in other tissues may be helpful to further test this hypothesis.

Three tissues were studied in all mouse models, i.e. liver, kidney and skeletal muscle. Comparisons of results from these commonly used tissues can avoid drawing premature conclusions about tissue-specific effects. In addition, previous work showed lower mTORC1 activity and partially higher mTORC2 activity in liver, kidney, muscle and heart of GHRKO and Snell dwarf mice [58]. Examining the same tissues is necessary to study mTOR regulation that leads to alteration in mTORC activity previously reported, and these considerations lay behind the strategy used here for exploring tissue-specific direct and indirect effects.

4.3 Feedback mechanisms of mTOR regulation

Regulation of mTOR complexes features sophisticated feedback mechanisms. For example the mTORC2 substrate AKT can phosphorylate and suppress TSC2 function to relieve inhibition on

mTORC1 activity [100,101]. S6K downstream of mTORC1 can phosphorylate mTORC2 component RICTOR to inhibit AKT activation [134]. In addition, both mTORC1 and S6K can directly phosphorylate and inhibit IRS-1, providing negative feedback for prolonged PI3K-AKT signaling [135–137]. As a component of both mTORC1 and mTORC2, DEPTOR inhibits the kinase activity of mTOR, acting as an internal negative regulator. Intriguingly, DEPTOR itself can be inhibited by mTORC1 and mTORC2 at both the mRNA and protein levels, and DEPTOR can also be phosphorylated in an mTORC1-dependent manner [62]. This phenomenon also exhibits feedback signaling but the underlying mechanisms are still unclear. The reduced expression of DEPTOR in GHRKO and Snell liver would not lead to observed decline in mTORC1 in these mice, and are thus in the opposite direction for mTORC1 regulation, but could reflect a feedback circuit of uncertain composition. Phosphatidic acid, a type of phospholipid generated in response to mitogenic treatment such as serum, can disrupt the interaction between DEPTOR and mTORC1 to activate mTORC1 under mitogenic signals such as serum and insulin [158]. Whether phosphatidic acid is involved in the regulation of DEPTOR in GHRKO and Snell dwarf mice would be interesting to study. Deeper investigation about the DEPTOR regulation, such as transcription, translation and phosphorylation, would help to resolve issues about the basis for lower DEPTOR and provide hints as to the functional effects of this decline.

Similarly, PRAS40 also functions as a negative component of mTORC1. The decreased PRAS40 expression in GHRKO and Snell liver might be under the same feedback signaling as DEPTOR. PRAS40 can be phosphorylated by AKT and mTORC1, releasing the inhibitory effects on mTORC1 [159,160]. Whether these phosphorylation events happen in GHRKO and Snell dwarf mice and whether they affect the level or function of PRAS40 would be worth investigating.

4.4 Phosphorylation of mTORC components

Proteins of mTOR complexes are widely phosphorylated for additional regulation of the complexes. The core mTOR kinase can be phosphorylated at Ser 2448 by S6K in response to serum stimulation [161]. mTOR within mTORC2 autophosphorylates at Ser 2481, indicating intact complex assembly [162]. In response to low ATP level, the mTORC1 scaffold protein RAPTOR is phosphorylated by AMPK at Ser 722 and Ser 792 to inhibit mTORC1 function [163]. The mTORC2 scaffold protein RICTOR is phosphorylated by the mTORC1 substrate S6K at Thr 1135 to inhibit mTORC2 substrate phosphorylation of AKT at Ser 473 [134]. Moreover, mSIN1 can be phosphorylated by AKT at Thr 86 to further activate mTORC2, leading to phosphorylation of AKT at Ser 473 [70]. The DEPTOR protein contains multiple phosphorylation sites [164], among which Ser 293, Thr 295 and Ser 299 are dependent on mTORC1 and mTORC2 [165,166], and phosphorylation of Ser 286, 287 and 291 is probably mediated by RSK1 and S6K [167]. This series of phosphorylation events facilitates the ubiquitylation and subsequent proteasome degradation of DEPTOR to release mTORC1 inhibition [164]. Both mTORC1 and AKT can phosphorylate PRAS40, leading to binding to protein 14-3-3 and releasing inhibition on mTORC1 [159,160]. Phosphorylation of these mTORC members display multiple aspects of mTORC1 and mTORC2 regulation. It would be informative to study whether these phosphorylation sites are present in GHRKO and Snell dwarf mice and how they might influence mTOR activity and substrate specificity.

4.5 TSC overexpression extends lifespan

The observation of increased TSC1 and TSC2 levels in long-lived mice is in line with previous reports about the beneficial effects of TSC overexpression in longevity. Overexpression of the *Drosophila* orthologs of TSC1 and TSC2, dTSC1 or dTSC2, in fruit flies led to lifespan extension [141]. TSC1 overexpression in mice improved their exercise performance and heart function, and extended the lifespan of female but not male mice [156]. It would be interesting to know whether overexpression of TSC2 has similar effects of increased health span and lifespan in mice. TSC1 and TSC2 proteins are also known as hamartin and tuberin, respectively, and function as tumor suppressers. Mutations in either TSC1 or TSC2 cause the disease Tuberous Sclerosis Complex featuring benign tumors that can occur across the body [168]. TSC2 mutations are more common than mutations of TSC1 in patients with this disorder. These loss-of-function mutations cause defective inhibition of Rheb, leading to hyperactive mTORC1 signaling. It is conceivable that TSC1 or TSC2 overexpression would inhibit tumor growth and cancer progression, which may partially account for extended health span and lifespan in Snell and GHRKO mice.

4.6 TSC phosphorylation

In addition to the two phosphorylation sites of TSC2 tested here, there are several other residues on TSC2 and TSC1 phosphorylated to transit upstream signals to mTORC1 [169]. For example, AMPK phosphorylates TSC2 at Thr 1227 and Ser 1345 to enhance TSC2 activity and inhibit mTORC1 functions in response to low ATP levels [129]. TSC2 is phosphorylated by ERK at Ser 664, and by RSK at Ser 1798, downstream of epidermal growth factor activation [126,127]. TSC1 is phosphorylated by cyclin-dependent kinase 1(CDK1) at three sites, Thr 417, Ser 584 and Thr

1047 during G2/M phase of the cell cycle, which somehow alters the activity of the complex and inhibits downstream S6K phosphorylation [170]. In cancer cells, inflammation induced TSC1 phosphorylation by IKKβ at Ser 487 and Ser 511 and resulted in inhibited TSC1 function, activated mTORC1 and subsequent tumor progression [171]. Whether the phosphorylation of TSC1 and TSC2 at these sites is altered and functioning in GHRKO and Snell dwarf mice would be worth following up.

4.7 TBC1D7 and Rheb

As a third subunit of the complex, TBC1D7 promotes the interaction between TSC1 and TSC2 [122]. However, the role of TBC1D7 in aging has not been well-established. Whether TBC1D7 is upregulated in GHRKO and Snell dwarf mice would be interesting to find out. Similarly, it would be useful to learn whether TBC1D7 overexpression also extends mouse lifespan. Another critical player in the TSC signaling pathway is Rheb, which functions as a direct activator of mTORC1. Studying the level and activity of Rheb in long-lived mice would add extra evidence to the current findings. The small molecule NR1 has been identified to be a Rheb inhibitor and selectively suppress mTORC1 activity [172]. This drug would be an interesting candidate to study in longevity experiments.

4.8 Regulation of mTORC1 localization

Apart from TSC signaling, mTORC1 is also regulated by the localization to the lysosomal membrane, which depends on amino acid levels in the cytoplasm and inside the lysosome. The results of whole liver lysates of GHRKO and Snell dwarf mice (Figures 3 and 5) did not find

difference in the levels of mTOR, RAPTOR and mLST8, suggesting similar numbers of mTOR complex 1 compared with controls. However, it remains unknown whether the amount of mTORC1 at the lysosome surface is also similar between long-lived and control mice. Therefore, analyzing mTORC components in purified lysosomes would be helpful to answer this question. Furthermore, other proteins in this pathway may also be involved in mTORC1 regulation, including the amino acid sensors, the Rag proteins that recruit mTORC1, and the anchor protein Regulator. A thorough examination of these players would give a more comprehensive understanding of mTORC1 regulation in long-lived mice.

4.9 mTORC2 substrate specificity

A previous study found that mTORC2 substrates are differentially regulated in GHRKO and Snell dwarf mice, with pAKT Ser 473 decreased, pAKT Thr 450 and pSGK unaltered in fed animals [58]. This interesting phenomenon suggests selective activity towards different mTORC2 substrates and may reflect differences in the kinases involved. The core kinase mTOR is responsible for the phosphorylation of AKT Thr 450 and SGK, whereas mSIN1, a subunit of mTORC2, also functions as a kinase to phosphorylate AKT at Ser 473 [49]. The kinase activity of mTOR and mSIN1 could be different and result in the substrate specificity observed in the long-lived mice. Moreover, substrates are recruited to mTORC2 by RICTOR, and the binding between mTORC2 and these targets may not be at the same intensity in control and mutant mice, leading to various effectiveness of recruitment and subsequent phosphorylation.

4.10 Significance

It is unknown why GHRKO and Snell dwarf mice are long-lived, and exploration of the many possible mechanistic linkages will require a diversity of approaches. Even if only one signaling pathway is critical for lifespan extension, it usually involves multiple upstream and downstream elements in the pathway. The complexity of mouse in vivo mechanisms slows down efforts to search for a fully comprehensive model to explain extended longevity. Therefore, this study was set with a realistic goal which is to find out why GHRKO and Snell dwarf mice have lower mTORC1 activity. Solving this problem is a small step towards understanding the slow-aging mechanisms of these mice. Given the fact that deficient mTORC1 signaling extends lifespan in different species including mice, the regulation of mTOR complexes is a highly relevant candidate mechanism for extended lifespan of GHRKO and Snell dwarf mice.

For this project, in vitro experiments were carried out to see whether TSC inhibits mTORC1 activity in GHRKO fibroblasts. Extending this to in vivo experiment would be more convincing about the negative regulatory role of TSC on mTORC1. This can be done by developing drugs to inhibit TSC2 activity or to disrupt the interaction between TSC components. This can also be done using a genetic approach to knock down TSC expression or introducing TSC mutations. Homozygous KO of TSC1 or TSC2 produces non-viable mice. Heterozygous TSC1 or TSC2 mice have tumors, and are used to study the human tuberous sclerosis diseases [173,174]. Thus the most informative manipulations should be tightly controlled to decrease TSC activity in treated GHRKO or Snell dwarf mice to similar levels as in wildtype mice without causing extra toxicity. Then phosphorylation of mTORC1 substrates in tissues can be measured to see whether TSC inhibition in GHRKO or Snell dwarf mice upregulates mTORC1 activity to the level comparable

with WT mice, which would suggest that higher TSC activity indeed inhibit mTORC1 activity in the long-lived mutant mice. Next, lifespan of these mice can be recorded to see whether the treatment would abolish the lifespan extension of these mice. If the extended lifespan is reversed to that of control mice, then this shows that higher TSC activity and lower mTORC1 activity lead to lifespan extension of GHRKO and Snell dwarf mice.

If these research projects provide promising results in mice, applications can be developed for human use in the future. Pharmaceutical approach is safer and easier to deliver than genetic approach for this purpose. Drugs can be developed as oral supplements for human, such as mTORC1 inhibitors that have better bioavailability than rapamycin, and TSC activitors that, unlike some mTOR inhibitors, would specially inhibit mTORC1 but not mTORC2 to avoid mTORC2 related side effects. It is imaginable that one day, such drugs would delay age-associated diseases and bring ten more years of lifespan to people taking them.

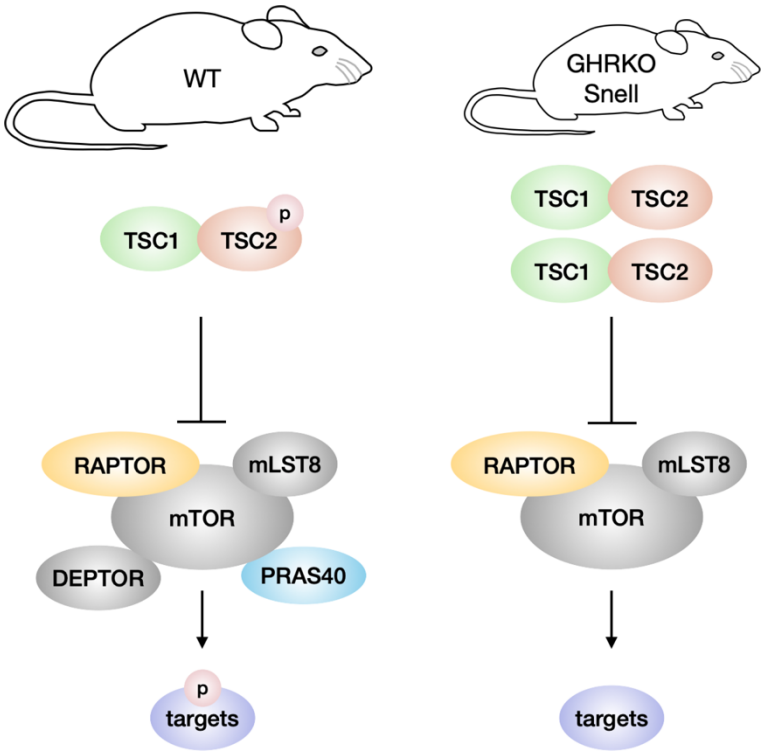

Figure 22 TSC and mTORC1 signaling in GHRKO/Snell dwarf mice
In comparison with wildtype mice, GHRKO and Snell dwarf mice have higher TSC activity, shown by increased TSC1 and TSC2 protein levels, and lower TSC2 phosphorylation. Elevated TSC signaling then inhibits the mTORC1 activity of phosphorylating downstream targets. GHRKO and Snell dwarf mice also showed lower DEPTOR and PRAS40 levels but these changes are not responsible for the lower mTORC1 activity observed in these mice.

References

1. Jin, K. Modern Biological Theories of Aging. *Aging and disease* **1**, 72–74 (2010).

2. Campisi, J. *et al.* From discoveries in ageing research to therapeutics for healthy ageing. *Nature* **571**, 183–192 (2019).

3. Friedman, D. B. & Johnson, T. E. A mutation in the age-1 gene in Caenorhabditis elegans lengthens life and reduces hermaphrodite fertility. *Genetics* **118**, 75–86 (1988).

4. Kenyon, C., Chang, J., Gensch, E., Rudner, A. & Tabtiang, R. A C. elegans mutant that lives twice as long as wild type. *Nature* **366**, 461–464 (1993).

5. Fu, Z., Gilbert, E. R. & Liu, D. Regulation of insulin synthesis and secretion and pancreatic Beta-cell dysfunction in diabetes. *Current diabetes reviews* **9**, 25–53 (2013).

6. de Meyts, P. *The Insulin Receptor and Its Signal Transduction Network*. (2000).

7. Watanabe, M., Hayasaki, H., Tamayama, T. & Shimada, M. Histologic distribution of insulin and glucagon receptors. *Brazilian Journal of Medical and Biological Research* **31**, 243–256 (1998).

8. Laron, Z. Insulin-like growth factor 1 (IGF-1): a growth hormone. *Molecular pathology : MP* **54**, 311–6 (2001).

9. Arcaro, A. Targeting the insulin-like growth factor-1 receptor in human cancer. *Frontiers in Pharmacology* **4**, (2013).

10. Belfiore, A., Frasca, F., Pandini, G., Sciacca, L. & Vigneri, R. Insulin Receptor Isoforms and Insulin Receptor/Insulin-Like Growth Factor Receptor Hybrids in Physiology and Disease. *Endocrine Reviews* **30**, 586–623 (2009).

11. Russell, S. J. & Kahn, C. R. Endocrine regulation of ageing. *Nature Reviews Molecular Cell Biology* **8**, 681–691 (2007).

12. Tatar, M. *et al.* A Mutant Drosophila Insulin Receptor Homolog That Extends Life-Span and Impairs Neuroendocrine Function. *Science* **292**, 107–110 (2001).

13. Joshi, R. L. *et al.* Targeted disruption of the insulin receptor gene in the mouse results in neonatal lethality. *The EMBO journal* **15**, 1542–7 (1996).

14. Accili, D. *et al.* Early neonatal death in mice homozygous for a null allele of the insulin receptor gene. *Nature Genetics* **12**, 106–109 (1996).

15. Blüher, M., Kahn, B. B. & Kahn, C. R. Extended Longevity in Mice Lacking the Insulin Receptor in Adipose Tissue. *Science* **299**, 572–574 (2003).

16. Holzenberger, M. *et al.* IGF-1 receptor regulates lifespan and resistance to oxidative stress in mice. *Nature* **421**, 182–187 (2003).

17. Selman, C. *et al.* Evidence for lifespan extension and delayed age–related biomarkers in insulin receptor substrate 1 null mice. *The FASEB Journal* **22**, 807–818 (2008).

18. Selman, C., Partridge, L. & Withers, D. J. Replication of Extended Lifespan Phenotype in Mice with Deletion of Insulin Receptor Substrate 1. *PLoS ONE* **6**, e16144 (2011).

19. Taguchi, A., Wartschow, L. M. & White, M. F. Brain IRS2 Signaling Coordinates Life Span and Nutrient Homeostasis. *Science* **317**, 369–372 (2007).

20. Olarescu, N. C., Gunawardane, K., Hansen, T. K., Møller, N. & Jørgensen, J. O. L. *Normal Physiology of Growth Hormone in Adults*. (2000).

21. Dehkhoda, F., Lee, C. M. M., Medina, J. & Brooks, A. J. The Growth Hormone Receptor: Mechanism of Receptor Activation, Cell Signaling, and Physiological Aspects. *Frontiers in Endocrinology* **9**, (2018).

22. Bartke, A. & Brown-Borg, H. Life Extension in the Dwarf Mouse. in 189–225 (2004).

23. Brown-Borg, H. M., Borg, K. E., Meliska, C. J. & Bartke, A. Dwarf mice and the ageing process. *Nature* **384**, 33–33 (1996).

24. EICHER, E. M. & BEAMER, W. G. Inherited Ateliotic Dwarfism in Mice: Characteristics of the mutation, little, on Chromosome 6. *Journal of Heredity* **67**, 87–91 (1976).

25. Godfrey, P. *et al.* GHRH receptor of little mice contains a missense mutation in the extracellular domain that disrupts receptor function. *Nature Genetics* **4**, 227–232 (1993).

26. Gaylinn, B. D. *et al.* The Mutant Growth Hormone-Releasing Hormone (GHRH) Receptor of the Little Mouse Does Not Bind GHRH1. *Endocrinology* **140**, 5066–5074 (1999).

27. Flurkey, K., Papaconstantinou, J., Miller, R. A. & Harrison, D. E. Lifespan extension and delayed immune and collagen aging in mutant mice with defects in growth hormone production. *Proceedings of the National Academy of Sciences* **98**, 6736–6741 (2001).

28. Sun, L. Y. *et al.* Growth hormone-releasing hormone disruption extends lifespan and regulates response to caloric restriction in mice. *eLife* **2**, (2013).

29. Snell, G. D. DWARF, A NEW MENDELIAN RECESSIVE CHARACTER OF THE HOUSE MOUSE. *Proceedings of the National Academy of Sciences* **15**, 733–734 (1929).

30. Ortman, R. A study of some cytochemical reactions and of the hormone content of the adenohypophysis in normal and in genetic dwarf mice. *Journal of Morphology* **99**, 417–431 (1956).

31. LEWIS, U. J., CHEEVER, E. v. & VANDERLAAN, W. P. Studies on the Growth Hormone of Normal and Dwarf Mice. *Endocrinology* **76**, 210–215 (1965).

32. CHEEVER, E. v., SEAVEY, B. K. & LEWIS, U. J. Prolactin of Normal and Dwarf Mice. *Endocrinology* **85**, 698–703 (1969).

33. Li, S. *et al.* Dwarf locus mutants lacking three pituitary cell types result from mutations in the POU-domain gene pit-1. *Nature* **347**, 528–533 (1990).

34. Pfaffle, R., Blankenstin, O., Wüller, S. & Kentrup, H. Combined pituirary hormone deficency: role of Pit-1 and Prop-1. *Acta Paediatrica* **88**, 33–41 (1999).

35. Cohen, L. E., Wondisford, F. E. & Radovick, S. ROLE OF PIT-1 IN THE GENE EXPRESSION OF GROWTH HORMONE, PROLACTIN, AND THYROTROPIN. *Endocrinology and Metabolism Clinics of North America* **25**, 523–540 (1996).

36. Murakami, S., Salmon, A. & Miller, R. A. Multiplex stress resistance in cells from long-lived dwarf mice. *The FASEB Journal* **17**, 1565–1576 (2003).

37. Salmon, A. B., Ljungman, M. & Miller, R. A. Cells From Long-Lived Mutant Mice Exhibit Enhanced Repair of Ultraviolet Lesions. *The Journals of Gerontology Series A: Biological Sciences and Medical Sciences* **63**, 219–231 (2008).

38. Swindell, W. R. *et al.* Endocrine regulation of heat shock protein mRNA levels in long-lived dwarf mice. *Mechanisms of Ageing and Development* **130**, 393–400 (2009).

39. Zhou, Y. *et al.* A mammalian model for Laron syndrome produced by targeted disruption of the mouse growth hormone receptor/binding protein gene (the Laron mouse). *Proceedings of the National Academy of Sciences* **94**, 13215–13220 (1997).

40. Danilovich, N., Wernsing, D., Coschigano, K. T., Kopchick, J. J. & Bartke, A. Deficits in Female Reproductive Function in GH-R-KO Mice; Role of IGF-I. *Endocrinology* **140**, 2637–2640 (1999).

41. Dominici, F., Arostegui Diaz, G., Bartke, A., Kopchick, J. & Turyn, D. Compensatory alterations of insulin signal transduction in liver of growth hormone receptor knockout mice. *Journal of Endocrinology* **166**, 579–590 (2000).

42. Coschigano, K. T., Clemmons, D., Bellush, L. L. & Kopchick, J. J. Assessment of Growth Parameters and Life Span of GHR/BP Gene-Disrupted Mice. *Endocrinology* **141**, 2608–2613 (2000).

43. List, E. O. *et al.* Liver-Specific GH Receptor Gene-Disrupted (LiGHRKO) Mice Have Decreased Endocrine IGF-I, Increased Local IGF-I, and Altered Body Size, Body Composition, and Adipokine Profiles. *Endocrinology* **155**, 1793–1805 (2014).

44. Dominick, G. *et al.* Regulation of mTOR Activity in Snell Dwarf and GH Receptor Gene-Disrupted Mice. *Endocrinology* **156**, 565–575 (2015).

45. List, E. O. *et al.* Removal of growth hormone receptor (GHR) in muscle of male mice replicates some of the health benefits seen in global GHR−/− mice. *Aging* **7**, 500–512 (2015).

46. Nagarajan, A., Srivastava, H., Jablonsky, J. & Sun, L. Y. Tissue-Specific GHR Knockout Mice: An Updated Review. *Frontiers in Endocrinology* **11**, (2020).

47. Allard, J. B. & Duan, C. IGF-Binding Proteins: Why Do They Exist and Why Are There So Many? *Frontiers in Endocrinology* **9**, (2018).

48. Boldt, H. B. & Conover, C. A. Pregnancy-associated plasma protein-A (PAPP-A): A local regulator of IGF bioavailability through cleavage of IGFBPs. *Growth Hormone & IGF Research* **17**, 10–18 (2007).

49. Conover, C. A. *et al.* Metalloproteinase pregnancy-associated plasma protein A is a critical growth regulatory factor during fetal development. *Development* **131**, 1187–1194 (2004).

50. Conover, C. A. & Bale, L. K. Loss of pregnancy-associated plasma protein A extends lifespan in mice. *Aging Cell* **6**, 727–729 (2007).

51. Conover, C. A., Bale, L. K. & Marler, R. J. Pregnancy-associated plasma protein-A deficiency improves survival of mice on a high fat diet. *Experimental Gerontology* **70**, 131–134 (2015).

52. Wang, M. & Miller, R. A. Fibroblasts from long-lived mutant mice exhibit increased autophagy and lower TOR activity after nutrient deprivation or oxidative stress. *Aging Cell* **11**, 668–674 (2012).

53. Endicott, S. J., Boynton, D. N., Beckmann, L. J. & Miller, R. A. Long-lived mice with reduced growth hormone signaling have a constitutive upregulation of hepatic chaperone-mediated autophagy. *Autophagy* **17**, 612–625 (2021).

54. Li, X., Frazier, J. A., Spahiu, E., McPherson, M. & Miller, R. A. Muscle-dependent regulation of adipose tissue function in long-lived growth hormone-mutant mice. *Aging* **12**, 8766–8789 (2020).

55. Ozkurede, U. *et al.* Cap-independent mRNA translation is upregulated in long-lived endocrine mutant mice. *Journal of Molecular Endocrinology* **63**, 123–138 (2019).

56. Meyer, K. D. *et al.* 5′ UTR m6A Promotes Cap-Independent Translation. *Cell* **163**, 999–1010 (2015).

57. Zhou, J. *et al.* Dynamic m6A mRNA methylation directs translational control of heat shock response. *Nature* **526**, 591–594 (2015).

58. Dominick, G. *et al.* Regulation of mTOR Activity in Snell Dwarf and GH Receptor Gene-Disrupted Mice. *Endocrinology* **156**, 565–575 (2015).

59. Garratt, M., Bower, B., Garcia, G. G. & Miller, R. A. Sex differences in lifespan extension with acarbose and 17-α estradiol: gonadal hormones underlie male-specific improvements in glucose tolerance and mTORC2 signaling. *Aging Cell* **16**, 1256–1266 (2017).

60. Smith, G. C. M. & Jackson, S. P. The PIKK Family of Protein Kinases. in *Handbook of Cell Signaling* 575–580 (Elsevier, 2010).

61. Kim, D.-H. *et al.* GβL, a Positive Regulator of the Rapamycin-Sensitive Pathway Required for the Nutrient-Sensitive Interaction between Raptor and mTOR. *Molecular Cell* **11**, 895–904 (2003).

62. Peterson, T. R. *et al.* DEPTOR Is an mTOR Inhibitor Frequently Overexpressed in Multiple Myeloma Cells and Required for Their Survival. *Cell* **137**, 873–886 (2009).

63. Hara, K. *et al.* Raptor, a Binding Partner of Target of Rapamycin (TOR), Mediates TOR Action. *Cell* **110**, 177–189 (2002).

64. Nojima, H. *et al.* The Mammalian Target of Rapamycin (mTOR) Partner, Raptor, Binds the mTOR Substrates p70 S6 Kinase and 4E-BP1 through Their TOR Signaling (TOS) Motif. *Journal of Biological Chemistry* **278**, 15461–15464 (2003).

65. Sancak, Y. *et al.* PRAS40 Is an Insulin-Regulated Inhibitor of the mTORC1 Protein Kinase. *Molecular Cell* **25**, 903–915 (2007).

66. Wang, L., Harris, T. E., Roth, R. A. & Lawrence, J. C. PRAS40 Regulates mTORC1 Kinase Activity by Functioning as a Direct Inhibitor of Substrate Binding. *Journal of Biological Chemistry* **282**, 20036–20044 (2007).

67. dos D. Sarbassov *et al.* Rictor, a Novel Binding Partner of mTOR, Defines a Rapamycin-Insensitive and Raptor-Independent Pathway that Regulates the Cytoskeleton. *Current Biology* **14**, 1296–1302 (2004).

68. Frias, M. A. *et al.* mSin1 Is Necessary for Akt/PKB Phosphorylation, and Its Isoforms Define Three Distinct mTORC2s. *Current Biology* **16**, 1865–1870 (2006).

69. Pearce, L. R. *et al.* Identification of Protor as a novel Rictor-binding component of mTOR complex-2. *Biochemical Journal* **405**, 513–522 (2007).

70. Yang, G., Murashige, D. S., Humphrey, S. J. & James, D. E. A Positive Feedback Loop between Akt and mTORC2 via SIN1 Phosphorylation. *Cell Reports* **12**, 937–943 (2015).

71. Yuan, Y. *et al.* Characterization of Sin1 Isoforms Reveals an mTOR-Dependent and Independent Function of Sin1γ. *PLOS ONE* **10**, e0135017 (2015).

72. Woo, S.-Y. *et al.* PRR5, a Novel Component of mTOR Complex 2, Regulates Platelet-derived Growth Factor Receptor β Expression and Signaling. *Journal of Biological Chemistry* **282**, 25604–25612 (2007).

73. Kaizuka, T. *et al.* Tti1 and Tel2 Are Critical Factors in Mammalian Target of Rapamycin Complex Assembly. *Journal of Biological Chemistry* **285**, 20109–20116 (2010).

74. Hara, K. *et al.* Regulation of eIF-4E BP1 Phosphorylation by mTOR. *Journal of Biological Chemistry* **272**, 26457–26463 (1997).

75. Burnett, P. E., Barrow, R. K., Cohen, N. A., Snyder, S. H. & Sabatini, D. M. RAFT1 phosphorylation of the translational regulators p70 S6 kinase and 4E-BP1. *Proceedings of the National Academy of Sciences* **95**, 1432–1437 (1998).

76. Jackson, R. J., Hellen, C. U. T. & Pestova, T. v. The mechanism of eukaryotic translation initiation and principles of its regulation. *Nature Reviews Molecular Cell Biology* **11**, 113–127 (2010).

77. Gingras, A.-C. *et al.* Regulation of 4E-BP1 phosphorylation: a novel two-step mechanism. *Genes & Development* **13**, 1422–1437 (1999).

78. Gingras, A.-C. *et al.* Hierarchical phosphorylation of the translation inhibitor 4E-BP1. *Genes & Development* **15**, 2852–2864 (2001).

79. Magnuson, B., Ekim, B. & Fingar, D. C. Regulation and function of ribosomal protein S6 kinase (S6K) within mTOR signalling networks. *Biochemical Journal* **441**, 1–21 (2012).

80. Meyuhas, O. Ribosomal Protein S6 Phosphorylation. in 41–73 (2015).

81. Holz, M. K., Ballif, B. A., Gygi, S. P. & Blenis, J. mTOR and S6K1 Mediate Assembly of the Translation Preinitiation Complex through Dynamic Protein Interchange and Ordered Phosphorylation Events. *Cell* **123**, 569–580 (2005).

82. Raught, B. *et al.* Phosphorylation of eucaryotic translation initiation factor 4B Ser422 is modulated by S6 kinases. *The EMBO Journal* **23**, 1761–1769 (2004).

83. Yang, H.-S. *et al.* The Transformation Suppressor Pdcd4 Is a Novel Eukaryotic Translation Initiation Factor 4A Binding Protein That Inhibits Translation. *Molecular and Cellular Biology* **23**, 26–37 (2003).

84. Ben-Sahra, I., Hoxhaj, G., Ricoult, S. J. H., Asara, J. M. & Manning, B. D. mTORC1 induces purine synthesis through control of the mitochondrial tetrahydrofolate cycle. *Science* **351**, 728–733 (2016).

85. Robitaille, A. M. *et al.* Quantitative Phosphoproteomics Reveal mTORC1 Activates de Novo Pyrimidine Synthesis. *Science* **339**, 1320–1323 (2013).

86. Ben-Sahra, I., Howell, J. J., Asara, J. M. & Manning, B. D. Stimulation of de Novo Pyrimidine Synthesis by Growth Signaling Through mTOR and S6K1. *Science* **339**, 1323–1328 (2013).

87. Peterson, T. R. *et al.* mTOR Complex 1 Regulates Lipin 1 Localization to Control the SREBP Pathway. *Cell* **146**, 408–420 (2011).

88. Shimano, H. & Sato, R. SREBP-regulated lipid metabolism: convergent physiology — divergent pathophysiology. *Nature Reviews Endocrinology* **13**, 710–730 (2017).

89. Zachari, M. & Ganley, I. G. The mammalian ULK1 complex and autophagy initiation. *Essays in Biochemistry* **61**, 585–596 (2017).

90. Ganley, I. G. *et al.* ULK1·ATG13·FIP200 Complex Mediates mTOR Signaling and Is Essential for Autophagy. *Journal of Biological Chemistry* **284**, 12297–12305 (2009).

91. Jung, C. H. *et al.* ULK-Atg13-FIP200 Complexes Mediate mTOR Signaling to the Autophagy Machinery. *Molecular Biology of the Cell* **20**, 1992–2003 (2009).

92. Hosokawa, N. *et al.* Nutrient-dependent mTORC1 Association with the ULK1–Atg13–FIP200 Complex Required for Autophagy. *Molecular Biology of the Cell* **20**, 1981–1991 (2009).

93. Liang, C. *et al.* Beclin1-binding UVRAG targets the class C Vps complex to coordinate autophagosome maturation and endocytic trafficking. *Nature Cell Biology* **10**, 776–787 (2008).

94. Kim, Y.-M. *et al.* mTORC1 Phosphorylates UVRAG to Negatively Regulate Autophagosome and Endosome Maturation. *Molecular Cell* **57**, 207–218 (2015).

95. Settembre, C. *et al.* A lysosome-to-nucleus signalling mechanism senses and regulates the lysosome via mTOR and TFEB. *The EMBO Journal* **31**, 1095–1108 (2012).

96. Martina, J. A., Chen, Y., Gucek, M. & Puertollano, R. MTORC1 functions as a transcriptional regulator of autophagy by preventing nuclear transport of TFEB. *Autophagy* **8**, 903–914 (2012).

97. Manning, B. D. & Toker, A. AKT/PKB Signaling: Navigating the Network. *Cell* **169**, 381–405 (2017).

98. Facchinetti, V. *et al.* The mammalian target of rapamycin complex 2 controls folding and stability of Akt and protein kinase C. *The EMBO Journal* **27**, 1932–1943 (2008).

99. Liu, P. *et al.* Cell-cycle-regulated activation of Akt kinase by phosphorylation at its carboxyl terminus. *Nature* **508**, 541–545 (2014).

100. Inoki, K., Li, Y., Zhu, T., Wu, J. & Guan, K.-L. TSC2 is phosphorylated and inhibited by Akt and suppresses mTOR signalling. *Nature Cell Biology* **4**, 648–657 (2002).

101. Manning, B. D., Tee, A. R., Logsdon, M. N., Blenis, J. & Cantley, L. C. Identification of the Tuberous Sclerosis Complex-2 Tumor Suppressor Gene Product Tuberin as a Target of the Phosphoinositide 3-Kinase/Akt Pathway. *Molecular Cell* **10**, 151–162 (2002).

102. Lang, F. & Shumilina, E. Regulation of ion channels by the serum- and glucocorticoid-inducible kinase SGK1. *The FASEB Journal* **27**, 3–12 (2013).

103. García-Martínez, J. M. & Alessi, D. R. mTOR complex 2 (mTORC2) controls hydrophobic motif phosphorylation and activation of serum- and glucocorticoid-induced protein kinase 1 (SGK1). *Biochemical Journal* **416**, 375–385 (2008).

104. Pearce, L. R., Komander, D. & Alessi, D. R. The nuts and bolts of AGC protein kinases. *Nature Reviews Molecular Cell Biology* **11**, 9–22 (2010).

105. Garg, R. *et al.* Protein kinase C and cancer: what we know and what we do not. *Oncogene* **33**, 5225–5237 (2014).

106. Larsson, C. Protein kinase C and the regulation of the actin cytoskeleton. *Cellular Signalling* **18**, 276–284 (2006).

107. Sabatini, D. M. Twenty-five years of mTOR: Uncovering the link from nutrients to growth. *Proceedings of the National Academy of Sciences* **114**, 11818–11825 (2017).

108. Wolfson, R. L. *et al.* Sestrin2 is a leucine sensor for the mTORC1 pathway. *Science* **351**, 43–48 (2016).

109. Bar-Peled, L. *et al.* A Tumor Suppressor Complex with GAP Activity for the Rag GTPases That Signal Amino Acid Sufficiency to mTORC1. *Science* **340**, 1100–1106 (2013).

110. Wei, Y. *et al.* TORC1 regulators Iml1/GATOR1 and GATOR2 control meiotic entry and oocyte development in Drosophila. *Proceedings of the National Academy of Sciences* **111**, E5670–E5677 (2014).

111. Wolfson, R. L. *et al.* KICSTOR recruits GATOR1 to the lysosome and is necessary for nutrients to regulate mTORC1. *Nature* **543**, 438–442 (2017).

112. Sancak, Y. *et al.* The Rag GTPases Bind Raptor and Mediate Amino Acid Signaling to mTORC1. *Science* **320**, 1496–1501 (2008).

113. Bar-Peled, L., Schweitzer, L. D., Zoncu, R. & Sabatini, D. M. Ragulator Is a GEF for the Rag GTPases that Signal Amino Acid Levels to mTORC1. *Cell* **150**, 1196–1208 (2012).

114. Chantranupong, L. *et al.* The CASTOR Proteins Are Arginine Sensors for the mTORC1 Pathway. *Cell* **165**, 153–164 (2016).

115. Wang, S. *et al.* Lysosomal amino acid transporter SLC38A9 signals arginine sufficiency to mTORC1. *Science* **347**, 188–194 (2015).

116. Rebsamen, M. *et al.* SLC38A9 is a component of the lysosomal amino acid sensing machinery that controls mTORC1. *Nature* **519**, 477–481 (2015).

117. Gu, X. *et al.* SAMTOR is an S-adenosylmethionine sensor for the mTORC1 pathway. *Science* **358**, 813–818 (2017).

118. Durán, R. V. *et al.* Glutaminolysis Activates Rag-mTORC1 Signaling. *Molecular Cell* **47**, 349–358 (2012).

119. Jewell, J. L. *et al.* Differential regulation of mTORC1 by leucine and glutamine. *Science* **347**, 194–198 (2015).

120. Zoncu, R. *et al.* mTORC1 Senses Lysosomal Amino Acids Through an Inside-Out Mechanism That Requires the Vacuolar H+-ATPase. *Science* **334**, 678–683 (2011).

121. Benvenuto, G. *et al.* The tuberous sclerosis-1 (TSC1) gene product hamartin suppresses cell growth and augments the expression of the TSC2 product tuberin by inhibiting its ubiquitination. *Oncogene* **19**, 6306–6316 (2000).

122. Dibble, C. C. *et al.* TBC1D7 Is a Third Subunit of the TSC1-TSC2 Complex Upstream of mTORC1. *Molecular Cell* **47**, 535–546 (2012).

123. Inoki, K., Li, Y., Xu, T. & Guan, K.-L. Rheb GTPase is a direct target of TSC2 GAP activity and regulates mTOR signaling. *Genes & Development* **17**, 1829–1834 (2003).

124. Bai, X. *et al.* Rheb Activates mTOR by Antagonizing Its Endogenous Inhibitor, FKBP38. *Science* **318**, 977–980 (2007).

125. Wee, P. & Wang, Z. Epidermal Growth Factor Receptor Cell Proliferation Signaling Pathways. *Cancers* **9**, 52 (2017).

126. Ma, L., Chen, Z., Erdjument-Bromage, H., Tempst, P. & Pandolfi, P. P. Phosphorylation and Functional Inactivation of TSC2 by Erk. *Cell* **121**, 179–193 (2005).

127. Roux, P. P., Ballif, B. A., Anjum, R., Gygi, S. P. & Blenis, J. Tumor-promoting phorbol esters and activated Ras inactivate the tuberous sclerosis tumor suppressor complex via p90 ribosomal S6 kinase. *Proceedings of the National Academy of Sciences* **101**, 13489–13494 (2004).

128. Herzig, S. & Shaw, R. J. AMPK: guardian of metabolism and mitochondrial homeostasis. *Nature Reviews Molecular Cell Biology* **19**, 121–135 (2018).

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

130. Gwinn, D. M. *et al.* AMPK Phosphorylation of Raptor Mediates a Metabolic Checkpoint. *Molecular Cell* **30**, 214–226 (2008).

131. Shoshani, T. *et al.* Identification of a Novel Hypoxia-Inducible Factor 1-Responsive Gene, RTP801, Involved in Apoptosis. *Molecular and Cellular Biology* **22**, 2283–2293 (2002).

132. Brugarolas, J. *et al.* Regulation of mTOR function in response to hypoxia by REDD1 and the TSC1/TSC2 tumor suppressor complex. *Genes & Development* **18**, 2893–2904 (2004).

133. DeYoung, M. P., Horak, P., Sofer, A., Sgroi, D. & Ellisen, L. W. Hypoxia regulates TSC1/2–mTOR signaling and tumor suppression through REDD1-mediated 14–3–3 shuttling. *Genes & Development* **22**, 239–251 (2008).

134. Dibble, C. C., Asara, J. M. & Manning, B. D. Characterization of Rictor Phosphorylation Sites Reveals Direct Regulation of mTOR Complex 2 by S6K1. *Molecular and Cellular Biology* **29**, 5657–5670 (2009).

135. Copps, K. D. & White, M. F. Regulation of insulin sensitivity by serine/threonine phosphorylation of insulin receptor substrate proteins IRS1 and IRS2. *Diabetologia* **55**, 2565–2582 (2012).

136. Tzatsos, A. & Kandror, K. v. Nutrients Suppress Phosphatidylinositol 3-Kinase/Akt Signaling via Raptor-Dependent mTOR-Mediated Insulin Receptor Substrate 1 Phosphorylation. *Molecular and Cellular Biology* **26**, 63–76 (2006).

137. Harrington, L. S. *et al.* The TSC1-2 tumor suppressor controls insulin–PI3K signaling via regulation of IRS proteins. *Journal of Cell Biology* **166**, 213–223 (2004).

138. González, A. & Hall, M. N. Nutrient sensing and TOR signaling in yeast and mammals. *The EMBO Journal* **36**, 397–408 (2017).

139. Kaeberlein, M. *et al.* Regulation of Yeast Replicative Life Span by TOR and Sch9 in Response to Nutrients. *Science* **310**, 1193–1196 (2005).

140. Vellai, T. *et al.* Influence of TOR kinase on lifespan in C. elegans. *Nature* **426**, 620–620 (2003).

141. Kapahi, P. *et al.* Regulation of Lifespan in Drosophila by Modulation of Genes in the TOR Signaling Pathway. *Current Biology* **14**, 885–890 (2004).

142. Wu, J. J. *et al.* Increased Mammalian Lifespan and a Segmental and Tissue-Specific Slowing of Aging after Genetic Reduction of mTOR Expression. *Cell Reports* **4**, 913–920 (2013).

143. Chiu, M. I., Katz, H. & Berlin, V. RAPT1, a mammalian homolog of yeast Tor, interacts with the FKBP12/rapamycin complex. *Proceedings of the National Academy of Sciences* **91**, 12574–12578 (1994).

144. Liu, G. Y. & Sabatini, D. M. mTOR at the nexus of nutrition, growth, ageing and disease. *Nature Reviews Molecular Cell Biology* **21**, 183–203 (2020).

145. Powers, R. W., Kaeberlein, M., Caldwell, S. D., Kennedy, B. K. & Fields, S. Extension of chronological life span in yeast by decreased TOR pathway signaling. *Genes & Development* **20**, 174–184 (2006).

146. Robida-Stubbs, S. *et al.* TOR Signaling and Rapamycin Influence Longevity by Regulating SKN-1/Nrf and DAF-16/FoxO. *Cell Metabolism* **15**, 713–724 (2012).

147. Bjedov, I. *et al.* Mechanisms of Life Span Extension by Rapamycin in the Fruit Fly Drosophila melanogaster. *Cell Metabolism* **11**, 35–46 (2010).

148. Miller, R. A. *et al.* Rapamycin-mediated lifespan increase in mice is dose and sex dependent and metabolically distinct from dietary restriction. *Aging Cell* **13**, 468–477 (2014).

149. Harrison, D. E. *et al.* Rapamycin fed late in life extends lifespan in genetically heterogeneous mice. *Nature* **460**, 392–395 (2009).

150. Wilkinson, J. E. *et al.* Rapamycin slows aging in mice. *Aging Cell* **11**, 675–682 (2012).

151. Nguyen, L. S. *et al.* Sirolimus and mTOR Inhibitors: A Review of Side Effects and Specific Management in Solid Organ Transplantation. *Drug Safety* (2019).

152. Ferron, G. M. & Jusko, W. J. Species differences in sirolimus stability in humans, rabbits, and rats. *Drug metabolism and disposition: the biological fate of chemicals* **26**, 83–4 (1998).

153. Chen, Y. & Zhou, X. Research progress of mTOR inhibitors. *European Journal of Medicinal Chemistry* **208**, 112820 (2020).

154. Lamming, D. W. *et al.* Rapamycin-Induced Insulin Resistance Is Mediated by mTORC2 Loss and Uncoupled from Longevity. *Science* **335**, 1638–1643 (2012).

155. Selman, C. *et al.* Ribosomal Protein S6 Kinase 1 Signaling Regulates Mammalian Life Span. *Science* **326**, 140–144 (2009).

156. Zhang, H.-M., Diaz, V., Walsh, M. E. & Zhang, Y. Moderate lifelong overexpression of tuberous sclerosis complex 1 (TSC1) improves health and survival in mice. *Scientific Reports* **7**, 834 (2017).

157. Skarnes, W. C. *et al.* A conditional knockout resource for the genome-wide study of mouse gene function. *Nature* **474**, 337–342 (2011).

158. Yoon, M.-S. *et al.* Rapid Mitogenic Regulation of the mTORC1 Inhibitor, DEPTOR, by Phosphatidic Acid. *Molecular Cell* **58**, 549–556 (2015).

159. Fonseca, B. D., Smith, E. M., Lee, V. H.-Y., MacKintosh, C. & Proud, C. G. PRAS40 Is a Target for Mammalian Target of Rapamycin Complex 1 and Is Required for Signaling Downstream of This Complex. *Journal of Biological Chemistry* **282**, 24514–24524 (2007).

160. Haar, E. vander, Lee, S., Bandhakavi, S., Griffin, T. J. & Kim, D.-H. Insulin signalling to mTOR mediated by the Akt/PKB substrate PRAS40. *Nature Cell Biology* **9**, 316–323 (2007).

161. Chiang, G. G. & Abraham, R. T. Phosphorylation of Mammalian Target of Rapamycin (mTOR) at Ser-2448 Is Mediated by p70S6 Kinase. *Journal of Biological Chemistry* **280**, 25485–25490 (2005).

162. Copp, J., Manning, G. & Hunter, T. TORC-Specific Phosphorylation of Mammalian Target of Rapamycin (mTOR): Phospho-Ser 2481 Is a Marker for Intact mTOR Signaling Complex 2. *Cancer Research* **69**, 1821–1827 (2009).

163. Gwinn, D. M. *et al.* AMPK Phosphorylation of Raptor Mediates a Metabolic Checkpoint. *Molecular Cell* **30**, 214–226 (2008).

164. Catena, V. & Fanciulli, M. Deptor: not only a mTOR inhibitor. *Journal of Experimental & Clinical Cancer Research* **36**, 12 (2017).

165. Gao, D. *et al.* mTOR Drives Its Own Activation via SCFβTrCP-Dependent Degradation of the mTOR Inhibitor DEPTOR. *Molecular Cell* **44**, 290–303 (2011).

166. Duan, S. *et al.* mTOR Generates an Auto-Amplification Loop by Triggering the βTrCP- and CK1α-Dependent Degradation of DEPTOR. *Molecular Cell* **44**, 317–324 (2011).

167. Zhao, Y., Xiong, X. & Sun, Y. DEPTOR, an mTOR Inhibitor, Is a Physiological Substrate of SCFβTrCP E3 Ubiquitin Ligase and Regulates Survival and Autophagy. *Molecular Cell* **44**, 304–316 (2011).

168. Henske, E. P., Jóźwiak, S., Kingswood, J. C., Sampson, J. R. & Thiele, E. A. Tuberous sclerosis complex. *Nature Reviews Disease Primers* **2**, 16035 (2016).

169. Huang, J. & Manning, B. D. The TSC1–TSC2 complex: a molecular switchboard controlling cell growth. *Biochemical Journal* **412**, 179–190 (2008).

170. Astrinidis, A., Senapedis, W., Coleman, T. R. & Henske, E. P. Cell Cycle-regulated Phosphorylation of Hamartin, the Product of the Tuberous Sclerosis Complex 1 Gene, by Cyclin-dependent Kinase 1/Cyclin B. *Journal of Biological Chemistry* **278**, 51372–51379 (2003).

171. Lee, D.-F. *et al.* IKKβ Suppression of TSC1 Links Inflammation and Tumor Angiogenesis via the mTOR Pathway. *Cell* **130**, 440–455 (2007).

172. Mahoney, S. J. *et al.* A small molecule inhibitor of Rheb selectively targets mTORC1 signaling. *Nature Communications* **9**, 548 (2018).

173. Kobayashi, T. *et al.* A germ-line Tsc1mutation causes tumor development and embryonic lethality that are similar, but not identical to, those caused by Tsc2 mutation in mice. *Proceedings of the National Academy of Sciences* **98**, 8762–8767 (2001).

174. Onda, H., Lueck, A., Marks, P. W., Warren, H. B. & Kwiatkowski, D. J. Tsc2+/– mice develop tumors in multiple sites that express gelsolin and are influenced by genetic background. *Journal of Clinical Investigation* **104**, 687–695 (1999).