S6K regulates inflammageing, immunosenescence and lifespan through the endolysosomal system

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献给最疼爱我的姥姥和姥爷

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ABBREVIATIONS

4EBP1	the eukaryotic initiation factor 4E binding protein 1
ABC	ammonium bicarbonate
AD	Alzheimer's disease
AMP	antimicrobial peptide
AMPK	5' adenosine monophosphate (AMP)-activated protein kinase
ANOVA	analysis of variance
Arf1	ADP ribosylation factor 1
ATG	autophagy-related
BAD	BCL2 associated agonist of cell death
BCA	bicinchoninic acid
BSA	bovine serum albumin
CA	constitutively active
CAN	acetonitrile
CBC	the nuclear RNA cap-binding complex
CBP80	80 kDa nuclear cap-binding protein
CCTβ	chaperonin containing TCP-1β
cDNA	complementary DNA
CPH	Cox Proportional Hazards
CR	caloric restriction
CT	cycle threshold
DAMP	damage-associated molecular pattern
DAPI	4′,6-diamidino-2-phenylindole
Deptor	DEP domain-containing mTOR-interacting protein
DN	dominant negative
DNA	deoxyribonucleic acid
DptA	Diptericin A
dS6K	Drosophila S6 kinase
EDTA	ethylenediaminetetraacetic acid
eEF2	eukaryotic elongation factor 2
eEF2K	eukaryotic elongation factor 2 kinase
EGR2	early growth response protein 2
eIF4A	eukaryotic initiation factor 4A
eIF4B	eukaryotic initiation factor 4B
eIF4E	eukaryotic initiation factor 4E
EPRS	glutamyl-prolyl-tRNA synthetase
ER	endoplasmic reticulum
ERα	estrogen receptor α
FA	formic acid

FELASA	the Federation of European Laboratory Animal Science Association
FoxO1/3a	forkhead box protein O/O3A
GAP	GTPase-activating protein
GATOR	GTPase-activating protein activity toward Rags
Gfi1	growth factor independent 1
GFP	green fluorescent protein
GO	gene ontology
GRB10	growth factor bound-receptor protein 10
Grp78	78-kDa glucose-regulated protein
GS	GeneSwitch
GSK3	glycogen synthase kinase 3
GβL	G-protein β-protein subunit-like
h.p.i.	hour post-infection
HIF1α	hypoxia-inducible factor 1-alpha
HPLC	high performance/pressure liquid chromatography
IFITM3	interferon-induced transmembrane protein 3
IGF	insulin-like growth factor
IIS	insulin and insulin-like growth factor signalling
IMD	immune deficiency
IRS1	insulin receptor substrate 1
ISC	intestinal stem cell
Lamp1	lysosomal-associated membrane protein 1
LB	lysogeny broth
LC–MS/MS	liquid chromatography-tandem mass spectrometry
LSD	lysosomal storage disease
MAPK	mitogen-activated protein kinase
MDM2	murine double minute 2
mLST8	mammalian lethal with SEC13 protein
mSIN1	mammalian stress-activated MAPK-interacting protein 1
mTOR	mechanistic target of rapamycin
NF-κB	nuclear factor-kappa B
NLS	nuclear localization sequence
OD	optical density
PBS	phosphate-buffered saline
PBST	Triton-X/PBS
PCR	polymerase chain reaction
PDCD4	programmed cell death 4
pH3	phospho-histone H3
PI3K	phosphatidylinositol 3-kinase
PKA	cAMP-dependent protein kinase
PKC	protein kinase C

PKG	cGMP-dependent protein kinase
PRAS40	proline-rich Akt substrate of 40 kDa
Protor1/2	protein observed with Rictor 1/2
PVDF	polyvinylidene fluoride
qRT-PCR	quantitative real-time PCR
Rapa	rapamycin
Raptor	regulatory associated protein of mTOR
Rheb	Ras homolog enriched in the brain
Rictor	rapamycin-insensitive companion of mTOR
RIPA	radioimmunoprecipitation assay
RNA	ribonucleic acid
RNAi	RNA interference
RORγ	RAR-related orphan receptor γ
Rpl32	60S ribosomal protein L32
rpS6	40S ribosomal protein S6
s.e.m.	standard error of the mean
S6K	S6 kinase
SAMTOR	S-adenosylmethionine sensor upstream of TORC1
SAR1B	secretion associated Ras related GTPase 1B
SGK1	serum/glucocorticoid-regulated kinase 1
SKAR	S6K1 Aly/REF-like substrate
SNARE	soluble N-ethylmaleimide-sensitive-factor attachment protein receptor
SREBP1	sterol responsive element binding protein 1
Stx12	mammalian Syntaxin 12/13
Syx13	Drosophila Syntaxin 13
TAB1	transforming growth factor β-activated kinase-binding protein 1
TAG	triacylglyceride
TAK1	transforming growth factor β-activated kinase 1
TBC1D7	TBC1 domain family member 7
TBS	Tris-buffered saline
TCEP	Tris(2-carboxyethyl)phosphine hydrochloride
TEAB	triethylammonium bicarbonate
TFEB	transcription factor EB
Th17	T helper 17
TLR	toll-like receptor
TMT	tandem mass tag
TORC1	mTOR Complex 1
TORC2	mTOR Complex 2
TOS	TOR signalling
TSC	tuberous sclerosis complex
UAS	upstream activating sequence

ULK1	Unc-51-like autophagy activating kinase
w^{Dah}	white Dahomey
YFP	yellow fluorescent protein

SUMMARY

Suppression of Target of Rapamycin complex 1 (TORC1) induces longevity and healthspan in diverse species. Suppression of S6 kinase (S6K) is an essential downstream mediator of the effect of TORC1 on ageing in *Drosophila*, but the mechanisms at work remain largely obscure. In this thesis, the role of S6K activity in ageing was investigated in *Drosophila*. I showed that reducing S6K activity ubiquitously in adult flies, using the GeneSwitch system, increased lifespan. Tissue-specific analysis revealed that the lifespan-extending effect was only observed when S6K activity was suppressed specifically in the fat body, the tissue that is functionally equivalent to the liver and white adipose tissue in mammals. I also found that expression of a constitutively active S6K protein in the fat body was sufficient to block the lifespan-extending effect of the TOR inhibitor rapamycin, suggesting that the activity of S6K specifically in the *Drosophila* fat body is essential for rapamycin-mediated longevity.

Using proteomics profiling and network propagation analysis, I found that in young adults, the proteins directly affected by TORC1-S6K signalling were endosome/lysosome-, oogenesis-, and translation-related, while at later age immune-, translation-, and lipid-related processes were altered. Therefore, I tested the effect of TORC1-S6K signalling on fecundity, global translation and triacylglycerol homeostasis, but none of these were altered upon TORC1-S6K manipulation. Although the *Drosophila* intestine has recently been shown to play a crucial role in TORC1-dependent longevity, I found that S6K activity in the fat body does not affect gut health.

By using electron microscopy, lysosome-specific live cell staining and genetic reporter flies, I found that TORC1 inhibition by rapamycin treatment repressed enlarged multilamellar lysosomes in the *Drosophila* fat body, and that this effect was blocked by elevating S6K activity. Inhibition of autophagy by Atg5 RNAi did not affect lysosomal size, but impairment of late endosomes by expressing a dominant negative form of Rab7 induced lysosomal enlargement. By proteomic analysis, I found that the protein level of Syntaxin 13 (Syx13), a SNARE family protein, was elevated by the inhibition of TORC1-S6K signalling. Furthermore, repressing Syx13 induced enlarged lysosomes in the fat body and overexpressing Syx13 attenuated the S6K-induced enlarged multilamellar lysosomes. Thus, Syx13 is a downstream effector of the TORC1-S6K pathway that mediates the lysosomal structural changes in *Drosophila*. During ageing in *Drosophila* chronic inflammation (termed “inflammageing” and

mainly represented by the activation of the immune deficiency (IMD) pathway) increases, and pathogen clearance decreases (termed “immunosenescence”). Activation of the IMD pathway, represented by elevated nuclear translocalisation of the NF-κB like transcription factor Relish and expression of *Diptericin A*, and decline of pathogen clearance in old flies, were both suppressed by reduced S6K activity or rapamycin treatment, whereas activation of S6K in the fat tissue blocked this effect. Syx13 inhibition also blocked the rapamycin-related effect on IMD activation. S6K activation-related IMD activation and immune dysfunction were blocked by Syx13 overexpression. Furthermore, I found that ameliorating inflammageing by knocking down Relish level in the fat body from middle-adulthood on significantly improved bacterial clearance and extended fly lifespan. These results suggest that the TORC1-S6K-Syx13 signalling plays a crucial role in regulating the endolysosomal system and the aged immune system in the fat body of *Drosophila*.

To assess if the regulation of Syx13 by rapamycin is also conserved in mammals, I used mice as a model system. I found that the chronic rapamycin treatment significantly increased Syntaxin 12/13 (Stx12) protein levels in the liver. The impact of reduced TORC1-S6K signalling on immunoageing was assessed in mice by proteomics profiling and network propagation analysis of liver samples from old mice treated with rapamycin and integrating these data with previous published transcriptome datasets of livers of aged mice with rapamycin treatment or S6K1 deficiency. I revealed that immune-related processes, such as inflammation and leukocyte proliferation, were commonly down-regulated by S6K1 deficiency or rapamycin treatment. These findings suggest that the regulation of Syntaxin 12/13 expression and its effect on immune ageing by TORC1-S6K signalling are conserved between flies and mammals.

In summary, my work established that suppression of the TORC1-S6K-Syx13 axis ameliorates both inflammageing and immunosenescence in hepatic tissues through the endolysosomal system, and thereby extends longevity in *Drosophila*, providing a mechanistic explanation for the effects of rapamycin and suppression of S6K on immune function and lifespan in model organisms and, potentially, humans.

CONTRIBUTIONS

Parts of this thesis were the basis of a manuscript that was published on bioRxiv (Zhang et al., 2022), and data that were not generated by me were clearly indicated. Details were given below:

Title: Inhibition of S6K lowers age-related inflammation and immunosenescence and increases lifespan through the endolysosomal system

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Author contributions:

Pingze Zhang, Sebastian Grönke and Linda Partridge conceived and designed the study.

Pingze Zhang performed most of the experiments. James H. Catterson conducted the lifespan assays in Figure 3-2.

Pingze Zhang analysed and visualised the data.

Pingze Zhang generated all schematic diagrams with BioRender.com.

Pingze Zhang, Sebastian Grönke and Linda Partridge wrote the manuscript.

1 INTRODUCTION

1.1 Targeting ageing

1.1.1 Ageing: a global public health challenge

The world's population is ageing. Over the past 200 years, average human life expectancy has increased dramatically in virtually every country, significantly changing the age structure of the global population (Oeppen & Vaupel, 2002). Furthermore, according to the UN World Population Prospects 2022 (https://population.un.org/wpp), by 2030 the proportion of people aged over 65 will be twice as high as it was fifty years ago.

Although human life expectancy has largely increased, the length of time people live in good health (healthspan) has not increased as much because we now live far longer than in our evolutionary past and reach ages that have not been shaped by natural selection. Advanced age is now the primary risk factor for many chronic diseases, including diabetes, cancer, cardiovascular and neurodegenerative diseases (Partridge et al., 2018). Understanding the biology of ageing could therefore lead to more appropriate and timely treatments for many major human diseases, improving the quality of life of the elderly and reducing the economic burden of the ageing society.

1.1.2 Ageing is malleable

Understanding and delaying the ageing process has been a challenge for biologists for decades. Although McCay et al. (1939) observed that dietary restriction extended lifespan in rodents, the first genetic evidence that ageing could be modified came out in the 1980s with the discovery that a single gene in *Caenorhabditis elegans* could determine lifespan (Friedman & Johnson, 1988; Klass, 1983). These findings sparked an intensive study of the molecular biology of ageing, and since then a growing body of evidence suggests that dietary, pharmacological, and genetic interventions improve health during ageing and ensure longevity.

Based on the current understanding of the fundamental mechanisms of ageing, Lopez-Otin et al. (2023) recently proposed twelve hallmarks of ageing: genomic instability, telomere attrition, epigenetic alterations, loss of proteostasis, disabled macroautophagy, deregulated nutrient-sensing, mitochondrial dysfunction, cellular senescence, stem cell exhaustion, altered intercellular communication, chronic inflammation, and dysbiosis. Experimentally targeting any one of these hallmarks could decelerate ageing and ameliorate age-related disorders. Moreover, these hallmarks are also interconnected because ageing is a complex process.

Modification of one specific hallmark tends to affect other hallmarks as well. For example, dietary restriction or genetic inhibition of the nutrient-sensing "insulin and insulin-like growth factor-1 (IGF-1) signalling" (IIS) pathway could delay ageing, and this effect is conserved across multiple model organisms and even in rhesus monkeys (Colman et al., 2009; Fontana et al., 2010). Alteration of the IIS pathway triggers downstream mechanistic (formerly "mammalian") Target of Rapamycin (mTOR) signalling and other signalling events, which impact on macroautophagy and genome instability (Hansen et al., 2008; Tain et al., 2021).

1.2 The mTOR signalling network and ageing

1.2.1 mTOR complexes

The mTOR network senses nutrients and various stressors and plays a key role in the regulation of growth, metabolism, and longevity. mTOR is a serine/threonine protein kinase which is a crucial component of mTOR Complex 1 (TORC1) and 2 (TORC2). The former includes three core components: mTOR, Raptor (regulatory associated protein of mTOR), mLST8 (mammalian lethal with SEC13 protein)/GβL (G-protein β-protein subunit-like), and two inhibitory subunits: Deptor (DEP domain-containing mTOR-interacting protein) and PRAS40 (proline-rich Akt substrate of 40 kDa); while the latter consists of mTOR, Rictor (rapamycin-insensitive companion of mTOR), mLST8/GβL, Protor1/2 (protein observed with Rictor 1/2), mSIN1 (mammalian stress-activated MAPK-interacting protein 1), and the inhibitory subunit Deptor (DEP domain-containing mTOR-interacting protein) (Figure 1-1) (Laplante & Sabatini, 2012; Saxton & Sabatini, 2017).

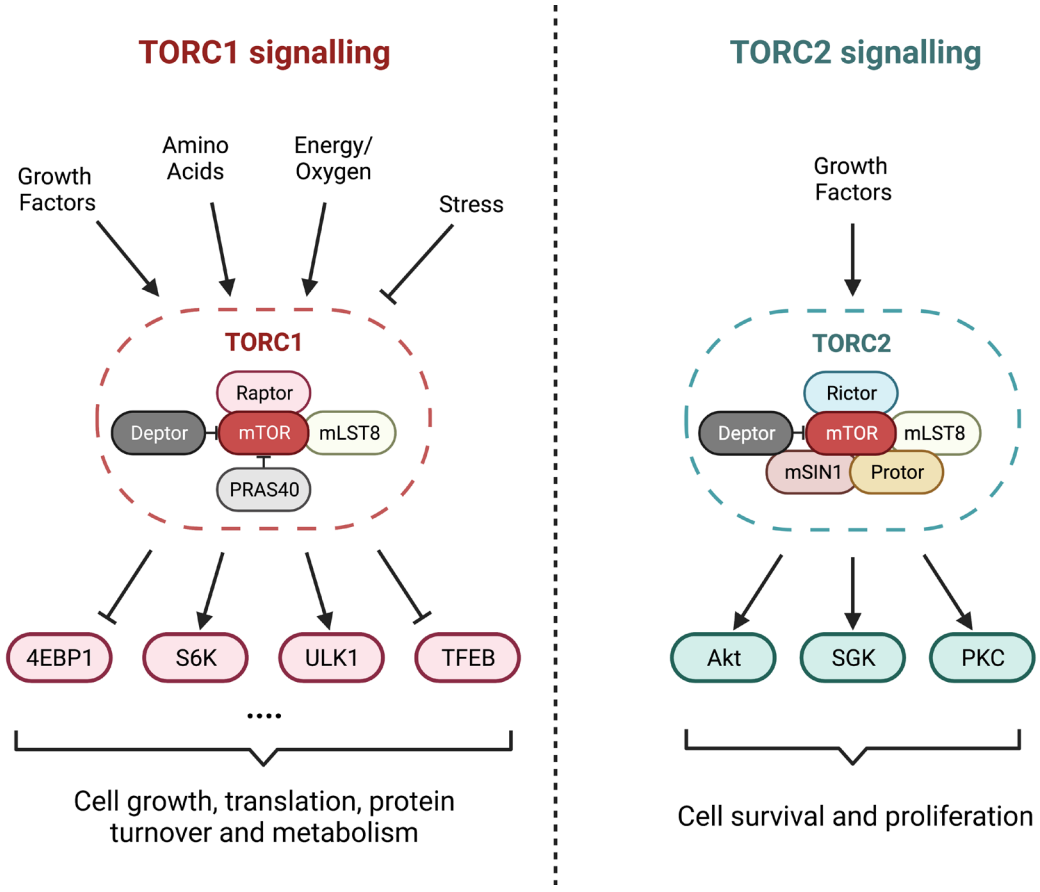

Figure 1-1: The TORC1 and TORC2 signalling pathways.

1.2.2 Upstream regulation of mTOR

TORC1 complex promotes anabolism and growth in the presence of a variety of signals, including growth factors, amino acids and energy, and its regulation is largely dependent on diet. In response to insulin or IGF (insulin-like growth factor), the PI3K (phosphatidylinositol 3-kinase)/Akt pathway becomes active and triggers the Akt-dependent multisite phosphorylation of TSC2 (tuberous sclerosis complex 2) to suppress the inhibitory effect of the TSC1-TSC2-TBC1D7 (TBC1 domain family member 7) heterotrimeric complex (Dibble et al., 2012; Inoki et al., 2002; Manning et al., 2002). TSC1-TSC2-TBC1D7 complex functions as a GAP (GTPase-activating protein) that inhibits the Rheb (Ras homolog enriched in the brain) GTPase, which directly binds and activates TORC1 (Garami et al., 2003; Inoki, Li, et al., 2003; Tee et al., 2003). In addition to phosphorylating TSC2, Akt phosphorylates PRAS40 to disrupt the inhibitory Raptor–PRAS40 interaction, thus promoting TORC1 signalling by allowing the recruitment of TORC1 substrates (Oshiro et al., 2007; Sancak et al., 2007; Vander Haar et al., 2007).

TORC1 is sensitive to diet-induced changes in amino acid concentrations. Amino acids inside the lysosomal lumen convert the Ras-related GTPases (Rags) to their active nucleotide-bound state through a vacuolar-type ATPase-dependent mechanism, allowing them to bind TORC1 via Raptor and recruit TORC1 to the lysosomal surface, where it is activated by the lysosome-localized Rheb (Condon & Sabatini, 2019; Kim et al., 2008; Sancak et al., 2008; Takahara et al., 2020). Leucine and arginine in the cytoplasm determine TORC1 activity through a distinct pathway involving GATOR1 (GTPase-activating protein activity toward Rags 1) and GATOR2 complexes (Bar-Peled et al., 2013). GATOR1 is a negative regulator of TORC1 signalling by acting as a GAP for the Ras-related GTPases, and is inhibited by GATOR2, a complex which is negatively regulated by the leucine-specific sensors Sestrin2 (Saxton, Knockenhauer, et al., 2016; Wolfson et al., 2016) and SAR1B (secretion associated Ras related GTPase 1B) (Chen et al., 2021), as well as arginine-specific sensor CASTOR1 (Cellular Arginine Sensor for mTORC1) (Chantranupong et al., 2016; Gai et al., 2016; Saxton, Chantranupong, et al., 2016; Xia et al., 2016). Several additional amino acid sensing mechanisms that modulate the TORC1 pathway have recently been described, including the S-adenosylmethionine sensor upstream of TORC1 (SAMTOR)-dependent methionine sensing and ADP ribosylation factor 1 (Arf1)-dependent glutamine sensing (Gu et al., 2017; Jewell et al., 2015).

TORC1 also responds to diverse forms of cell stresses that are incompatible with growth such as low ATP levels induced by glucose deprivation. A reduction in cellular ATP levels leads to an increase in the cellular AMP:ATP ratio, leading to the activation of AMP-activated protein kinase (AMPK) (Gonzalez et al., 2020). AMPK inhibits TORC1 both indirectly, by phosphorylating and activating TSC2 (Inoki, Zhu, et al., 2003), and directly, by phosphorylating Raptor, which prevents TORC1 activation (Gwinn et al., 2008; Van Nostrand et al., 2020).

TORC2 activity is regulated by the mSIN1 subunit, which contains a phosphoinositide-binding PH domain critical for insulin/PI3K-dependent regulation of TORC2 activity. mSIN1 inhibits TORC2 activity in the absence of insulin, and this inhibition is relieved by binding to PI3K-generated PIP3 at the plasma membrane, thereby releasing mTOR and allowing activation of TORC2 (Liu et al., 2015). Akt can also phosphorylate mSIN1, which creates a positive-feedback loop whereby partial activation of Akt enhances TORC2 activity, leading to the phosphorylation and the subsequent full activation of Akt (Yang et al., 2015). PI3K also promotes the association of TORC2 with ribosomes to activate its kinase activity (Zinzalla et al., 2011). TORC2 activity is also negatively regulated by TORC1 through a feedback loop. TORC1 and its downstream substrate S6 kinase (S6K) phosphorylate insulin receptor substrate 1 (IRS1) and growth factor bound-receptor protein 10 (GRB10, a negative regulator of insulin/IGF-1 receptor signalling), inhibiting insulin/PI3K signalling (Hsu et al., 2011; Yu et al., 2011). S6K also destabilizes TORC2 by phosphorylating Rictor and mSIN1 (Dibble et al., 2009; Julien et al., 2010; Liu et al., 2013). Besides, AMPK phosphorylates TORC2 components (mTOR and Rictor) to increase TORC2 activity and its substrate Akt, thereby promoting cell survival (Kazyken et al., 2019).

1.2.3 Downstream effects of mTOR

TORC1 mainly regulates cell growth and translation through phosphorylation of two key downstream effectors: S6K and 4EBP1 (the eukaryotic initiation factor 4E (eIF4E) binding protein 1). When TORC1 is active under an amino acid-rich diet, the phosphorylated S6K phosphorylates several substrates that promote mRNA translation initiation, including eIF4B, a positive regulator of eIF4A helicase activity, and PDCD4 (programmed cell death 4), an inhibitor of eIF4A (Dorrello et al., 2006; Holz et al., 2005). In a parallel pathway, TORC1-mediated phosphorylation of 4EBP1 triggers its association with the eIF4E, allowing the latter to activate 5′cap-dependent mRNA translation (Ma & Blenis, 2009).

Moreover, TORC1 promotes cell growth through suppression of protein degradation, most notably autophagy. Under starvation, TORC1 phosphorylates ULK1 (Unc-51-like autophagy activating kinase)/ATG1 (autophagy-related 1), a kinase that drives autophagosome formation (Kim et al., 2011). TORC1 also phosphorylates and inhibits the nuclear translocation of the transcription factor EB (TFEB), which controls the expression of genes involved with lysosomal biogenesis and the autophagy machinery (Martina et al., 2012; Roczniak-Ferguson et al., 2012; Settembre et al., 2012).

TORC1 is a central regulator of glucose and lipid metabolic processes. TORC1 promotes de novo lipid synthesis by activating the transcription factor sterol responsive element binding protein 1 (SREBP1) to drive the expression of genes involved in fatty acid and cholesterol biosynthesis (Porstmann et al., 2008). TORC1 also increases the transcription factor hypoxia-inducible factor 1-alpha (HIF1α) translation, which leads to the expression of several glycolytic enzymes to promote glycolysis (Duvel et al., 2010).

TORC2 controls cell proliferation and survival by phosphorylating AGC family kinases, including Akt and SGK1 (serum/glucocorticoid-regulated kinase 1) (Garcia-Martinez & Alessi, 2008; Guertin et al., 2006). For example, TORC2 activates Akt to promote cell survival and growth by phosphorylating several Akt substrates such as FoxO1/3a (forkhead box protein O/O3A) transcription factors, the metabolic regulator GSK3β (glycogen synthase kinase 3β), and the TORC1 inhibitor TSC2 (Guertin et al., 2006).

1.2.4 Role of mTOR in ageing and age-associated diseases

mTOR has been implicated in several age-related processes, including cellular senescence, regulation of stem cells, autophagy, and mitochondrial function (Ben-Sahra et al., 2016; Herranz et al., 2015; Juricic et al., 2022; Laberge et al., 2015; Y. X. Lu et al., 2021; Yilmaz et al., 2012). Dietary longevity interventions, such as caloric restriction (CR), have also been shown to involve the TORC1 signalling pathway (Lopez-Otin et al., 2023).

Suppression of TORC1 signalling genetically, or pharmacologically by feeding with the FDA-licensed TORC1-specific inhibitor rapamycin, promotes lifespan and healthspan in various model organisms, including nematode worms, fruit flies and mice (Bjedov et al., 2010; Harrison et al., 2009; Robida-Stubbs et al., 2012). Mechanically, rapamycin modulates lifespan via two evolutionarily conserved TORC1 downstream effector mechanisms: autophagy and

S6K (Bjedov et al., 2010). Autophagy is a lysosome-dependent cellular clearance pathway that is tightly linked with cellular and tissue homeostasis (Aman et al., 2021). Promoting autophagy enhances longevity and suppresses age-related tissue deterioration in *Drosophila* and mice (Aman et al., 2021; Y. X. Lu et al., 2021; Pyo et al., 2013). Moreover, recent findings implicate the intestine as a crucial organ mediating the effects of increased autophagy on health during ageing (Bjedov et al., 2020; Gelino et al., 2016; Y. X. Lu et al., 2021). S6K is an AGC family kinase that regulates various fundamental cellular processes, including translation, lipid metabolism and immunity (Magnuson et al., 2012). Female mice deficient of S6K1 function are long-lived and resistant to age-related pathologies such as immune and motor dysfunction (Selman et al., 2009).

Moreover, TORC1 inhibition also ameliorates several age-related pathologies, including muscle atrophy, cardiac dysfunction in mice (Dai et al., 2014; Flynn et al., 2013; Tang et al., 2019), and, more importantly, immune disorders in mice and even in humans (Chen et al., 2009; Mannick et al., 2014; Mannick et al., 2018). Age-associated changes in the immune system include chronic inflammation, termed "inflammageing", and deterioration of immune function, termed "immunosenescence" (Fulop et al., 2017; Muller et al., 2013). Age-related changes in the immune system contribute to a wide range of age-associated diseases, such as cancer, diabetes, and cardiovascular diseases (Ferrucci & Fabbri, 2018; Fulop et al., 2017; Lopez-Otin et al., 2013). The nuclear factor-kappa B (NF-κB) pathway plays a crucial role in innate immunity and rapamycin treatment ameliorates senescence-associated NF-κB activation (Laberge et al., 2015). The effects of rapamycin on inflammageing and lifespan are diminished in mice with genetically enhanced NF-κB activity (*nfκb1*$^{-/-}$) (Correia-Melo et al., 2019), suggesting the age-prolonging effects of rapamycin are partially mediated by its ability to limit NF-κB signalling. Moreover, late-life TORC1 inhibition using a rapamycin derivative improves immune function in older people after influenza vaccination without significant adverse effects (Mannick et al., 2018). Interestingly, TORC1 pathway activity also shows significant associations with the load of bacteria in old flies (Felix et al., 2012; Schinaman et al., 2019), suggesting that enhanced immune function from suppression of TORC1 is conserved in flies.

1.3 Role of S6K in physiology and diseases

1.3.1 Structure and regulation of S6K

S6K is a member of the AGC kinase family, named for its three representative families cAMP-dependent protein kinase (PKA), cGMP-dependent protein kinase (PKG), and protein kinase C (PKC). These kinases share several conserved structural features and play critical roles in various cellular processes (Figure 1-2).

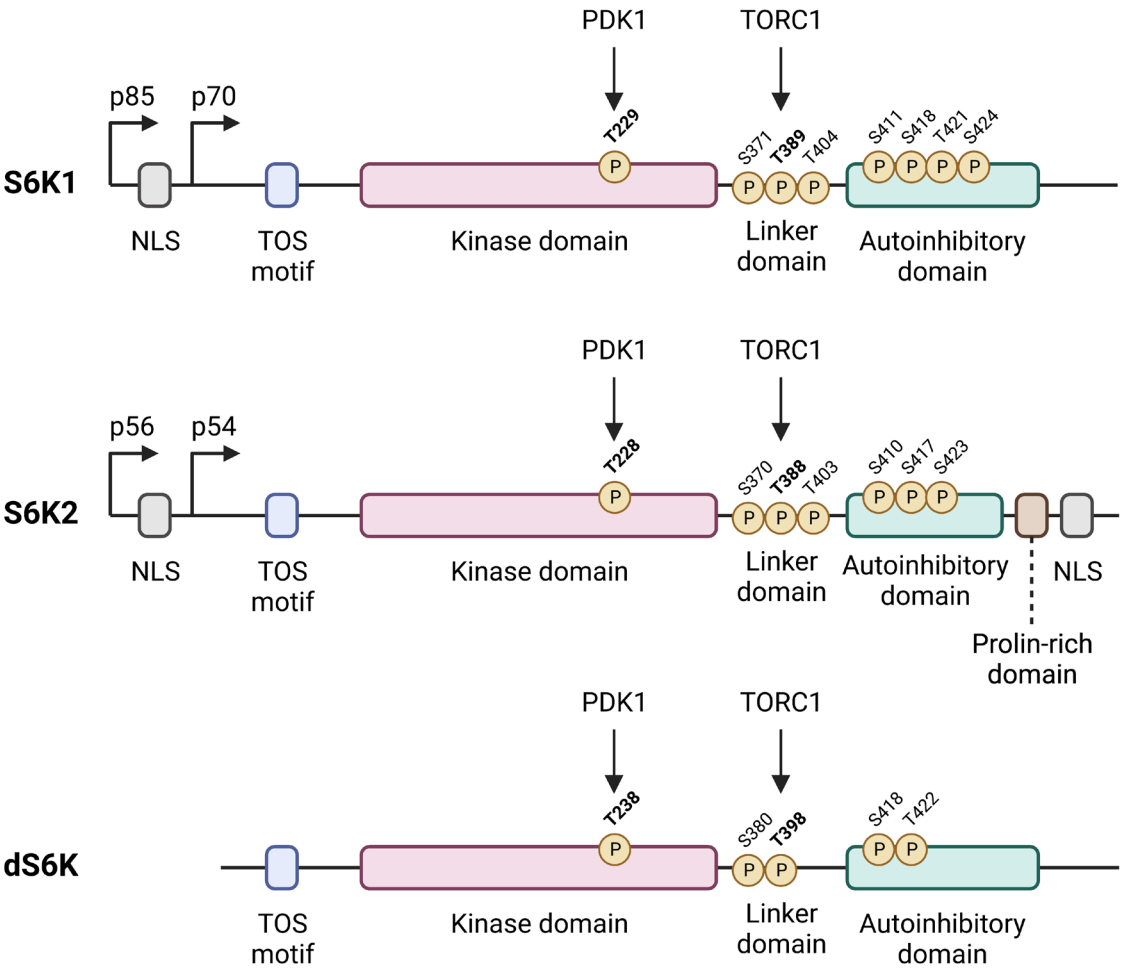

Figure 1-2: Domain structure of mammalian and *Drosophila* S6K.
Shown are schematic diagrams of the mammalian S6K1 (top), mammalian S6K2 (middle), and *Drosophila* S6K (dS6K, bottom). TORC1 phosphorylates the hydrophobic motif of S6Ks (S6K1 at T389, S6K2 at T388, and dS6K at T398) in the linker region and PDK1 phosphorylates the activation loop site (S6K1 at T229, S6K2 at T228, and dS6K at T238) within the kinase domain.

In mammals, there are two forms of S6K: S6K1 (*RPS6KB1*, also known as S6Kα) and S6K2 (*RPS6KB2*, also known as S6Kβ), which share a high level of sequence homology but are encoded by distinct genes (Lee-Fruman et al., 1999). As a result of the alternative translation start sites, S6K1 and S6K2 each encode two protein isoforms: p70-S6K and p85-S6K for S6K1 (Grove et al., 1991), as well as p54-S6K and p56-S6K for S6K2 (Gout et al., 1998). Although both S6K1 and S6K2 are required for full rpS6 (40S ribosomal protein S6) phosphorylation, studies on S6Ks have mainly focused on S6K1 (Magnuson et al., 2012; Pende et al., 2004). In contrast to mammalian rpS6, the phosphorylation of *Drosophila* rpS6 is carried out by *Drosophila* S6K (dS6K), which is encoded by a single gene. *Drosophila* S6K has also been shown to phosphorylate mammalian rpS6 in a mitogen-dependent, wortmannin- and rapamycin-sensitive manner, suggesting that its regulation is similar to that of mammalian p70-S6K (Watson et al., 1996).

S6K1 protein can be subdivided into several important regulatory domains: an N-terminus that contains a NLS (nuclear localization sequence); the TOS (TOR signalling) motif, which binds directly to Raptor for TORC1-dependent activation; a kinase domain that contains the activation loop; a linker region that contains the hydrophobic motif; and a C-terminal autoinhibitory domain, which blocks the activation loop from TORC1 and PDK1 (Figure 1-2). In S6K2, the additional C-terminal NLS and the proline-rich domain may enable diverse cellular localization and protein interactions (Gout et al., 1998).

It is widely accepted that S6K1 activation requires mitogen-induced phosphorylation of the C-terminal autoinhibitory domain on multiple sites. This relieves inhibition by inducing a conformational change that allows TORC1 access to the linker domain and thus phosphorylates T389. Phosphorylated T389 serves as a docking site for PDK1, which then phosphorylates the activation loop on T229, finally leading to a full S6K1 activation (Magnuson et al., 2012). Notably, the domain structure and phosphorylation sites are highly conserved in *Drosophila* S6K (Stewart et al., 1996; Watson et al., 1996). While phosphorylation is the most well-understood mechanism that regulates S6K1, other post-translational modifications such as phosphatase-mediated dephosphorylation (Hahn et al., 2010; Liu et al., 2011), acetylation (Fenton, Gwalter, Cramer, et al., 2010; Fenton, Gwalter, Ericsson, et al., 2010), and ubiquitination (Panasyuk et al., 2008; Wang et al., 2008) have also been suggested to play a role.

1.3.2 Downstream substrates and effectors of S6K

The discovery of several substrates for S6K has expanded its role beyond rpS6 in controlling protein production and has implicated S6K as a regulator of mRNA and protein synthesis, cell survival, inflammation and immune responses, and the retrograde control of insulin and mTOR signalling (Figure 1-3) (Magnuson et al., 2012; Wu et al., 2022).

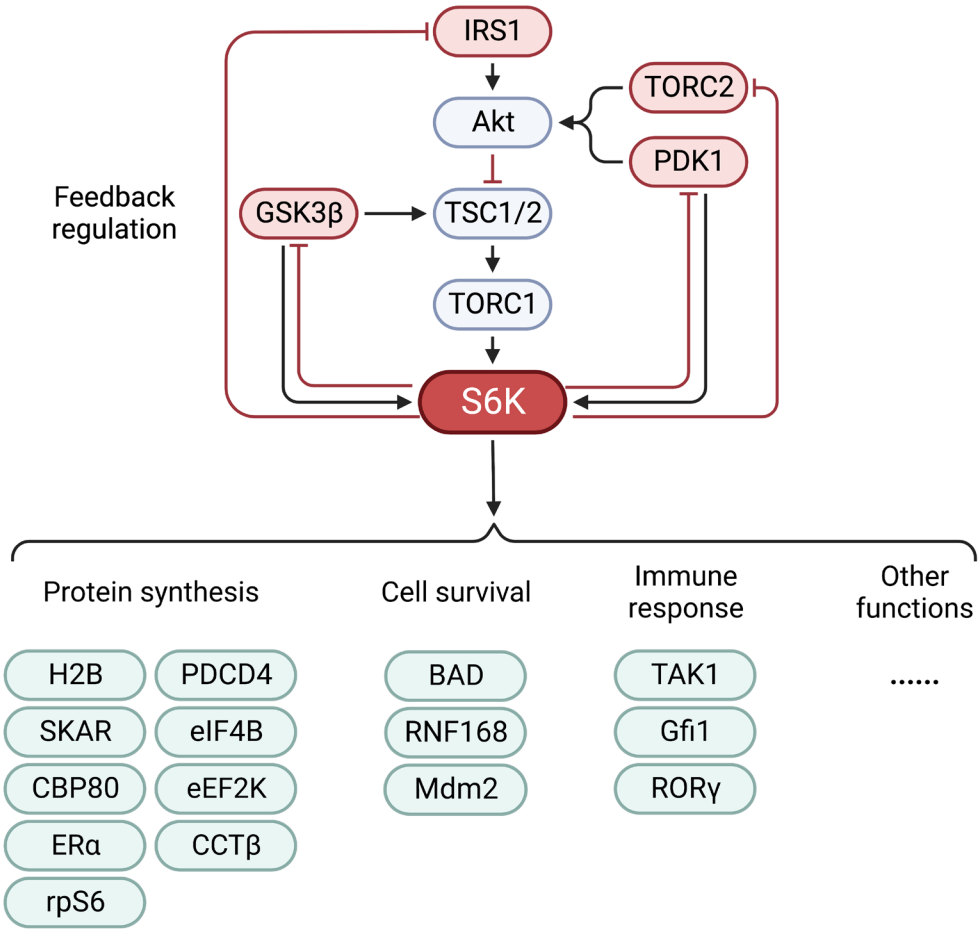

Figure 1-3: Key upstream regulators and downstream effectors of S6K.
After phosphorylation and activation by both TORC1 and PDK1, S6K promotes various cellular processes, including mRNA and protein synthesis, cell survival, and inflammation, through the regulation of multiple downstream substrates. S6K also drives feedback regulation on PI3K-Akt-mTOR signalling.

S6K targets several substrates involved in mRNA synthesis and processing. S6K phosphorylates histone H2B, which leads to trimethylation of lysine 27 on histone H3. This epigenetic mark suppresses *Wnt* gene expression and promotes adipogenesis (Yi et al., 2016).

S6K1 phosphorylates ERα (estrogen receptor α) directly, resulting in the activation of its transcriptional activity (Yamnik et al., 2009). Notably, ERα can also stimulate the transcriptional expression of S6K1 (Maruani et al., 2012). Upon growth factor stimulation, S6K1 directly binds and phosphorylates the CBC (the nuclear RNA cap-binding complex) subunit CBP80 (80 kDa nuclear cap-binding protein), resulting in increased RNA cap binding activity, which may stimulate efficient pre-mRNA splicing and subsequent mRNA translation (Wilson et al., 2000). Additionally, S6K1 phosphorylates SKAR (S6K1 Aly/REF-like substrate) to increase the translational efficiency of newly spliced mRNA (Ma et al., 2008; Richardson et al., 2004).

As the most well-studied substrate of S6K, S6 plays a crucial role in promoting protein synthesis through its phosphorylation and activation. While both S6K1 and S6K2 are necessary for the full phosphorylation of S6, the latter has a greater impact (Pende et al., 2004). Although the precise function of this phosphorylation remains unclear, the activation of S6 is vital for protein synthesis and cell cycle advancement, implying that S6Ks influence cell growth by affecting the translation process (Chauvin et al., 2014; Puighermanal et al., 2017). In addition to S6, S6K regulates protein synthesis by phosphorylating several substrates involved in translation initiation and elongation, as well as other steps. S6K1 phosphorylates eIF4B, which recruits eIF4B to eIF4A and enhances eIF4A helicase activity (Raught et al., 2004; Shahbazian et al., 2006). In parallel, S6K1-mediated phosphorylation of the eIF4A inhibitor PDCD4 further enhances eIF4A helicase activity by promoting its degradation by the proteasome (Dorrello et al., 2006). S6K phosphorylates eEF2K (eukaryotic elongation factor 2 (eEF2) kinase), in turn inactivating eEF2, thereby increasing eEF2 activity, which leads to enhanced translation elongation (Wang et al., 2001). S6K also phosphorylates CCTβ (chaperonin containing TCP-1β) and may regulate protein folding (Abe et al., 2009).

S6K1 plays an essential role in the regulation of cell survival, partially through the regulation of cellular apoptosis and DNA damage response. S6K1 has been shown to phosphorylate BAD (BCL2 associated agonist of cell death), a pro-apoptotic protein, which inactivates its pro-apoptotic function (Harada et al., 2001). Upon encountering DNA damage, the p38 MAPK (mitogen-activated protein kinase) pathway triggers the activation of TORC1-S6K1. Subsequently, S6K1 binds to and phosphorylates MDM2 (murine double minute 2), thereby impeding its nuclear translocation and its ability to ubiquitinate the tumour suppressor protein p53 (Lai et al., 2010). As a consequence, the increased p53 protein level leads to apoptosis.

Additionally, S6K phosphorylates the E3 ubiquitin ligase RNF168, which leads to its degradation by the E3 ubiquitin-protein ligase TRIP12 and impairing its function in the DNA damage response, ultimately resulting in genome instability (Xie et al., 2018).

S6K has also been linked to a variety of other cellular processes, including inflammation and immune cell differentiation. S6K1 negatively regulates TLR (toll-like receptor) signalling pathway and pro-inflammatory cytokine production via TAK1 (transforming growth factor β (TGF-β)-activated kinase 1), a key regulator of inflammatory signalling transduction. S6K1 hinders the interaction between TAK1 and TAB1 (TAK1-binding protein 1), a crucial regulator for TAK1 catalytic function, thereby impeding TAK1 activity (Kim et al., 2014). Th17 (T helper 17) cell differentiation, which is crucial in maintaining mucosal barriers and clearing pathogens at mucosal surfaces, is controlled by two essential transcription factors, namely RORγ (RAR-related orphan receptor γ) and Gfi1 (growth factor independent 1). S6Ks play a crucial role in controlling Th17 differentiation by regulating the expression and nuclear translocation of these transcription factors. S6K1 promotes Th17 differentiation by suppressing Gfi1 expression through the expression of EGR2 (early growth response protein 2), while S6K2 binds to RORγ and helps in transporting it to the nucleus (Kurebayashi et al., 2021).

Several core components of the insulin/mTOR signalling network are affected by an S6K-mediated feedback loop. S6K represses the expression of the IRS1 gene, as well as directly binds and phosphorylates IRS1, which impairs its function as an adaptor and thereby makes it insensitive to upstream stimuli. (Carracedo et al., 2008; Harrington et al., 2004; Tremblay et al., 2007; Um et al., 2004). S6K plays a dual role in the regulation of Akt activation. On the one hand, it directly phosphorylates PDK1 and impairs PDK1 interaction with and activation of Akt (Jiang et al., 2022). On the other hand, S6K regulates the activity of TORC2 by phosphorylating key components of TORC2, mSIN1 and Rictor, may leading to the phosphorylation and subsequent full activation of Akt (Dibble et al., 2009; Julien et al., 2010; Liu et al., 2013). In addition to direct activation of S6K1, GSK3 can also be phosphorylated and inhibited by S6K1 under nutrient deprivation conditions (Shin et al., 2011; Zhang et al., 2006).

1.3.3 Function of S6K in physiology and diseases

S6K has a diverse range of functions in metabolism, diseases, and ageing. The most striking change in S6K1-deficient animals is the dramatic decrease in body and organ size, which is

caused by the cell-autonomous reduction in cell size (Montagne et al., 1999; Pende et al., 2004). S6K also mediates metabolic homeostasis. Deletion of S6K1 causes hypoinsulinemia, glucose intolerance and reduced β-cell mass in mice (Pende et al., 2000). Strikingly, a TORC1–S6K1-mediated negative-feedback loop determines IRS1 activity, and thereby regulates glucose tolerance and insulin sensitivity (Bae et al., 2012; Tremblay et al., 2007; Um et al., 2004). *S6K1*$^{-/-}$ mice also have reduced body fat mass due to the impaired de novo adipocyte generation (Carnevalli et al., 2010). Deletion of S6K activates AMPK signalling in multiple tissues to control the energy state of the cell and the AMPK-dependent metabolic program (Aguilar et al., 2007). Besides, mediobasal hypothalamic S6K1 activity plays a role in controlling feeding and metabolic responses that are necessary for maintaining energy balance (Blouet et al., 2008).

S6K1 plays a crucial role in shaping the tumour microenvironment to promote tumour growth. It controls hematopoietic stem cell self-renewal and the maintenance of leukaemia (Ghosh et al., 2016). The absence of S6K1 in the tumour microenvironment hinders tumour angiogenesis by impairing the expression of HIF1α and its target genes, and thus suppresses tumour growth (Lee et al., 2020). In addition to cancer, S6K1 also contributes to muscle hypertrophy in airway smooth muscle and skeletal muscle (Deng et al., 2010; Ohanna et al., 2005).

As an essential downstream substrate of TORC1, S6K has been implicated in ageing and age-associated disorders. The expression of S6K1 is elevated in the brains of old patients with Alzheimer's disease (AD) (Caccamo et al., 2015). In a mouse model of AD, S6K1 repression has been shown to improve synaptic plasticity and spatial memory deficits and reduce the accumulation of amyloid-β and tau, which are the two neuropathological hallmarks of AD (Caccamo et al., 2015). Ubiquitous deletion of S6K increases lifespan in worms and deletion of S6K1 does so in mice, implicating S6K in the regulation of longevity (McQuary et al., 2016; Selman et al., 2009). Mice deficient of S6K1 function are not only long-lived, but are also resistant to age-related pathologies, such as immune and motor dysfunction, with a gene expression pattern that is similar to caloric restriction or pharmacological activation of AMPK (Selman et al., 2009). However, mechanistic understanding of how TORC1-S6K signalling promotes lifespan and healthspan remains limited.

1.4 Aim of the thesis

TORC1 is an evolutionarily conserved regulator of longevity and healthspan. S6K is an essential downstream mediator for the effect of TORC1 on longevity. However, mechanistic insights on how TORC1-S6K signalling promotes lifespan and healthspan are still limited. *Drosophila* provides an ideal model organism to dissect the role of S6K in regulating the ageing process as it has only a single S6K, which bears significant homology to the mammalian p70-S6K1 (Stewart et al., 1996). In this thesis, I aimed to uncover the mechanisms by which suppression of S6K activity ameliorates age-associated dysfunction and extends lifespan. My specific aims were:

- Determine the effect of S6K on longevity in *Drosophila*
- Identify the fly tissue critical for S6K-dependent lifespan extension
- Characterize the effects of TORC1-S6K signalling in early and late adulthood
- Elucidate the molecular mechanisms mediating TORC1-S6K-dependent longevity
- Verify the findings in rapamycin-treated mice

2 MATERIALS AND METHODS

2.1 Fly work

2.1.1 Fly stock and husbandry

All transgenic fly lines were backcrossed for at least six generations into the outbred wild-type strain, white Dahomey (w^{Dah}) (Grandison et al., 2009). For experiments, flies were maintained on 10% (w/v) brewer's yeast, 5% (w/v) sucrose and 1.5% (w/v) agar food at 25°C, 60% humidity, on a 12 h:12 h light:dark cycle. Rapamycin (LC Laboratories, #R-5000) was dissolved in ethanol and added to the food at a concentration of 200 µM. RU486 (Sigma, #M8046) was dissolved in ethanol and added to the food at a concentration of 200 µM to induce gene expression using the GeneSwitch system (Roman et al., 2001). The corresponding control food contained only ethanol. Female flies were used in all experiments. Fly genotypes are indicated in the figure legend or in the panel body. Fly stocks are listed as below.

Fly strain	Source	Identifier
w^{Dah}	This lab	
w^{Dah};*Lsp2GS*	Ragheb et al. (2017)	
w^{Dah};*daGS*	This lab	
w^{Dah};*ElavGS*	This lab	
w^{Dah};*MHCGS*	This lab	
w^{Dah};*HandGS*	This lab	
w^{Dah};*TiGS*	This lab	
w^{Dah};*UAS-S6K*^{CA}	This lab	
w^{Dah};*UAS-S6K*^{RNAi}	VDRC	18126
w^{Dah};*UAS-Atg5*^{RNAi}	This lab	
w^{Dah};*UAS-Syx13*	FlyORF	F003008
w^{Dah};*UAS-Syx13*	This lab	
w^{Dah};*UAS-Syx13*^{RNAi}	VDRC	102432
w^{Dah};*UAS-YFP-Rab5*^{DN}	BDSC	9771
w^{Dah};*UAS-YFP-Rab7*^{DN}	BDSC	9778
w^{Dah};*UAS-YFP-Rab5*	BDSC	23270
w^{Dah};*UAS-YFP-Rab7*	BDSC	24616
w^{Dah};*UAS-GFP-Lamp1*	Pulipparacharuvil et al. (2005)	
w^{Dah};*Lamp1-3xmCherry*	Hegedus et al. (2016)	
w^{Dah};*UAS-Relish*^{RNAi}	VDRC	49413

2.1.2 Lifespan, fecundity and starvation assays

For lifespan assays, parental flies were crossed in cages with grape juice agar plates and fresh yeast paste. Flies were allowed to lay eggs for 18 h, embryos were collected in PBS and dispensed into bottles at 20 µL per bottle to achieve standard larval density. Flies that enclosed within a 24 h time window were then transferred to fresh bottles where they were allowed to mate for 48 h. Subsequently, flies were anaesthetised with CO_2 and 20 female flies were sorted to vials. Flies were transferred to fresh food vials every two to three days and scored for death. For fecundity assays, flies were treated as described above for the lifespan assay, but only five female flies were used per vials. Eggs laid within 20 h were collected and counted twice a week in the first four weeks. For starvation assays, female flies were reared and maintained as for lifespan assay. 10-day-old flies were transferred to 1% agar and scored for deaths at least twice per day.

2.1.3 Antibiotic treatment

For antibiotic treatment, tetracycline and ampicillin were dissolved in water and added to the food in a concentration of 100 mg/L and 50 mg/L, respectively. In order to test whether the treatment was efficient at removing bacteria, flies were dipped in 70% ethanol for 3 min and rinsed with sterile PBS. Individual flies were mashed in sterile PBS. Samples were plated on LB plates, cultured at room temperature, and the number of bacterial colonies was scored at day 7.

2.1.4 Infection assays

For infection assays, flies were infected by pricking the thorax with a fine needle dipped in freshly cultured *Ecc15* (OD^{600} = 200). At 0 or 12 hour post-infection (h.p.i.), flies were surface sterilized with 70% ethanol, rinsed with sterile PBS, and then three flies were homogenized in sterile PBS by FastPrep-24 Matrix-D. Tenfold-series dilutions of fly homogenates were plated on LB plates as spots, cultured at room temperature, and the number of bacterial colonies was scored the next day. 0 h.p.i. represented the initial infectious dose and was not included in the statistical analysis. For *ex vivo* infection assay, fat bodies were dissected in sterile PBS and immediately incubated with PBS containing freshly cultured *Ecc15* (final concentration OD^{600} = 0.2) at room temperature for 1 h. For sham control, tissues were incubated with sterile PBS at room temperature for 1 h. Samples were then processed as described for immunofluorescence assay.

2.2 Mouse husbandry

The mouse rapamycin study was performed in accordance with the recommendations and guidelines of the Federation of the European Laboratory Animal Science Association (FELASA), with all protocols approved by the Landesamt für Natur, Umwelt und Verbraucherschutz, Nordrhein-Westfalen, Germany (reference no. 81-02.04.2019.A313). Female F_1 hybrid mice (C3B6F1) were generated in-house by crossing C3H/HeOuJ females with C57BL/6NCrl males (strain codes 626 and 027, respectively, Charles River Laboratories). Animals were housed in groups of five females in individually ventilated cages under specific-pathogen-free conditions with constant temperature (21 °C), 50-60% humidity and a 12 h:12 h light:dark cycle. For environmental enrichment, mice had constant access to nesting material and chew sticks. Rapamycin treatment was initiated at 6 months of age and was administrated continuously. Encapsulated rapamycin was obtained from Rapamycin Holdings and was added to the food (ssniff R/M-low phytoestrogen, ssniff Spezialdiäten) at a concentration of 42 mg/kg. Control food contained corresponding amounts of the encapsulation material Eudragit S100. Both rapamycin and control food contained 3.2 mL/kg PEG-400. For tissue collection, mice were killed by cervical dislocation, and tissues were rapidly collected and snap-frozen using liquid nitrogen.

2.3 Biochemistry and molecular biology methods

2.3.1 Generation of an *UAS-Syx13* transgenic fly line

To generate the transgenic *UAS-Syx13* fly line, full-length *Syx13* sequence was PCR amplified with primers (forward: *TTTTTTCTCGAGCACCatgtccaaggccttgaaca* and reverse: *TTTTTTTCTAGAttaactgttcagtttggcaacga*) using cDNA clone LD27581 (*Drosophila* Genomics Resource Center) as template. The PCR product was digested with *XhoI* and *XbaI* (NEB) and cloned into the *pUAST-attB* vector (Bischof et al., 2007). To increase expression efficiency, a *CACC* Kozak sequence was inserted directly before the *ATG* start codon. The phiC31-mediated integrase system (Bischof et al., 2007) was used to generate transgenic flies, using the *attP40* insertion site (Markstein et al., 2008). Embryos were injected by Jacqueline Eßer at the Max Planck Institute for Biology of Ageing.

2.3.2 Peptide preparation for LC-MS/MS analysis

Dissected fat bodies from young (day 10) and old (day 50) female flies (five fat bodies were pooled as a replicate, four replicates per group) or 50 mg 24-month-old mouse livers were lysed in 6 M guanidinium chloride, 2.5 mM TCEP (Tris(2-carboxyethyl)phosphine hydrochloride), 10 mM chloroacetamide, 100 mM Tris-HCl for 10 min at 95°C. Lysates were further disrupted by using a Bioruptor plus (Diagenode) with 30 s sonication and 30 s break for 10 cycles. Samples were then diluted 10fold with 20 mM Tris and trypsin (Trypsin Gold, Promega, #V5280, 1:200 w/w) and digested overnight at 37°C. Digestion was stopped by adding formic acid to a final concentration of 1%. Peptide cleaning was performed by using in-house C18-SD (Empore) StageTips (Li et al., 2021). Therefore, StageTips were washed with methanol and 40% acetonitrile (ACN)/0.1% formic acid (FA) and finally equilibrated with 0.1% FA. Digested peptides were loaded on the equilibrated StageTips. StageTips were washed twice with 0.1% FA and peptides were eluted with 40% ACN/0.1% FA and then dried in a Speed-Vac (Eppendorf) at 45°C for 45 min.

2.3.3 TMT labelling and fractionation

Note: This part of the experiment was conducted by the Proteomics Core Facility at the Max Planck Institute for Biology of Ageing.

Eluted peptides were reconstituted in 0.1M triethylammonium bicarbonate (TEAB). TMTpro 16plex (ThermoFisher, #A44522) labelling was carried out according to the manufacturer's instruction with the following changes: 0.5 mg of TMT reagent was re-suspended with 33 µL of anhydrous ACN. 7 µL of TMT reagent in ACN was added to 9 µL of clean peptide in 0.1M TEAB. The final ACN concentration was 43.75% and the ratio of peptides to TMT reagent was 1:20. All 16 samples were labelled in one TMT batch. After 60 min of incubation the reaction was quenched with 2 µL of 5% hydroxylamine. Labelled peptides were pooled, dried, re-suspended in 0.1% FA, and desalted using StageTips. Samples were fractionated on a 1 mm x 150 mm, 130Å, 1.7 µm ACQUITY UPLC Peptide CSH C18 Column (Waters, #186006935), using an Ultimate 3000 UHPLC (ThermoFisher). Peptides were separated at a flow of 30 µL/min with an 88 min segmented gradient from 1% to 50% buffer B for 85 min and from 50% to 95% buffer B for 3 min; buffer A was 5% ACN, 10 mM ammonium bicarbonate (ABC), buffer B was 80% ACN, 10 mM ABC. Fractions were collected every three minutes, and fractions were pooled in two passes (1 + 17, 2 + 18 … etc.) and dried in a Speed-Vac (Eppendorf).

2.3.4 LC-MS/MS analysis and protein identification

Note: This part of the experiment was conducted by the Proteomics Core Facility at the Max Planck Institute for Biology of Ageing.

Dried fractions were re-suspended in 0.1% FA and separated on a 500 mm, 0.075 mm Acclaim PepMap 100 C18 HPLC column (ThermoFisher, #164942) and analysed on a Orbitrap Lumos Tribrid mass spectrometer (ThermoFisher) equipped with a FAIMS device (ThermoFisher) that was operated in two compensation voltages, -50 V and -70 V. Synchronous precursor selection based MS3 was used for TMT reporter ion signal measurements. Peptides were separated by EASY-nLC1200 using a 90 min linear gradient from 6% to 31% buffer; buffer A was 0.1% FA, buffer B was 0.1% FA, 80% ACN. The analytical column was operated at 50°C. . Raw files corresponding to the mouse livers were split based on the FAIMS compensation voltage using FreeStyle (ThermoFisher). Fly fat body proteomics data were analysed using Proteome Discoverer (version 2.4.1.15); mouse liver proteomics data were analysed using MaxQuant (version 1.6.17.0). Isotope purity correction factors, provided by the manufacturer, were included in the analysis. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (Perez-Riverol et al., 2022) partner repository with the dataset identifier PXD035293.

2.3.5 Electron microscopy

Note: This part of the experiment was conducted by the Imaging Core Facility at CECAD, Cologne.

Fat bodies were fixed in 4% formaldehyde (Science Services) and 2,5% glutaraldehyde (Merck) in 0.1M Cacodylate buffer (AppliChem) for 48 h at 4°C. After washing in 0.1M cacodylate buffer, tissues were treated with 2% osmiumtetroxid (Science Services) in 0.1M Cacodylate buffer for 2 h. After dehydration of the sample with ascending ethanol concentrations followed by propylenoxid, samples were embedded in Epon (Sigma). Ultrathin sections (70 nm) were cut (EM-UC7, Leica Microsystems), collected onto mesh copper grids (Electron Microscopy Sciences), and contrasted with uranyl acetate (Plano GMBH) and lead citrate (Sigma). At least ten images per sample were acquired with a transmission electron microscope (JEM 2100 Plus, JEOL), a OneView 4K camera (Gatan) with DigitalMicrograph software at 80 KV at room temperature.

2.3.6 Lysotracker staining

Tissues were dissected in PBS and stained with LysoTracker Red DND-99 (ThermoFisher, #L7528, 1:2000) and Hoechst 33342 (Enzo, #ENZ-52401, 0.5 ng/µL). Samples were rinsed with PBS and mounted with VECTASHIELD Antifade Mounting Medium (Vector Laboratories, #H-1000). Three images per sample were captured using a Leica TCS SP8 DLS confocal microscope with a 20x objective and 6x digital zoom in. Images were processed by background subtraction, median filtering, and spot quantification using Imaris 9 (Bitplane). Settings of the confocal microscope were kept consistent between images of an experiment. Lysostracker-positive puncta were defined as large, when the diameter was greater than 1.5 µm, which is the 75th percentile of the diameter of rapamycin-induced Lysotracker-positive puncta.

2.3.7 Immunofluorescence

Tissues were dissected in PBS and fixed 30 min with 4% formaldehyde, methanol-free (ThermoFisher, #28908) at room temperature. Samples were washed in 0.3% Triton-X/PBS (PBST), blocked in 5% bovine serum albumin (BSA)/PBST for 1 at room temperature, incubated in primary antibody overnight at 4°C, and in secondary antibody for 2 h at room temperature. The following primary antibodies were used: anti-Relish (Developmental Studies

Hybridoma Bank, #21F3, 1:100), anti-Relish (RayBiotech, #RB-14-0004-200, 1:200), anti-phospho-Histone H3 (Cell Signaling Technology, #9701, 1:200). The following secondary antibodies were used: Alexa Flour 488 goat anti-mouse IgG (ThermoFisher, #A11001, 1:1,000), Alexa Flour 633 goat anti-mice IgG (ThermoFisher, #A21050, 1:1,000), Alexa Flour 594 goat anti-rabbit IgG (ThermoFisher, #A11012, 1:1,000), Alexa Flour 633 goat anti-rabbit IgG (ThermoFisher, #A21070, 1:1,000). Samples were mounted with VECTASHIELD Antifade Mounting Medium with DAPI (Vector Laboratories, #H-1200). Three images per sample were captured using a Leica TCS SP8 DLS confocal microscope with 20x objective and 6x digital zoom. For Relish protein localisation, images were processed by background subtraction and median filtering using Imaris 9 (Bitplane). The number of nuclei with high Relish fluorescence intensity was normalized by the total number of nuclei. Confocal settings were kept consistent between images of the same experiment.

2.3.8 Immunoblotting

Tissues were homogenized and lysed in ice-cold RIPA buffer supplemented with cOmplete, Mini, EDTA-free, Protease Inhibitor Cocktail (Roche, #11836170001) and PhosSTOP phosphatase inhibitor tablet (Roche, #049068370001) using a hand-held homogenizer. Extracts were centrifuged and protein concentrations were determined using Pierce BCA Protein Assay Kit (ThermoFisher, #23225). Extracts were mixed with 4x Laemmli loading buffer and boiled for 5 min at 95°C. 10 µg (fly tissues) or 25 µg (mouse tissues) of proteins were loaded per lane on Any kD or 4-20% Criterion TGX stain-free precast gels (Bio-Rad) and transferred to Immobilon-FL PVDF membrane (Millipore, #IPFL00010). Membranes were blocked by Intercept TBS Blocking Buffer (LI-COR, #927-60001) for 1 h and probed with the following primary antibodies diluted in Intercept T20 TBS Antibody Diluent (LI-COR, #927-65001): anti-S6K(Bjedov et al., 2010) (1:1,000), anti-pS6K T398 (Cell Signaling Technology, #9209, 1:1,000), anti-Relish (Developmental Studies Hybridoma Bank, #21F3, 1:100), anti-tubulin (Sigma, #T9026,1:5,000), anti-puromycin (Sigma, #MABE343, 1:1,000), anti-Stx12 (Synaptic Systems, #110 132, 1:1000). The following secondary antibodies were used: IRDye 800CW Goat anti-Mouse IgG (H + L) (LI-COR, #926-32210, 1:15,000) and IRDye 680RD Goat anti-Rabbit IgG (H + L) (LI-COR, #926-68071, 1:15,000). Total protein on the membrane was visualized as stain-free signal using ChemiDoc MP Imagers (Bio-Rad). Immunoblotting images were captured using Odyssey Infrared Imaging system with application software

V3.0.30 (LI-COR) and were analysed using Fiji (Schindelin et al., 2012) (US National Institutes of Health).

2.3.9 RNA isolation and qRT-PCR

Total RNA was extracted from tissues using the RNeasy Mini Kit (Qiagen, #74106) following the manual. RNA concentration was measured using the Qubit RNA Broad Range Assay Kit (ThermoFisher, #Q10211). cDNA synthesis was performed using SuperScript VILO Master Mix (ThermoFisher, #11755-250) with 600 ng total RNA as input. qRT-PCR was performed using SYBR Green Master Mix (ThermoFisher, #4367659) on a QuantStudio6 Flex Real-Time PCR System (ThermoFisher). Relative expression was determined using the ΔΔCT method and *Rpl32 (ribosomal protein L32)* as normalization control. The following primers were used:

DptA-forward: CCACGAGATTGGACTGAATG

DptA-reverse: GGTGTAGGTGCTTCCCACTT

Syx13-forward: GCGGCAGGTCGAGCAAATA

Syx13-reverse: AGTTCCGAGGTGCCATCCT

Rpl32-forward: ATATGCTAAGCTGTCGCACAAATGG

Rpl32-reverse: GATCCGTAACCGATGTTGGGCA

2.3.10 Triacylglyceride (TAG) assay

Five whole flies were homogenized in 0.05% Tween-20 and incubated at 70°C for 5 min. Extracts were centrifuged, the supernatant was mixed with prewarmed Infinity Triglycerides reagent (ThermoFisher, #TR22421) and incubated at 37°C for 5 min. Protein content was determined by Pierce BCA Protein Assay Kit (ThermoFisher, #23225). Triglyceride levels were determined by plate reader at the absorbance of 540 nm using a triglyceride standard (Sigma, #17811-1AMP). Protein levels were used for normalization.

2.3.11 Puromycin incorporation assay

Fat bodies were dissected in PBS and immediately transferred to Schneider's Drosophila media (Biowest, #L0207-500) with 5 µg/mL puromycin (Sigma, #P8833) in combination with 50 nM rapamycin (LC Laboratories, #R-5000) and/or 50 nM RU486 (Sigma, #M8046). As negative control, no puromycin was added in the Schneider's *Drosophila* media. Sample were incubated for 45 min with 400 rpm at 25°C and then lysed according to the immunoblotting assay.

2.3.12 Gut dysplasia assay

Guts were dissected in PBS and immediately fixed 30 min with 4% formaldehyde, methanol-free (ThermoFisher, #28908). Samples were washed with PBS and mounted with VECTASHIELD Antifade Mounting Medium with DAPI (Vector Laboratories, #H-1200). The R2 region of the gut was captured using a Leica TCS SP8 DLS confocal microscope with a 40x objective. Images were analysed using Fiji (Schindelin et al., 2012) (US National Institutes of Health). The percentage of dysplasia was calculated as the ratio between the length of multi-layer nuclei region and the total length of the R2 region of the gut.

2.4 Computational and biostatistical methods

2.4.1 Proteomics data analysis

Peptide intensity values were log2- and z-transformed. The results were rescaled by multiplying the z-intensity of each individual peptide with the global standard deviation of the log2-transformed data and adding back the global mean of the log2-transformed data. Only proteins that were detected in at least three out of the four replicates for each treatment were considered for downstream analyses. Missing values were imputed using the impute package (version 1.66.0) in R. Differential expression analysis was performed using the limma package (version 3.48.3) in R. For principal component analysis and plotting, batch effects from different dissection timepoints were removed from the normalized data using the limma package.

2.4.2 Network propagation

High confidence interactions of the STRING protein-protein association network database for *Drosophila melanogaster* or *Mus musculus* (Szklarczyk et al., 2019) were extracted as background for later propagation (combined score > 899). Network propagation was performed using the BioNetSmooth package (version 1.0.0) in R. Log2 fold changes of comparisons were imported into the network and propagated with $\alpha = 0.5$ for 26 iterations (for flies) or 25 iterations (for mice). Top and bottom 5% proteins, which contain more than four protein-protein interactions were used for further analysis.

2.4.3 Gene ontology term enrichment

Gene ontology information was retrieved from org.Dm.eg.db package (version 3.13.0) or Uniprot-GOA database (http://www.ebi.ac.uk/GOA/, version 2022-04-30) in R. The high confidence interactions of STRING database (combined score > 899) were set as background and the results of network propagation were used for gene ontology term enrichment analysis using Fisher tests with a minimal node size of five from ViSEAGO package (version 1.6.0) in R. For *Lsp2GS>S6K^{CA}* dataset, S6K^{CA}-dependent Rapa-induced annotations were selected if the p-value of Rapa vs Control annotation is at least 100 times than the p-value of Induced *vs* Induced+Rapa annotation (EtOHvsRapa..log10_pvalue - RUvsRURapa..log10_pvalue > 2). S6K^{CA}-independent Rapa-induced annotations were selected if the p-value of Rapa *vs* EtOH annotation is greater than 0.01 (EtOHvsRapa..log10_pvalue > 2) and if the p-value of Rapa *vs*

EtOH annotation is not 100 times than the p-value of Induce *vs* Induced+Rapa annotation (EtOHvsRapa..log10_pvalue - RUvsRURapa..log10_pvalue ≤ 2). For mouse liver analysis, the biological processes significantly regulated ($p<0.05$) as the same direction in all three datasets (hepatic proteome of 24m rapamycin-treated mice, hepatic microarray of 25m rapamycin-treated mice (Fok et al., 2014), and hepatic microarray of 20m S6K1 deficient mice (Selman et al., 2009)) are plotted as shared up- or down-regulated GO annotations after reducing redundancy via REVIGO (cutoff: "0.7", valueType: "pvalue", measure: "SIMREL"). The significance score was calculated by the -log10_p-value with the sign of regulation direction (positive values for up-regulated GOs and negative values for down-regulated GOs).

2.5 Statistics

All statistical analyses were performed in GraphPad Prism 9 and R 4.1.0. Linear mixed model and negative binomial generalized linear model were generated and analysed in R using lme4, lmertest, and emmeans package. Proteomics data were analysed in R using impute, limma, ViSEAGO package. Cox Proportional Hazards (CPH) test were performed in R using survival package. Log-rank tests were performed in Microsoft Excel for Mac (version 16.62) and R using survival package. For small sample sizes ($n < 8$), data are presented as individual points with mean ± s.e.m.. For large sample size ($n \geq 8$), box plots were used with median, 25th and 75th percentiles, and Tukey whiskers indicated. Sample sizes and statistical tests used are indicated in the figure legend.

3 RESULTS

3.1 S6K activity in the fat body is essential for longevity

3.1.1 S6K activity in adult flies is important for longevity

To address whether down-regulation of S6K activity prolongs lifespan in *Drosophila*, we used the inducible GeneSwitch system in combination with an S6K RNAi line to knock down S6K expression specifically in the adult stage. Ubiquitous downregulation of S6K activity using a ubiquitous *daGS* driver resulted in a small but significant increase in lifespan (Figure 3-1), demonstrating that, as in *Caenorhabditis elegans* and mice (McQuary et al., 2016; Selman et al., 2009), reduced S6K activity can also extend lifespan in *Drosophila*.

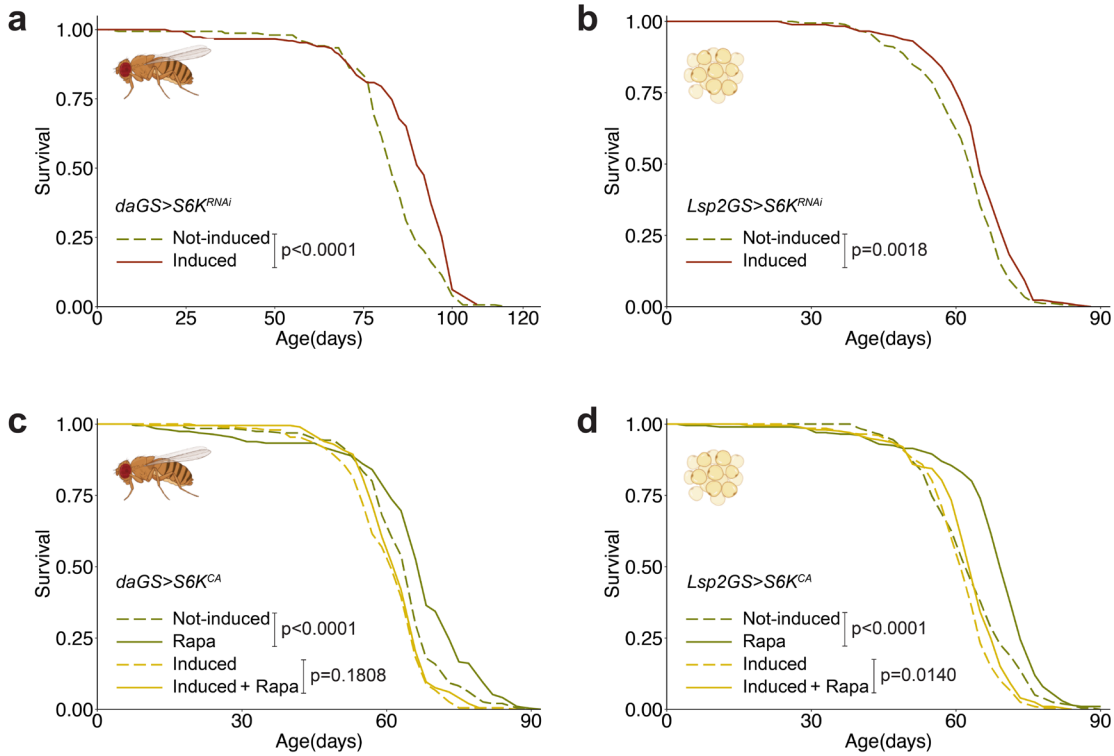

Figure 3-1: S6K activity in the fat body of adult flies determines longevity.

a, Adult-onset repression of S6K ubiquitously using *daGS>S6K^{RNAi}* extended lifespan (n=150). **b,** Adult-onset repression of S6K in the fat body using *Lsp2GS>S6K^{RNAi}* extended lifespan (n=180). **c,** Rapamycin extended lifespan of control flies, but not of flies with ubiquitous over-expression of constitutively active S6K (*daGS>S6K^{CA}*). Ubiquitous over-expression of constitutively active S6K significantly attenuated the response to rapamycin treatment (rapamycin: p<0.0001, *daGS>S6K^{CA}* induction: p<0.0001, interaction p=0.0236, n=200). **d,** Adult-onset S6K activation in the fat body (*Lsp2GS>S6K^{CA}*) significantly attenuated rapamycin-

related longevity (rapamycin: p<0.0001, *Lsp2GS>S6K^{CA}* induction: p<0.0001, interaction p=0.0331, n=200). Log-rank test and Cox Proportional Hazards (CPH) test.

3.1.2 *Drosophila* fat body is a central tissue for S6K-dependent longevity

To identify the tissue(s) in which reduced S6K activity acted to extend lifespan, we employed tissue-specific GeneSwitch drivers to suppress S6K activity in fat body tissue (*Lsp2GS*), neurons (*ElavGS*), muscle (*MHCGS*), heart tube (*HandGS*) and intestine (*TiGS*) of adult flies. Repression of S6K activity in neurons and intestine did not affect lifespan (Figure 3-2a,d), while in muscle and heart tube it shortened it (Figure 3-2b,c). Lifespan was extended only upon fat-body-specific repression of S6K activity (Figure 3-1b) indicating that, in *Drosophila*, activity of S6K in the fat body is limiting for lifespan. As the lifespan-extending effect of the TOR inhibitor rapamycin can be blocked by ubiquitous S6K activation (Bjedov et al., 2010), we next tested whether activating S6K just in fat body tissue was sufficient to block the effect of rapamycin on survival. We administered rapamycin to flies that over-expressed a constitutively active S6K protein (S6K^{CA}) (Barcelo & Stewart, 2002) under the control of the fat-body-specific *Lsp2GS* GeneSwitch driver (Ragheb et al., 2017) and measured their survival. Similar to the ubiquitous activation (Figure 3-1c), expression of S6K^{CA} specifically in the fat tissue was sufficient to block rapamycin-mediated lifespan extension (Figure 3-1d). In summary, these results implicate the fat body as a key tissue regulating survival downstream of TORC1 and S6K.

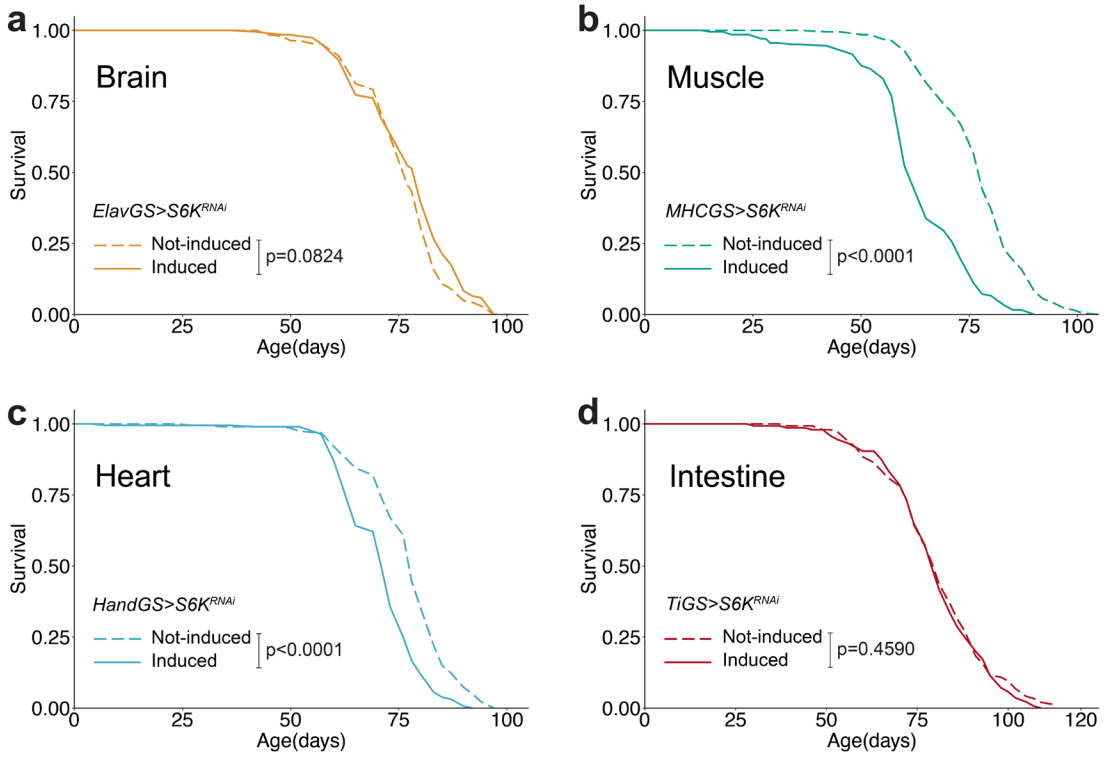

Figure 3-2: Downregulation of S6K activity in the brain, muscle, heart, or intestine does not extend lifespan.

a, Adult-onset repression of S6K in the brain using *elavGS>S6K^{RNAi}* did not affect lifespan (n=200). **b,** Adult-onset repression of S6K in the muscle using *MHCGS>S6K^{RNAi}* shortened lifespan (n=200). **c,** Adult-onset repression of S6K in the heart using *HandGS>S6K^{RNAi}* shortened lifespan (n=200). **d,** Adult-onset repression of S6K in the intestine using *TiGS>S6K^{RNAi}* did not affect lifespan (n=150). Log-rank test.

Note: These experiments were performed by James H. Catterson. Analysis and visualisation were carried out by Pingze Zhang.

3.2 Effects of TORC1-S6K signalling in early adulthood

3.2.1 Proteomics analysis of fat body

The *Drosophila* fat body is functionally equivalent to mammalian liver and white adipose tissue and is the central organ for metabolism and immune responses (Zheng et al., 2016). To gain insight into the physiological actions of TORC1-S6K signalling in the fat body during ageing, we used unbiased TMT-based proteomics profiling of fat bodies from day 10 (young)

and day 50 (old) flies that over-expressed a constitutively active S6K protein ($S6K^{CA}$) under the control of the fat body-specific *Lsp2GS* GeneSwitch driver (*Lsp2GS>S6K*^{CA}) in combination with rapamycin treatment, or flies that over-expressed a S6K RNAi line to knock down S6K in the fat body (*Lsp2GS>S6K*^{RNAi}) (Figure 3-3a). Principal component analysis (PCA) revealed clear separation among age and treatment (Figure 3-3b). We identified a total of 4101 proteins from the *Lsp2GS>S6K*^{CA} dataset and 4809 proteins from the *Lsp2GS>S6K*^{RNAi} dataset. To explore S6K-dependent protein alterations, we filtered the proteins that were significantly changed ($p<0.05$) by both rapamycin treatment and S6K manipulation. Proteins regulated by TORC1-S6K signalling were associated with endocytosis, RNA binding processes and extracellular matrix in young fat bodies, while in old fat bodies mitochondria-, translation- and immune-related proteins were significantly altered (Figure 3-3c).

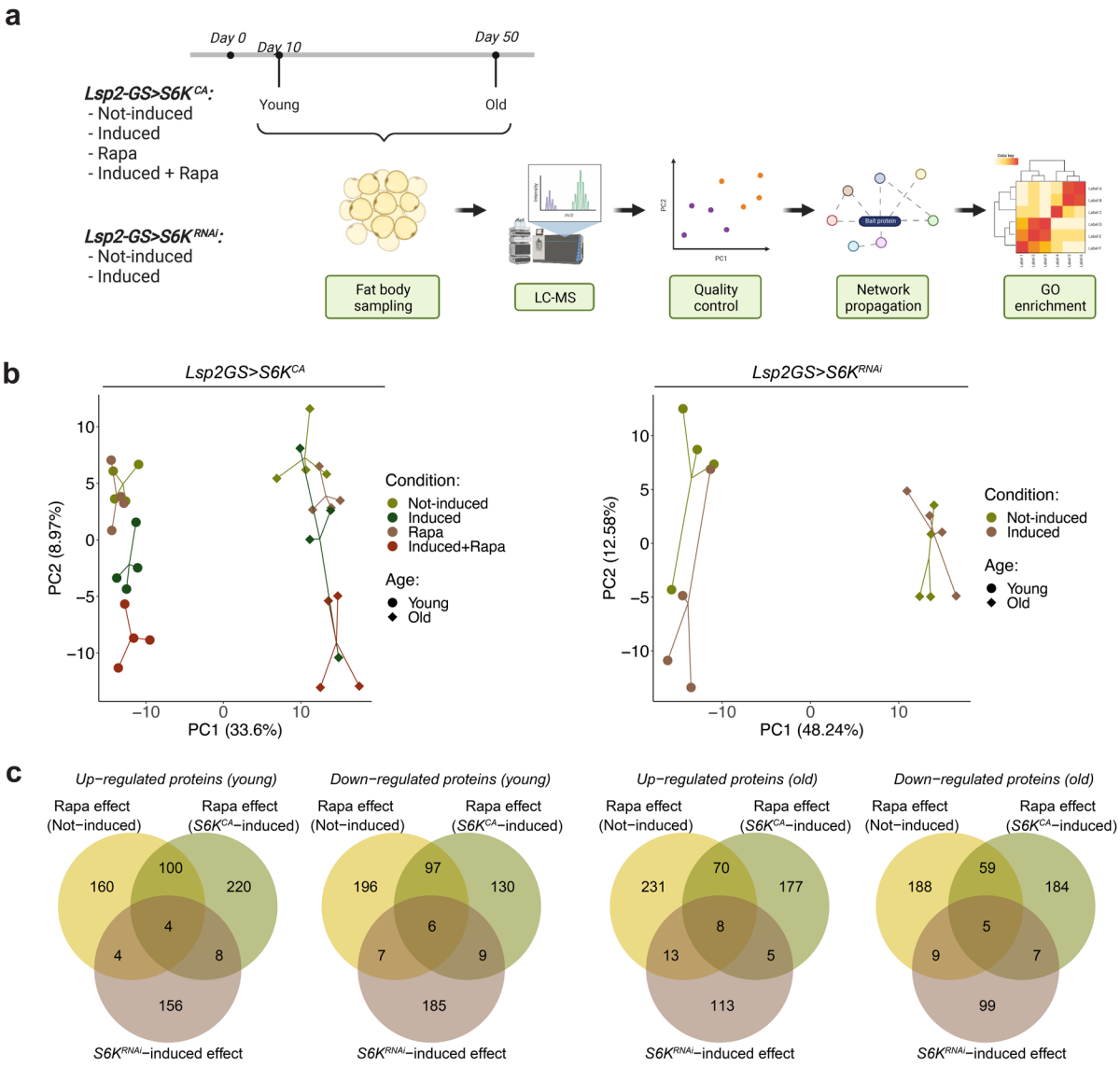

Figure 3-3: Proteomics analysis of TORC1-S6K dependent changes in the fly fat body. **a,** Outline of the proteomics experiment. **b,** Principal component analysis projections of proteomic replicates of *Lsp2GS>S6K^{CA}* (left) and *Lsp2GS>S6K^{RNAi}* (right), showing separation according to treatments and age. Each replicate represents a pool of five fat bodies**. c,** Venn diagram showing the number of up- and down-regulated proteins ($p<0.05$) of each comparison at young and old age. "Rapamycin effect" (not-induced) comprises differentially expressed proteins between *S6K^{CA} not-induced vs S6K^{CA} not-induced+Rapa*; "Rapamycin effect" (*S6K^{CA}*-induced) comprises *S6K^{CA}-induced vs S6K^{CA}-induced+Rapa;* and *S6K^{RNAi}*-induced effect comprises differentially expressed proteins between *S6K^{RNAi} not-induced vs S6K^{RNAi}-induced.*

To further enhance the resolution of our analysis, we conducted network propagation analysis (Vanunu et al., 2010) to incorporate information on protein-protein interactions. We then

identified functional categories and clustered them into rapamycin-induced, S6K-dependent terms and S6K inhibition-induced terms (Figure 3-4). Proteins directly affected by TORC1-S6K signalling were endosome/lysosome- oogenesis-, and translation-related, while at later age immune-, translation- and lipid-related processes were altered.

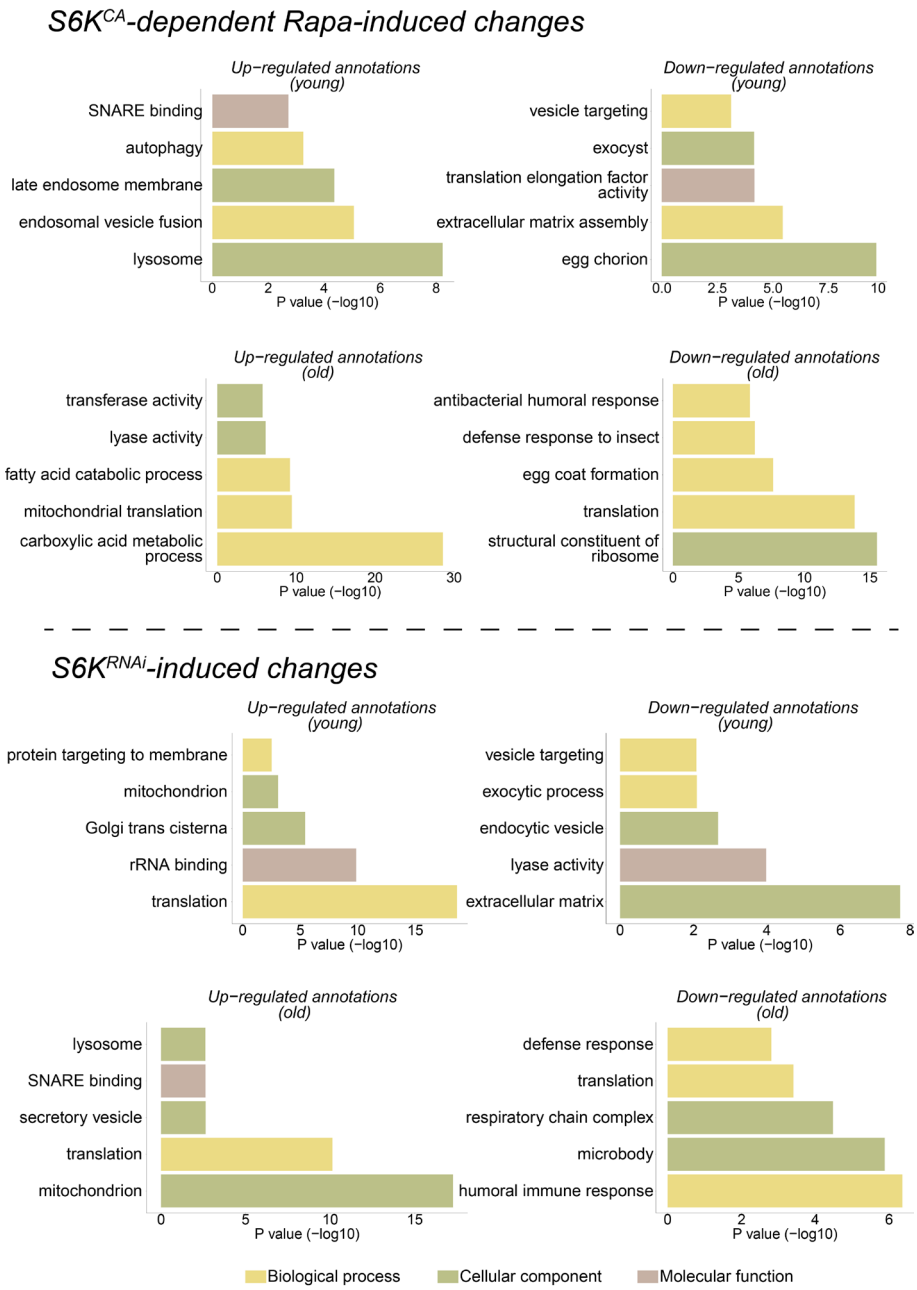

Figure 3-4: Functional enrichment and clustering analysis of the S6K-dependent targets. Representation of gene ontology (GO) terms in *Lsp2GS>S6K^CA* dataset (left) and *Lsp2GS>S6K^RNAi* dataset (right) after network propagation. S6K^{CA}-dependent Rapa-induced annotations were selected if the p-value of *S6K^CA not-induced vs S6K^CA not-induced+Rapa* annotation is at least 100 times greater than the p-value of *S6K^CA-induced vs S6K^CA-*

induced+Rapa annotation (EtOH vs Rapa..log10_pvalue – RU vs RURapa..log10_pvalue > 2). Colour indicates the categories of the terms.

3.2.2 S6K activity in the fat body does not affect fecundity, global translation, or triacylglycerol homeostasis

The fat body plays an important role in oogenesis and reproduction in flies and reduced reproduction is often associated with increased longevity (Flatt, 2011). As oogenesis was identified as a significantly enriched term in the proteomics analysis, we measured egg laying of females with fat body specific S6K downregulation and found that fecundity was not affected (Figure 3-5). This result suggests that reduced reproduction is not a causal factor in S6K-related lifespan-extension, consistent with the findings that rapamycin can extend lifespan in sterile flies (Bjedov et al., 2010).

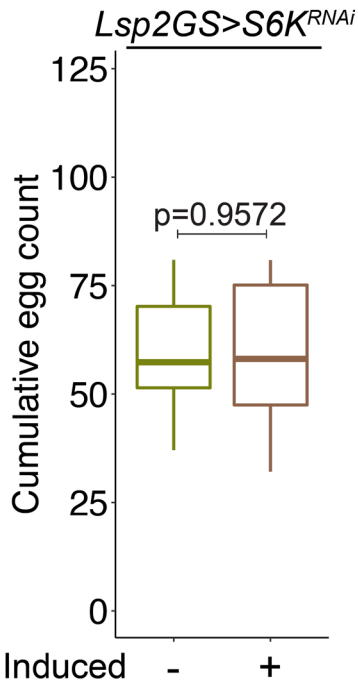

Figure 3-5: S6K inhibition in the fat body does not affect egg laying.
Adult-onset repression of S6K in the fat body using *Lsp2GS>S6K*^{RNAi} did not affect cumulative egg laying of flies from day 3 to day 25 (n=10 vials with 5 females). Data are displayed as Tukey box plot**.** Two-sided Student's t-test.

S6K is a regulator of protein synthesis, and protein translation was identified as an enriched term in the proteomics analysis. Furthermore, reduced protein synthesis has been associated with longevity in several invertebrate longevity models. Thus, we addressed whether S6K or rapamycin affect global protein synthesis in the fly fat body by performing puromycin incorporation assays. Interestingly, puromycin incorporation was affected neither by rapamycin treatment nor by S6K activation upon rapamycin feeding (Figure 3-6), suggesting that chronic rapamycin treatment and S6K activity do not change global translation rates in the fly fat body. Consistently, chronic rapamycin feeding also had no effect on global translation rates in multiple mouse tissues (Garelick et al., 2013).

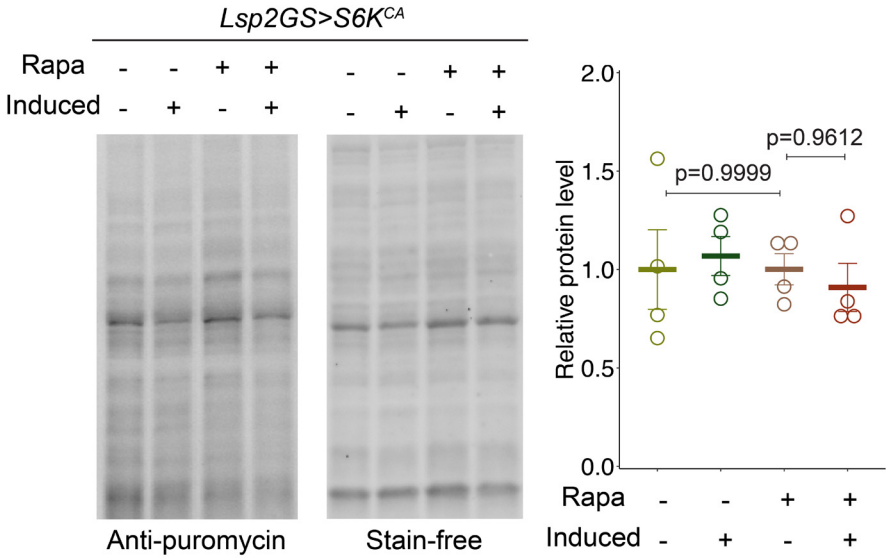

Figure 3-6: Rapamycin treatment does not affect global translation rates in the fat body. Rapamycin treatment or overexpression of constitutively active S6K specifically in the fat body (*Lsp2GS>S6K*^{CA}) did not affect the overall level of newly synthesized proteins in the fat body of young flies, depicted by puromycin immunoblotting (rapamycin: p=0.5660, *Lsp2GS>S6K*^{CA} induction: p=0.9320, interaction p= 0.5610, n=4). Stain-free blot served as loading control. Data are mean ± s.e.m.. Each data point represents an average value per five fat bodies. Linear mixed model followed by Tukey's multiple comparison test.

Lipid metabolism has been implicated in the ageing process (Johnson & Stolzing, 2019) and rapamycin increases lipid storage in flies (Bjedov et al., 2010). However, S6K activation did not alter rapamycin-induced triglyceride accumulation in the fat body (Figure 3-7a). In addition, repressing S6K in the fat body did not affect survival under starvation (Figure 3-7b), and

ubiquitous S6K activation did not attenuate rapamycin-induced starvation resistance (Figure 3-7c). Thus, TORC1-S6K-dependent longevity cannot be explained by an effect on triglyceride accumulation.

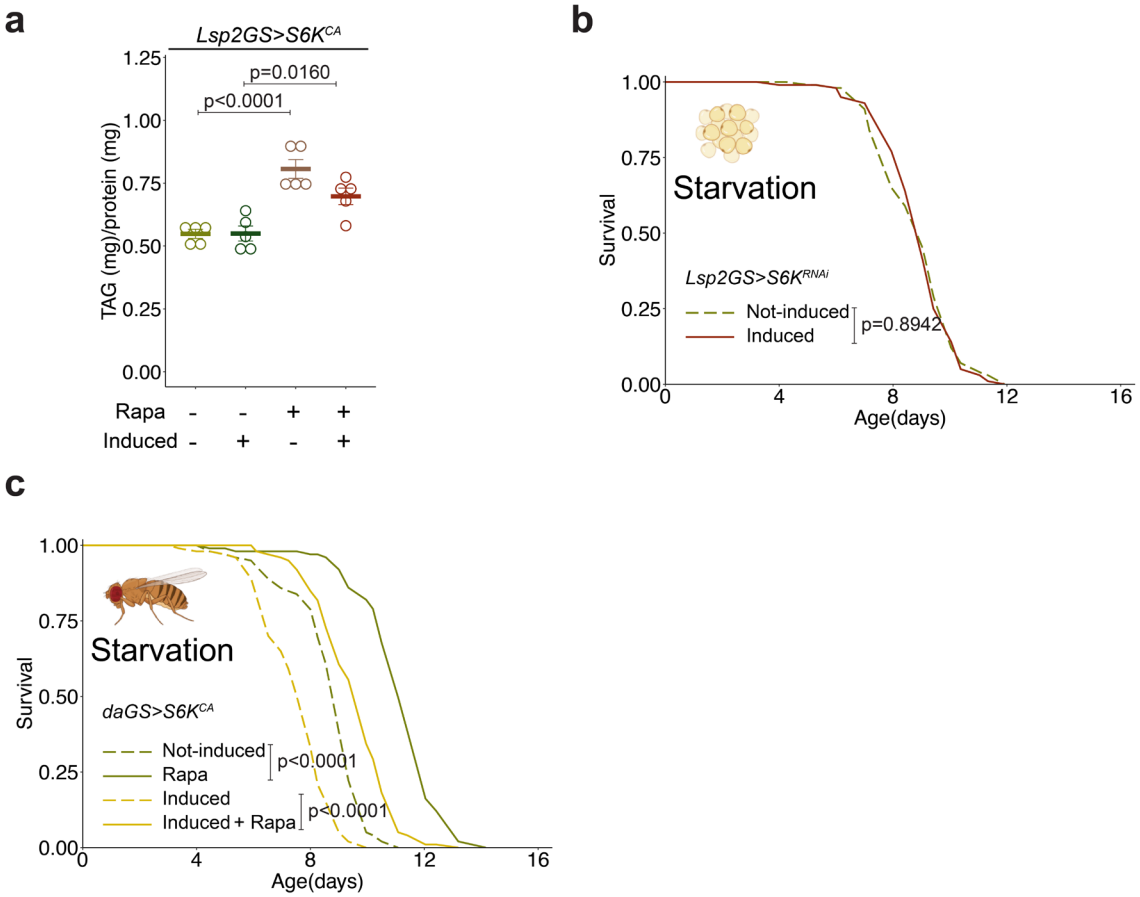

Figure 3-7: Overexpression of constitutively active S6K does not block the effect of rapamycin on TAG storage and starvation resistance in the fly fat body.

a, Rapamycin increased triglyceride (TAG) storage of young flies. Expression of constitutively active S6K in the fat body (*Lsp2GS>S6K^{CA}*) did not block the effect of rapamycin on triglyceride storage (rapamycin: p<0.0001, *Lsp2GS>S6K^{CA}* induction: p=0.0957, interaction p=0.0890, n=5). **b,** Starvation resistance was not affected by RNAi mediated downregulation of S6K activity in the fat body (*Lsp2GS>S6K^{RNAi}*, n=100). **c,** Rapamycin increased starvation resistance of young flies. Expression of constitutively active S6K ubiquitously (*daGS>S6K^{CA}*) did not block the effect of rapamycin on starvation resistance (rapamycin: p<0.0001, *daGS>S6K^{CA}* induction: p<0.0001, interaction p=0.5770, n=100). Data are mean ± s.e.m. **(a)**. Each data point represents an average value per five whole flies **(a)**. Linear mixed model followed by Tukey's multiple comparison test **(a)**; log-rank test and CPH analysis **(b-c)**.

3.2.3 TORC1-S6K-related longevity is independent of intestinal health

The *Drosophila* intestine has recently been shown to play a crucial role in TOR dependent longevity (J. Lu et al., 2021; Y. X. Lu et al., 2021; Luis et al., 2016; Regan et al., 2016; Regan et al., 2022). Treating flies with rapamycin improved gut health by increasing intestinal autophagy, reducing intestinal stem cell (ISC) turnover and age-related gut dysplasia (J. Lu et al., 2021; Y. X. Lu et al., 2021; Luis et al., 2016; Regan et al., 2016). Considering the existing crosstalk between the fat body and the gut (Chen et al., 2014; Scopelliti et al., 2019), we asked whether modifying S6K activity in the fat body affects intestinal health. Therefore, we measured gut autophagy levels, ISC proliferation and gut dysplasia in flies with fat body specific downregulation of S6K and in flies treated with rapamycin and fat body specific overexpression of $S6K^{CA}$. As expected, rapamycin treatment increased the number of lysotracker-positive puncta in the gut, however activation of S6K in the fat body did not block this increase (Figure 3-8a), suggesting that S6K activity in the fat body does not affect intestinal autophagy. Next, we performed phospho-histone H3 (pH3) immunostaining of fly guts, as a proxy for proliferating ISCs (Micchelli & Perrimon, 2006). Neither elevated S6K activity in combination with rapamycin (Figure 3-8b) nor reduced S6K level (Figure 3-8c) in the fat body affected the number of proliferating ISCs when compared to the respective controls. Finally, we measured age-associated gut dysplasia in these flies. Consistent with previous findings, rapamycin treatment protected against age-related gut dysplasia, however, enhancing S6K activity in the fat body did not block this beneficial effect (Figure 3-8d). In addition, gut dysplasia was not improved by fat body specific S6K repression (Figure 3-8e). In summary, these results suggest that S6K activity in the fat body does not regulate longevity by improving gut health.

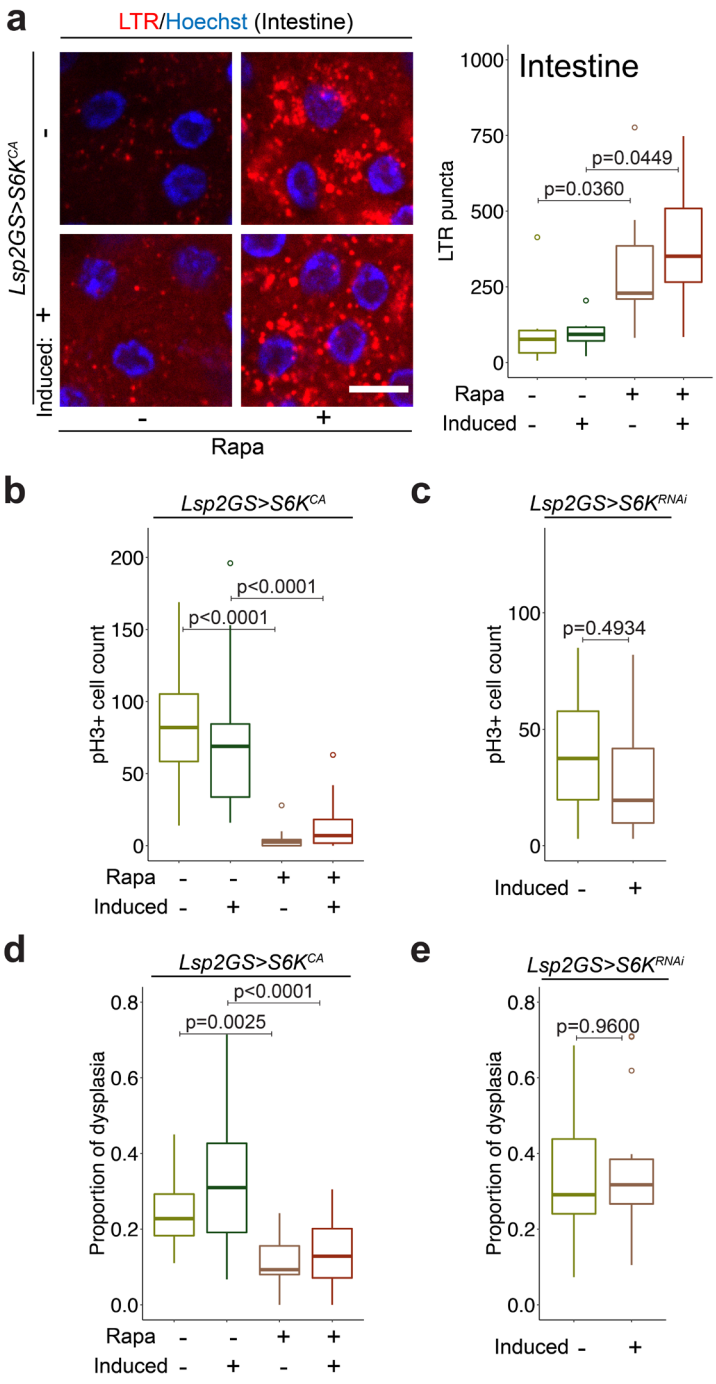

Figure 3-8: S6K activity in the fat body does not affect intestinal health.

a, Rapamycin treatment induced acid organelles in the intestine of young (day 20) flies. Overexpression of constitutively active S6K specifically in the fat body (*Lsp2GS>S6K*^{CA}) did not affect this phenotype, depicted by lysotracker staining (rapamycin effect p<0.0001, *Lsp2GS>S6K*^{CA} induction effect p=0.8463, interaction p=0.9474, linear mixed model followed by Tukey's multiple comparison test, n=9). **b,** Rapamycin treatment suppressed dividing intestinal stem cells of young (day 20) flies. Overexpression of constitutively active S6K specifically in the fat body (*Lsp2GS>S6K*^{CA}) did not affect this phenotype, depicted by pH3

staining in the intestine (rapamycin effect p<0.0001, *Lsp2GS>S6K^{CA}* induction effect p=0.8310, interaction p=0.1610, linear mixed model followed by Tukey's multiple comparison test, n=21 in control group, 23 in induced group, 20 in Rapa group and Rapa+induced group). **c,** RNAi mediated downregulation of S6K activity specifically in the fat body (*Lsp2GS>S6K^{RNAi}*) did not affect dividing intestinal stem cells of young (day 20) flies, depicted by pH3 staining in the intestine (linear mixed model, n=21 in control group and n=23 in induced group). **d,** Rapamycin treatment suppressed intestinal dysplasia of old (day 50) flies. Overexpression of constitutive active S6K specifically in the fat body (*Lsp2GS>S6K^{CA}*) did not affect this phenotype (rapamycin effect p<0.0001, *Lsp2GS>S6K^{CA}* induction effect p=0.1340, interaction p=0.3960, linear mixed model followed by Tukey's multiple comparison test, n=19 in control group, 18 in induced group, 17 in Rapa group and 20 in Rapa+induced group). **e,** RNAi mediated downregulation of S6K activity specifically in the fat body (*Lsp2GS>S6K^{RNAi}*) did not affect intestinal dysplasia of old (day 50) flies (linear mixed model, n=15). Data are displayed as Tukey box plot. Each data point represents an average value per intestine**.** Scale bar, 10 μm.

3.2.4 TORC1-S6K signalling in the fat body affects lysosomal morphology

Proteomics profiling showed that lysosome-related annotations were associated with TORC1-S6K activity in the young fat body (Figure 3-4). To explore the role of TORC1-S6K signalling on lysosomes, we stained fat bodies with lysotracker, a fluorescent, acidotropic dye that stains lysosomes and autophagy-associated autolysosomes (DeVorkin & Gorski, 2014). In line with the role of TORC1 in lysosomal biogenesis and autophagy, rapamycin treatment increased the number of lysotracker-positive puncta in fat body cells. Interestingly, S6K activation did not attenuate rapamycin-induced, lysotracker-positive puncta but led to a substantial accumulation of enlarged acidic organelles in fat body cells (Figure 3-9a). These S6K-dependent enlarged organelles were further identified as lysosomes, indicated by a *GFP-Lamp1* reporter (Pulipparacharuvil et al., 2005), and late endosomes, indicated by a *YFP-Rab7* reporter (Figure 3-9b).

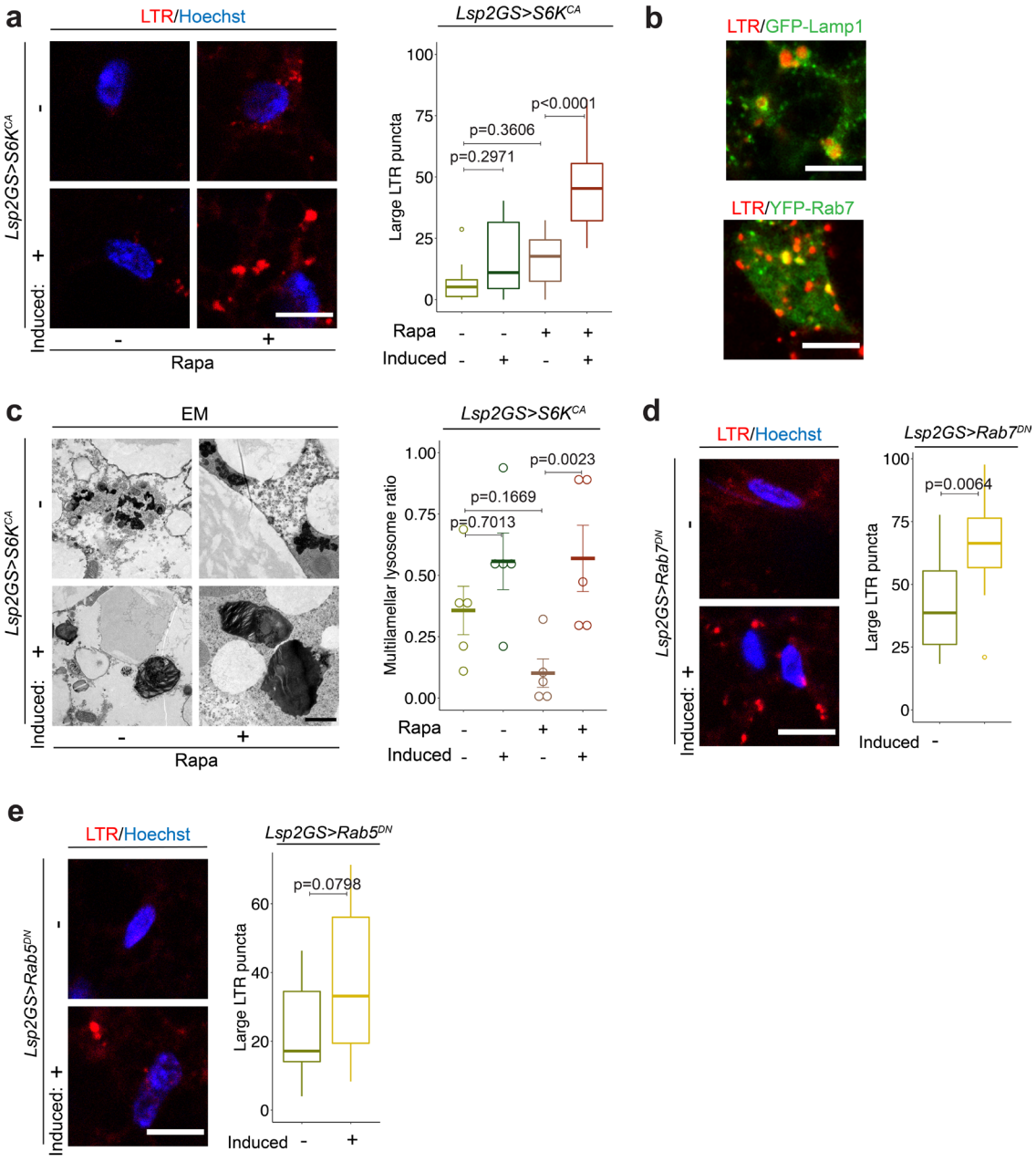

Figure 3-9: TORC1-S6K signalling affects lysosomal morphology in the fat body.

a, Lysotracker staining of fat bodies from young (day 10) flies treated with rapamycin and over-expressing constitutively active S6K (*Lsp2GS>S6K^{CA}*). Fat body-specific, adult-onset over-expression of constitutively active S6K significantly increased acidic organelle size in response to rapamycin treatment (rapamycin: p<0.0001, *Lsp2GS>S6K^{CA}* induction: p<0.0001, interaction p=0.0246, n=12). **b,** Lysotracker-positive enlarged acidic organelles (red) colocalized with the lysosomal marker Lamp1 (*Lsp2GS>S6K^{CA};GFP-Lamp1*, green, upper panel) and partially colocalized with Rab7 (*Lsp2GS>S6K^{CA};YFP-Rab7,* green, lower panel), a marker for late endosomes, in young flies treated with rapamycin and over-expressing S6K^{CA}. **c,** Electron microscopy images of fat bodies of young flies treated with rapamycin and over-expressing

S6K^{CA}. Over-expression of S6K^{CA} in the fat body attenuated the effect of rapamycin on multilamellar lysosomes (rapamycin: p=0.0395, *Lsp2GS>S6K^{CA}* induction: p=0.2798, interaction p=0.0388, n=5). **d,** Lysotracker staining of young fat bodies over-expressing dominant negative Rab7 (*Lsp2GS>Rab7^{DN}*). Fat body-specific over-expression of Rab7^{DN} significantly increased acidic organelle size (p=0.0064, n=12). **e,** Fat body-specific over-expression of dominant negative Rab5 (*Lsp2GS>Rab5^{DN}*) also increased acidic organelle size (p=0.0798, n=12). Data are displayed as Tukey box plot **(a, d-e)** or mean ± s.e.m. **(c)**. Each data point represents an average value per fat body**.** Scale bar, 10 µm **(a-b, d-e)** or 1 µm **(c).** Linear mixed model **(a, d-e)** or negative binomial generalized linear model **(c)** followed by Tukey's multiple comparison test.

To measure the change in lysosomes directly, we used a *Lamp1-3xmCherry* reporter (Hegedus et al., 2016), which marks lysosomes by an endogenous, promoter-driven, C-terminally mCherry-tagged Lamp1. Consistent with the results from lysotracker staining, mCherry-labelled lysosomes were enriched as large puncta in the fat bodies with S6K activation co-treated with rapamycin (Figure 3-10a). In addition, these enlarged lysosomes presented as multilamellar structures when analysed by electron microscopy (Figure 3-9c). Of note, the ratio of multilamellar lysosomes was ameliorated by rapamycin treatment in an S6K-dependent manner. Thus, TORC1-S6K signalling regulated changes in lysosomal morphology in the fat body.

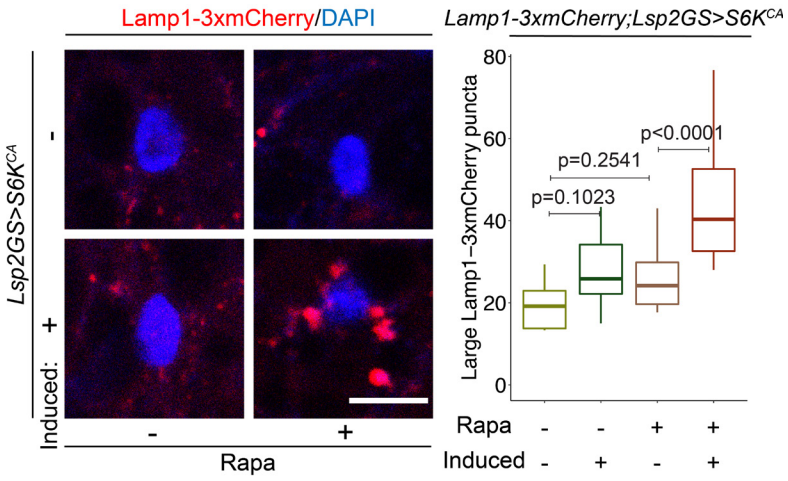

Figure 3-10: TORC1-S6K signalling affects lysosomal size in the fly fat body.
Visualization of enlarged lysosomes in the fat body of young flies treated with rapamycin and overexpressing constitutively active S6K (*Lsp2GS>S6K^{CA}*) using a *3xmCherry-Lamp1* reporter

(rapamycin: p<0.0001, *Lsp2GS>S6K^{CA}* induction: p<0.0001, interaction p=0.0468, n=14). Data are displayed as Tukey box plot. Each data point represents an average value per fat body. Scale bar, 10 μm. Linear mixed model followed by Tukey's multiple comparison test.

The lysosome is the terminal compartment for autophagy and endocytosis (Inpanathan & Botelho, 2019). As autophagy plays a vital role during ageing (Aman et al., 2021), we firstly investigated if a defective autophagy process caused the TORC1-S6K-dependent lysosomal changes. Consistent with previous findings (Bjedov et al., 2010), repressing autophagy induction by *Lsp2GS>Atg5^{RNAi}* blocked rapamycin-induced accumulation of lysotracker-positive organelles in the fat body (Figure 3-11a), indicative of perturbed autophagy. Surprisingly, the number of enlarged lysotracker-positive organelles induced by S6K activation with rapamycin treatment was not changed upon inhibition of Atg5 in the fat body (Figure 3-11b), suggesting that the S6K-dependent lysosomal enlargement was not caused by autophagy dysfunction.

TORC1-S6K-dependent enlarged lysotracker-positive organelles were partially colocalised with late endosomes (Figure 3-9b). Thus, we next investigated if the endocytosis pathway might contribute to the S6K-dependent lysosomal enlargement. We impaired late endosome formation by expressing dominant-negative (DN) Rab7 (*Lsp2GS>Rab7^{DN}*), which led to enlarged lysotracker-positive organelles (Figure 3-9d). Moreover, perturbation of early endosomes by Rab5 DN (*Lsp2GS>Rab5^{DN}*) also mildly induced lysotracker-positive organelle enlargement (Figure 3-9e). These results suggest that the endocytosis pathway could contribute to TORC1-S6K-related lysosomal morphological changes in the fat body.

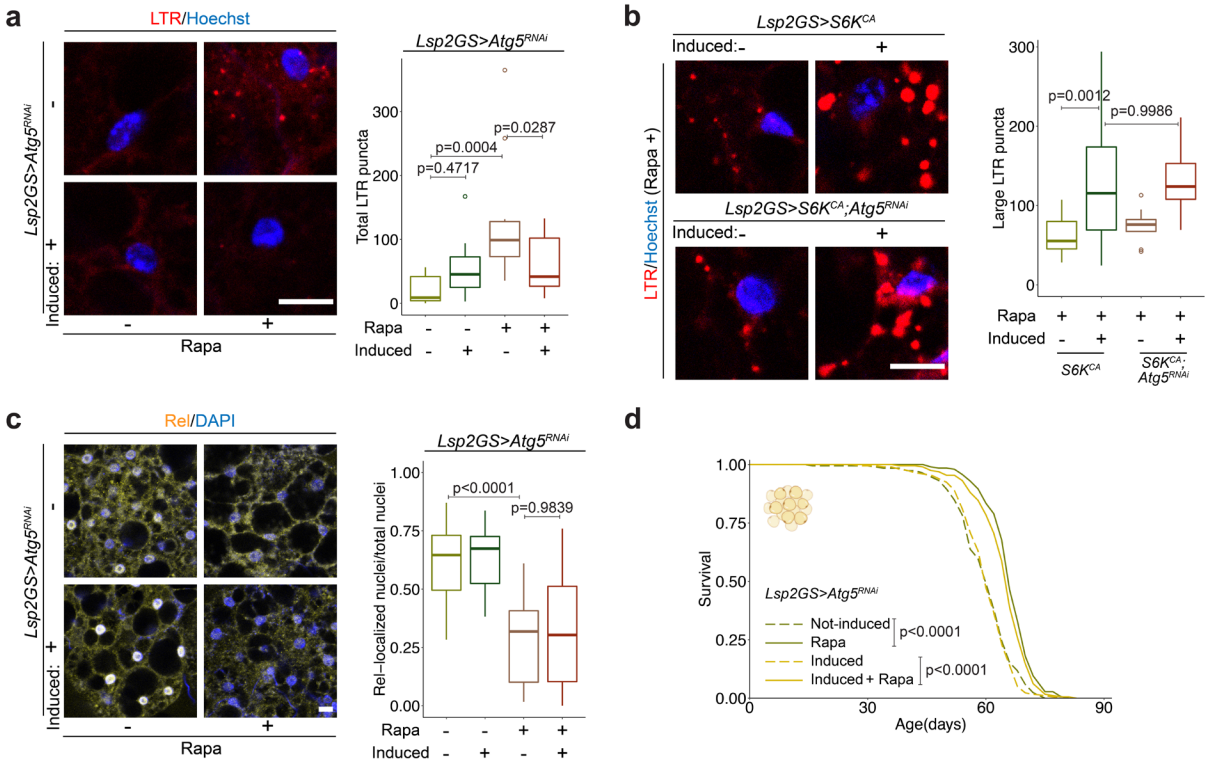

Figure 3-11: Autophagy in the fat body does not regulate lysosomal morphology or rapamycin-related longevity.

a, Rapamycin treatment induced acid organelles in the fat body of young flies. RNAi mediated knock down of Atg5 specifically in the fat body (*Lsp2GS>Atg5^{RNAi}*) blocked this phenotype, depicted by lysotracker staining (rapamycin: p=0.0025, *Lsp2GS>Atg5^{RNAi}* induction: p=0.2947, interaction p=0.0035, n=12). **b,** RNAi mediated knock down of Atg5 specifically in the fat body (*Lsp2GS>S6K^{CA};Atg5^{RNAi}*) did not affect the enlarged lysosome phenotype of fat body tissue of S6K overexpression flies (*Lsp2GS>S6K^{CA}*) treated with rapamycin, depicted by lysotracker staining (*Lsp2GS>Atg5^{RNAi}* induction: p=0.6766, *Lsp2GS>S6K^{CA}* induction: p<0.0001, interaction p=0.5220, n=12). **c,** RNAi mediated knock down of Atg5 specifically in the fat body (*Lsp2GS>Atg5^{RNAi}*) did not affect Relish localisation in old fat bodies treated with rapamycin (rapamycin: p<0.0001, *Lsp2GS>Atg5^{RNAi}* induction: p=0.6189, interaction p=0.9932, n=14). **d,** RNAi mediated knock down of Atg5 specifically in the fat body (*Lsp2GS>Atg5^{RNAi}*) did not block lifespan extension upon rapamycin treatment (rapamycin: p<0.0001, *Lsp2GS>Atg5^{RNAi}* induction: p=0.4130, interaction p=0.4320, n=200). Data are displayed as Tukey box plot. Each data point represents an average value per fat body. Scale bar, 10 µm. Linear mixed model followed by Tukey's multiple comparison test **(a-c)** or log-rank test and CPH analysis **(d)**.

3.2.5 Syntaxin 13 is a downstream effector of TORC1-S6K signalling regulating lysosomal structure

The presence of multilamellar lysosomes suggested impaired lysosomal fusion or defective membrane function, which is typical of lysosomal storage diseases (Platt et al., 2012). As vesicle fusion and vesicle membrane-related processes were negatively regulated by TORC1-S6K signalling (Figure 3-4), Syntaxin 13 (Syx13), the most differentially expressed S6K-dependent SNARE protein, was identified as a promising TORC1-S6K downstream effector (Figure 3-12a-b). Interestingly, Syx13 transcription was only mildly increased by rapamycin treatment in the fly fat body (Figure 3-13), suggesting that TORC1-S6K probably regulate Syx13 expression via a post-transcriptional mechanism. However, the exact mechanism by which TORC1-S6K regulates Syx13 protein levels is currently unknown.

RNAi-mediated repression of Syx13 in young fat bodies recapitulated S6K-associated lysosomal enlargement (Figure 3-12c). However, over-expressing Syx13 did not alter lysosomal morphology under basal condition (Figure 3-12d). To determine whether Syx13 was causally associated with the S6K-induced changes in lysosomal morphology, we increased both Syx13 expression and S6K activity in the fat body of flies that were treated with rapamycin. Strikingly, S6K-induced enlarged lysosomes (Figure 3-12e) and the associated multilamellar structure (Figure 3-12f) were diminished by Syx13 over-expression. Therefore, TORC1-S6K-Syx13 signalling mediated the lysosomal structural changes.

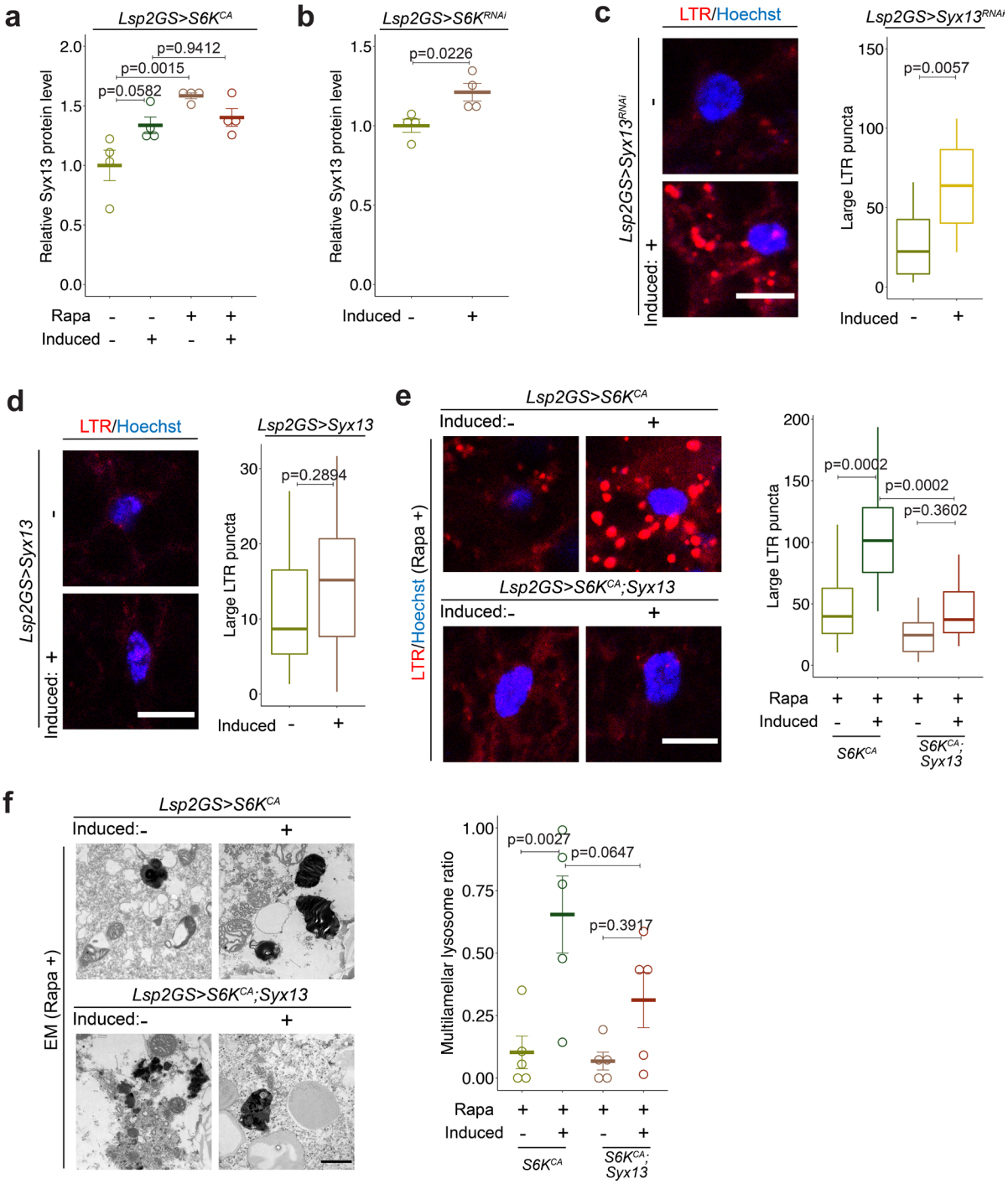

Figure 3-12: Syntaxin 13 is a downstream effector of TORC1-S6K signalling that regulates lysosomal structure in the fly fat body.

a, Syx13 protein level was increased upon rapamycin treatment in young fat body cells and this increase was partly reverted by S6K over-expression (*Lsp2GS>S6K^{CA};* rapamycin: p=0.0003, *Lsp2GS>S6K^{CA}* induction: p=0.0138, interaction p=0.0086, n=5). **b,** Syx13 protein level was increased upon S6K repression (*Lsp2GS>S6K^{RNAi};* p=0.0226, n=5) in young fat body cells. Syx13 protein levels were measured by mass spectrometry-based proteomics. **c,** Knock-down

of Syx13 expression (*Lsp2GS>Syx13*^{RNAi}) resulted in enlarged lysosomes in the fat body of young flies, depicted by lysotracker staining (p=0.0057, n=14). **d,** Over-expression of Syx13 (*Lsp2GS>Syx13*) did not affect lysosomal enlargement (p=0.2894, n=12) in young fat bodies. **e,** Over-expression of Syx13 (*Lsp2GS>S6K*^{CA}*;Syx13*) rescued the enlarged lysosomes of flies over-expressing S6K (*Lsp2GS>S6K*^{CA}) treated with rapamycin (*Lsp2GS>S6K*^{CA} induction: p<0.0001, *Lsp2GS>Syx13* induction: p<0.0001, interaction p=0.0429, n=12). **f,** Overexpression of Syx13 (*Lsp2GS>S6K*^{CA}*;Syx13*) partially rescued the multilamellar lysosomes of S6K overexpressing (*Lsp2GS>S6K*^{CA}) flies treated with rapamycin, depicted by electron microscopy (*Lsp2GS>S6K*^{CA} induction: p=0.0005, *Lsp2GS>Syx13* induction: p=0.7989, interaction p=0.3068, n=5). Data are displayed as mean ± s.e.m. **(a, b, f)** or displayed as Tukey box plot **(c-e).** Each data point represents an average value per five fat bodies **(a-b)** or per fat body **(c-f).** Scale bar, 10 µm **(c-e)** or 1 µm **(f).** Linear mixed model **(a-e)** or negative binomial generalized linear model **(f)** followed by Tukey's multiple comparison test.

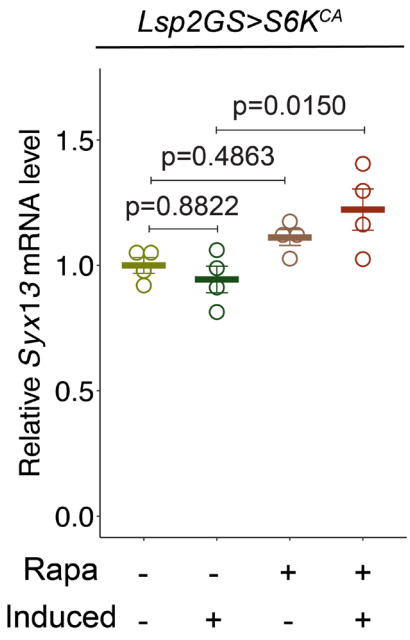

Figure 3-13: TORC1-S6K signalling affects Syx13 transcription in the fly fat body.
Fat body-specific, adult-onset overexpression of constitutively active S6K significantly increased Syx13 transcription in response to rapamycin treatment in young fat bodies (rapamycin: p=0.1467, *Lsp2GS>S6K*^{CA} induction: p=0.6155, interaction p=0.0035, n=5). Data are displayed as mean ± s.e.m.. Each data point represents an average value per five fat bodies. Two-way ANOVA followed by Tukey's multiple comparison test.

3.3 The role of TORC1-S6K signalling in ageing

3.3.1 Age-associated inflammation and immune functional decline are modulated by TORC1-S6K signalling

Lysosomal function plays a crucial role in inflammatory and autoimmune disorders (Bonam et al., 2019). A growing body of evidence has established that the ageing immune system can lead to a low-grade inflammation that is associated with increased mortality (Badinloo et al., 2018; Ferrucci & Fabbri, 2018; Kounatidis et al., 2017). The fat body is a major immune organ of *Drosophila*, and the proteins in old fat bodies that were altered by TORC1-S6K signalling showed significant enrichment of immune-related GO annotations (Figure 3-4). The primary immune response pathway in *Drosophila* is immune deficiency (IMD) signalling. Upon activation of the IMD pathway, the NF-κB-like transcription factor Relish is cleaved in the cytoplasm, and the activated fragment of Relish translocates to the nucleus and induces the transcription of antimicrobial peptides (AMPs), such as *Diptericin A (DptA)* (Chen et al., 2014; Kim et al., 2006; Myllymaki et al., 2014). In line with previous reports (Chen et al., 2014; Ren et al., 2007; Zhan et al., 2007), aged fat bodies exhibited elevated Relish cleavage (Figure 3-14a), Relish-positive nuclei (Figure 3-14b) and *DptA* expression (Figure 3-14c), indicating that IMD signalling was activated during ageing. These changes were significantly ameliorated by reduced S6K activity in the fat body (Figure 3-14d-f). Furthermore, rapamycin treatment greatly suppressed the age-associated changes in Relish localisation and *DptA* expression in the fat body, while activation of S6K blocked rapamycin-related reduction in IMD signalling (Figure 3-14g-h). Another common feature of the aged immune system is immunosenescence, represented by a declining ability of old flies to clear pathogens (Kim et al., 2001; Ramsden et al., 2008). We therefore investigated whether S6K plays a role in bacterial clearance upon systemic infection with *Ecc15*, a gram-negative bacterium widely used to study *Drosophila* immune responses (Neyen et al., 2014). Rapamycin treatment increased bacterial clearance in old flies, and expression of $S6K^{CA}$ specifically in the fat tissue blocked this effect (Figure 3-14i). These results suggest that TORC1-S6K signalling regulates age-associated activation of the IMD pathway and the decline of pathogen clearance.

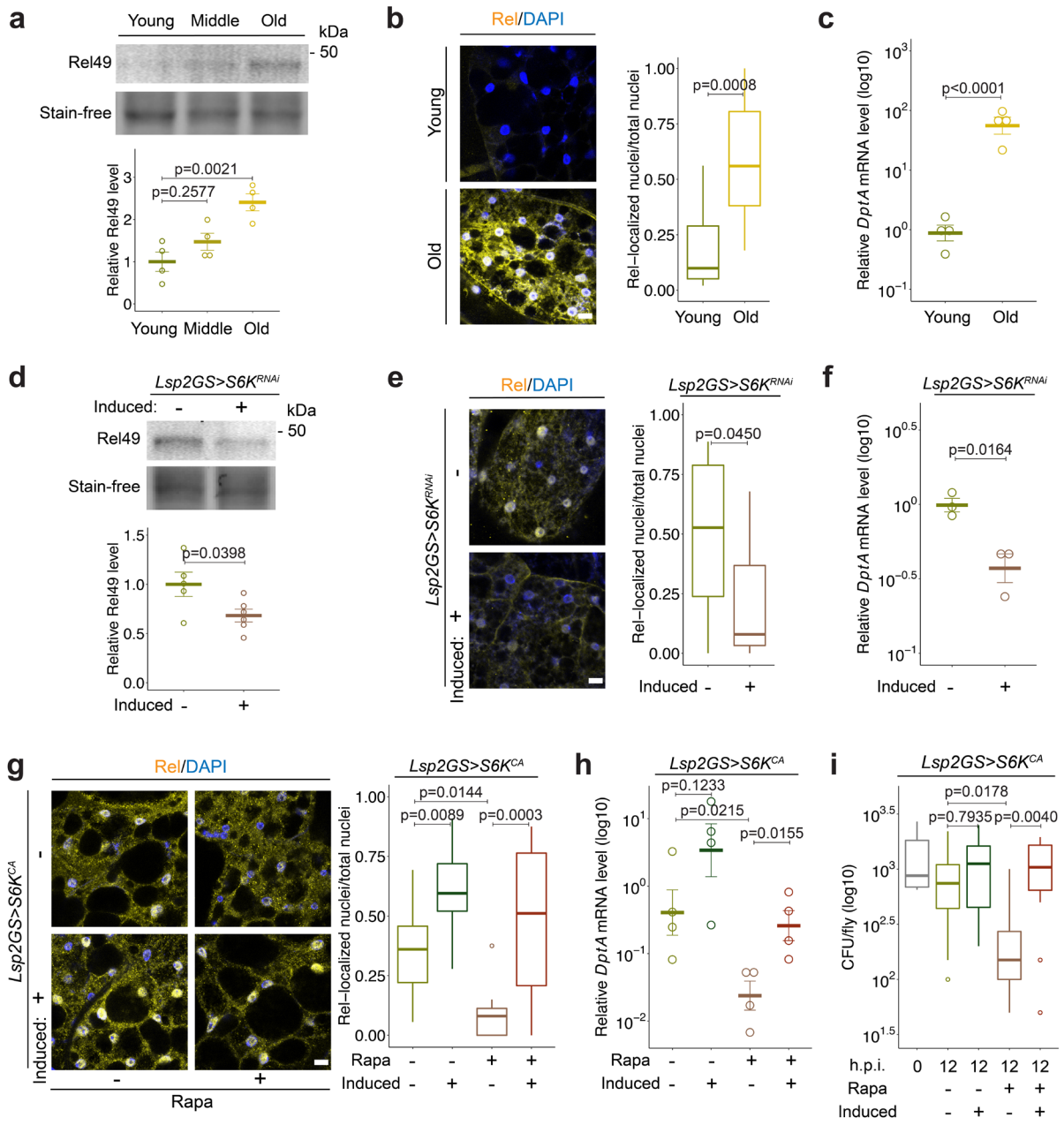

Figure 3-14: Immune ageing is modulated by TORC1-S6K signalling in the fat body.

a, Cleaved Relish (Rel49, 49 kDa) in fat bodies of young (day 10), middle (day 30), and old (day 50) flies (age effect p=0.0034, n=4). **b-c,** Relish protein localisation (**b,** n=9 in young and n=13 in old) and *DptA* transcript expression (**c**, n=4) in fat bodies of young and old flies. **d-f,** S6K inhibition (*Lsp2GS>S6K*^{RNAi}) suppressed the age-related increase in activated Rel49 (**d**, n=5), accumulation of Relish in the nucleus (**e**, n=14), and the increase in *DptA* expression (**f**, n=3) in fat bodies of old (day 50) flies. **g-h,** Rapamycin treatment suppressed age-related Relish localisation **(g)** and the increase in *DptA* **(h)**. Over-expression of S6K (*Lsp2GS>S6K*^{CA}) blocked the effect of rapamycin on Relish localisation (**g,** rapamycin: p=0.0025, *Lsp2GS>S6K*^{CA} induction: p<0.0001, interaction p=0.2345, n=14) and *DptA* expression (**h,** n=4) in fat bodies of

old (day 50) flies. **i,** Rapamycin treatment improved bacterial clearance in old (day 50) flies infected with *Ecc15*. This effect was blocked by S6K overexpression (*Lsp2GS>S6K^{CA}*) (rapamycin: p=0.0185, *Lsp2GS>S6K^{CA}* induction: p=0.0024, interaction p=0.0620, n=12). Data are displayed as mean ± s.e.m. **(a, c-d, f, h)** or Tukey box plot **(b, e, g, i).** Each data point represents an average value per fat body **(b, e, g**), per five fat bodies **(a, c, d, f, h)**, or per three whole flies **(i).** Scale bar, 10 µm. One-way ANOVA followed by Dunnett's multiple comparison test **(a);** linear mixed model **(b, e, g)** or two-way ANOVA with log transformation **(i)** followed by Tukey's multiple comparison test; two-sided Student's t-test with **(c, f, h)** or without log-transformation **(d).**

Potential mechanisms of age-associated immune activation include increased intestinal permeability, chronic infection, and "sterile inflammation" caused by internal factors such as cellular senescence and oxidative stress (Ferrucci & Fabbri, 2018; Garschall & Flatt, 2018). We next investigated whether S6K directly regulates IMD activation upon microbial infection. We therefore challenged young fat bodies *ex vivo* with *Ecc15*. Although *Ecc15* infection led to a clear induction in the number of Relish-positive nuclei under basal conditions, knock-down of S6K failed to repress this induction in the young fat body (Figure 3-15a), suggesting that the effect of S6K on the IMD pathway is indirect and dependent on age-dependent changes in the fly fat body. We next explored whether activation of the IMD pathway by TORC1-S6K signalling is dependent on age-associated accumulation of bacteria (Grenier & Leulier, 2020). Therefore, flies were treated with an antibiotic cocktail from young age to eliminate bacterial accumulation during ageing. Colony unit forming assays indicated that the antibiotic cocktail dramatically reduced the total number of microbes present (Figure 3-15b). However, rapamycin treatment still repressed the number of Relish-positive nuclei and *DptA* expression in the fat body in an S6K-dependent manner (Figure 3-15c-d). Taken together, these results show that TORC1-S6K signalling regulates age-associated activation of the IMD pathway in the fat body independent of bacterial load.

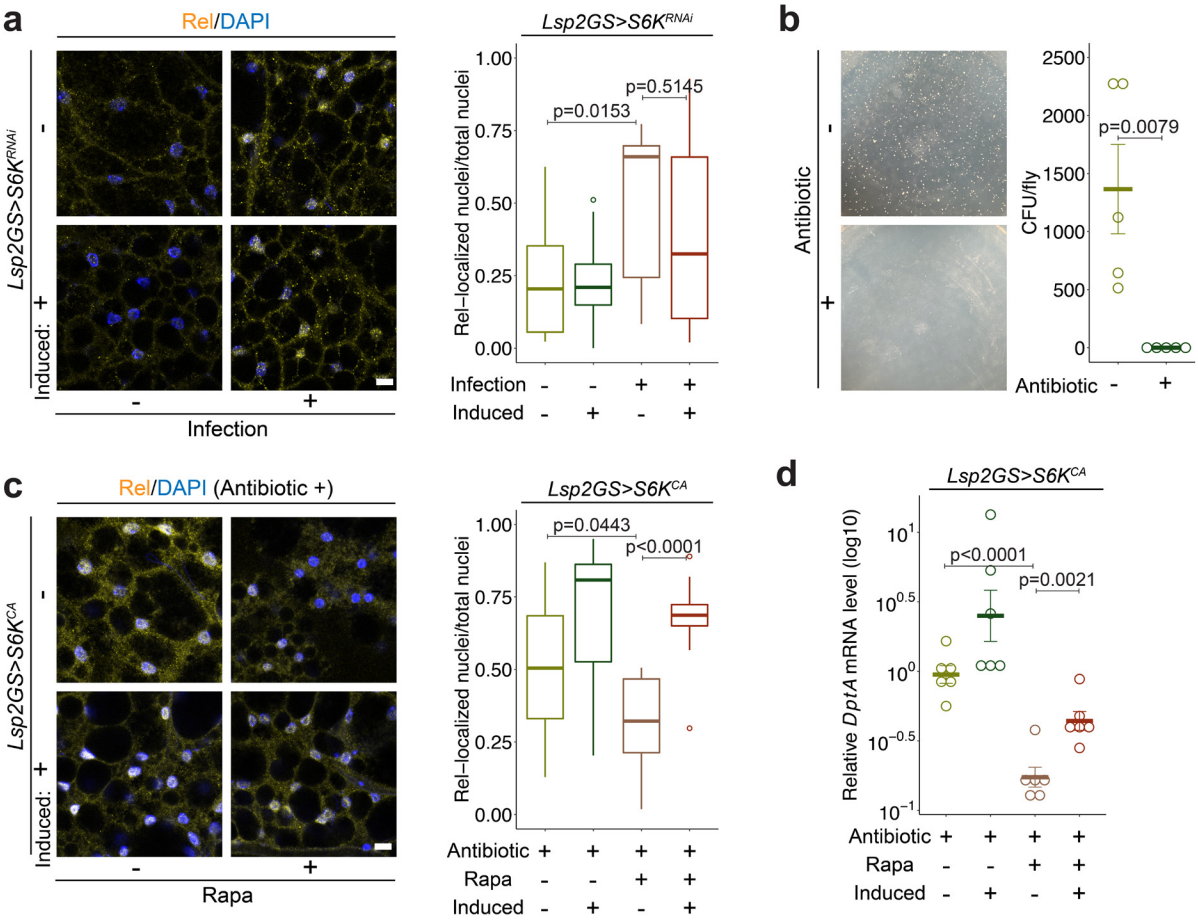

Figure 3-15: TORC1-S6K-related benefits on inflammageing are independent of bacterial load.

a, Infection with *Ecc15* induced nuclear accumulation of Relish in young fat body cells. RNAi-mediated knockdown of S6K (*Lsp2GS>S6K*^{RNAi}) did not affect the infection-induced localisation of Relish to the nucleus (infection: p=0.0013, *Lsp2GS>S6K*^{RNAi} induction: p=0.3499, interaction p=0.3151, n=12). **b,** Colony forming unit (CFU) assay confirmed effective bacterial growth repression by the antibiotic treatment with tetracycline and ampicillin (n=5). **c,** Rapamycin treatment suppressed accumulation of Relish in the nucleus in the fat body of old flies treated with antibiotic. Overexpression of constitutively active S6K (*Lsp2GS>S6K*^{CA}) blocked the effect of rapamycin on Relish localisation (rapamycin: p=0.0264, *Lsp2GS>S6K*^{CA} induction: p<0.0001, interaction p=0.1300, n=14). **d,** Rapamycin treatment suppressed *DptA* expression in old fat body cells. Overexpression of constitutively active S6K (*Lsp2GS>S6K*^{CA}) induced *DptA* expression of flies treated with rapamycin and antibiotics (n=6). Data are displayed as Tukey box plot **(a, c)** or mean ± s.e.m. **(b, d)**. Each data point represents an average value per fat body **(a, c),** per whole fly **(b),** or per five fat bodies **(d).** Scale bar, 10 µm. Linear mixed model followed by Tukey's multiple comparison test **(a, c)**; Mann-Whitney test **(b)**; or two-sided Student's t-test with log transformation **(d)**.

3.3.2 The endolysosomal system mediates the effects of rapamycin on inflammageing and immunosenescence

Given that TORC1-S6K-Syx13 signalling regulated lysosomal morphology, we hypothesised that age-related IMD activation in the fat body could be an outcome of endolysosomal dysfunction. We therefore first investigated if the endocytosis pathway contributes to TORC1-S6K-related regulation of Relish. Over-expression of dominant-negative Rab7 blocked the rapamycin-induced decrease in the number of Relish-positive nuclei in the aged fat body (Figure 3-16a). Moreover, impairment of early endosomes by expression of dominant-negative Rab5 also attenuated TORC1-related Relish repression (Figure 3-16b), suggesting that the endolysosomal system acts downstream of TORC1-S6K in regulating IMD activity.

We next evaluated if Syx13 acted downstream of TORC1-S6K to regulate immunosenescence. Knock down of Syx13 caused a robust induction in the number of Relish-positive nuclei in the old fat body and blocked the effects of rapamycin on Relish-positive nuclei and bacterial clearance (Figure 3-16c-d). Relish repression upon S6K knock down was also rescued by reducing Syx13 expression (Figure 3-16e). Furthermore, while S6K activation blocked the effects of rapamycin on Relish-positive nuclei and bacterial clearance in the old fat body, co-over-expression of Syx13 restored the effects of rapamycin (Figure 3-16f-g). Thus, Syx13 acts downstream of TORC1 and S6K to regulate immunosenescence in the ageing fat body. Despite the finding that autophagy can control inflammation and longevity (Deretic & Levine, 2018), blocking autophagy by Atg5 RNAi in the fat body did not block rapamycin-dependent repression of Relish-positive nuclei and longevity (Figure 3-11c-d). Taken together, these findings show that TORC1-S6K-Syx13 signalling regulates immune ageing via the endolysosomal system.

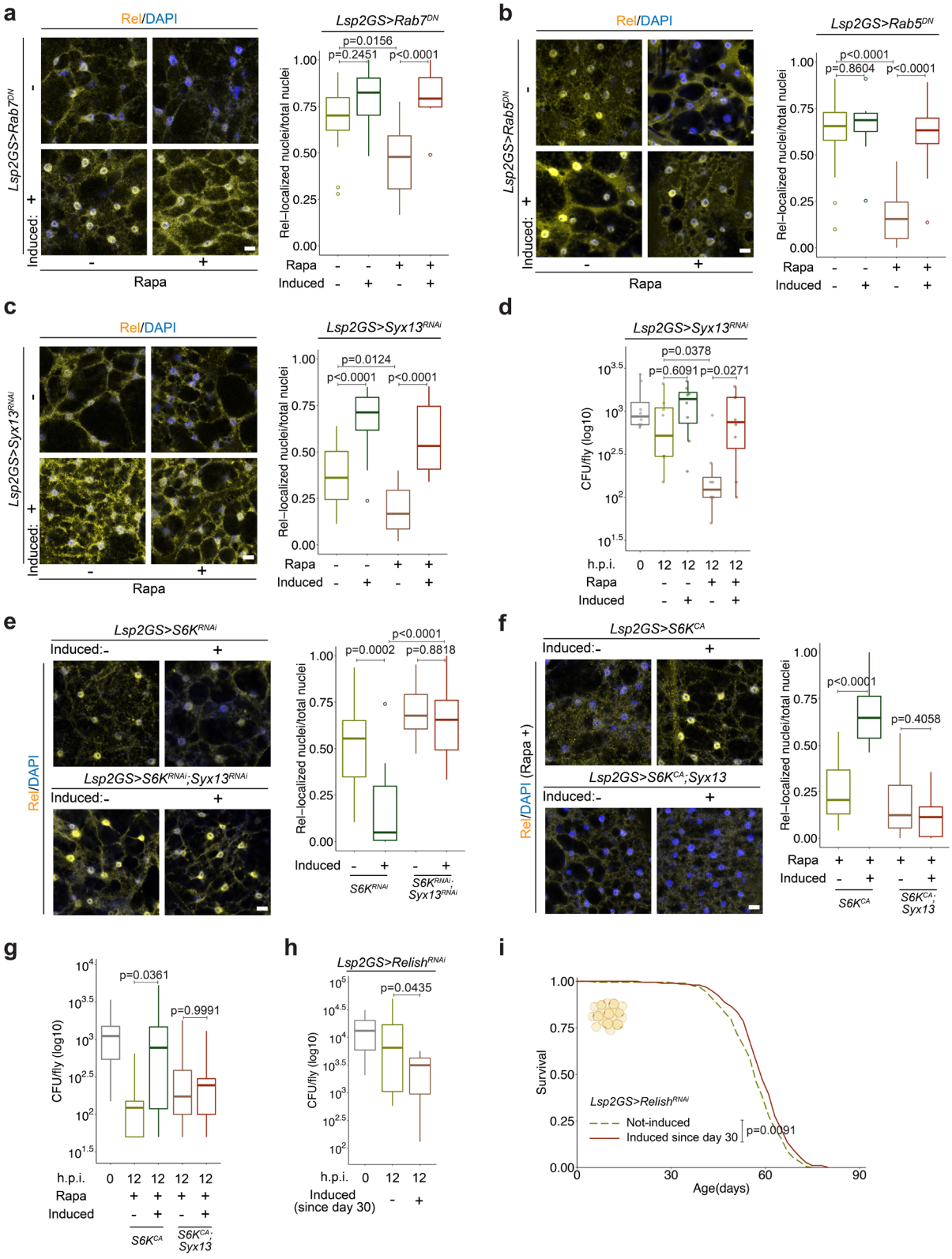

Figure 3-16: The TORC1-S6K-Syx13 axis regulates immunoageing via the endolysosomal system.

a-c, Rapamycin treatment suppressed the age-related nuclear localisation of Relish in old fat body cells. Dominant negative Rab7 (**a**, rapamycin: p=0.0638, *Lsp2GS>Rab7^{DN}* induction:

p<0.0001, interaction p=0.0160, n=14) and Rab5 (**b**, rapamycin: p<0.0001, *Lsp2GS>Rab5^{DN}* induction: p<0.0001, interaction: p=0.0003, n=14) blocked the effect of rapamycin on the age-related nuclear localisation of Relish. **c-d,** Knockdown of Syx13 (*Lsp2GS>Syx13^{RNAi}*) blocked the effect of rapamycin on (**c**) Relish nuclear localisation (rapamycin: p=0.0012, *Lsp2GS>Syx13^{RNAi}* induction: p<0.0001, interaction p=0.2930, n=14) and (**d)** bacterial clearance (rapamycin: p=0.0093, *Lsp2GS>Syx13^{RNAi}* induction: p=0.0057, interaction p=0.2226, n=8). **e,** Knockdown of Syx13 (*Lsp2GS>S6K^{RNAi};Syx13^{RNAi}*) blocked the effect of S6K knockdown on Relish nuclear localisation (*Lsp2GS>S6K^{RNAi}* induction: p=0.0004, *Lsp2GS>Syx13^{RNAi}* induction: p<0.0001, interaction: p=0.0088, n=14). **f-g,** Overexpression of Syx13 (*Lsp2GS>S6K^{CA};Syx13*) rescued the effect of S6K activation on Relish localisation (**f,** *Lsp2GS>S6K^{CA}* induction: p<0.0001, *Lsp2GS>Syx13* induction: p<0.0001, interaction p<0.0001, n=14) and bacterial clearance (**g,** *Lsp2GS>S6K^{CA}* induction: p=0.0644, *Lsp2GS>Syx13* induction: p=0.7042, interaction: p=0.0429, n=10) in old flies treated with rapamycin. **h-i,** Middle-age-onset repression of Relish using *Lsp2GS>Relish^{RNAi}* improved bacterial clearance (**h,** n=8 in 0 h.p.i. group and n=12 in 12 h.p.i. groups) and extended lifespan (**i,** p=0.0091, n=200). Data are displayed as Tukey box plot**.** Each data point represents an average value per fat body **(a-c, e-f)** or per three whole flies **(d, g-h).** Scale bar, 10 μm. Linear mixed model **(a-c, e-f)**, two-way ANOVA with log transformation **(d, g)** followed by Tukey's multiple comparison test; two-sided Student's t-test **(h)**; log-rank test **(i)**.

3.3.3 Reduced relish activity in the fat body prevents immunosenescence and increases longevity

Given that TORC1-S6K signalling regulated age-associated immune dysfunctions, we next addressed whether age-associated inflammation in the fly fat body affects pathogen clearance and longevity. Down-regulating Relish level via RNAi in the fat body from middle-age significantly improved bacterial clearance and extended fly lifespan (Figure 3-16h-i), demonstrating that ameliorating inflammageing may contribute to efficient clearance of bacteria at old age and lifespan extension.

3.4 TORC1-S6K signalling regulates Stx12 expression and immunoageing in mouse liver

Syx13 levels were up-regulated in the fly fat body upon down-regulation of TORC1-S6K signalling. Interestingly, Syx13 levels were also up-regulated in long-lived S6K mutant worms (McQuary et al., 2016) and in rapamycin-treated yeast (Paulo & Gygi, 2015), suggesting that this regulation is evolutionarily conserved, at least among yeast, worms and flies. To assess if the regulation of Syx13 by rapamycin is also conserved in mammals, we used western blot to measure Syntaxin 12/13 (Stx12) levels in the liver of female mice treated with rapamycin. In line with the findings in invertebrates, chronic rapamycin treatment significantly enhanced Stx12 protein levels in mouse liver (Figure 3-17a), suggesting that the TORC1-associated regulation of Syntaxin 12/13 is conserved from yeast to mammals. To address whether reduced TORC1-S6K signalling also affected immunoageing in mice, we performed proteomics profiling of old liver samples from long-lived mice treated with rapamycin and integrated these data with previously published transcriptome analyses of livers of aged mice with rapamycin treatment or S6K1 deficiency (Fok et al., 2014; Selman et al., 2009) (Figure 3-17b). Network propagation analysis (Vanunu et al., 2010) of the 4340 proteins shared between the three datasets identified immune-related processes, including inflammation and leukocyte proliferation, as commonly down-regulated by S6K deficiency or rapamycin treatment, while lipid- and translation-related processes were enhanced (Figure 3-17c). Taken together, these findings indicate that these effects of TORC1-S6K signalling on Syntaxin 12/13 expression and immune ageing are conserved between flies and mammals.

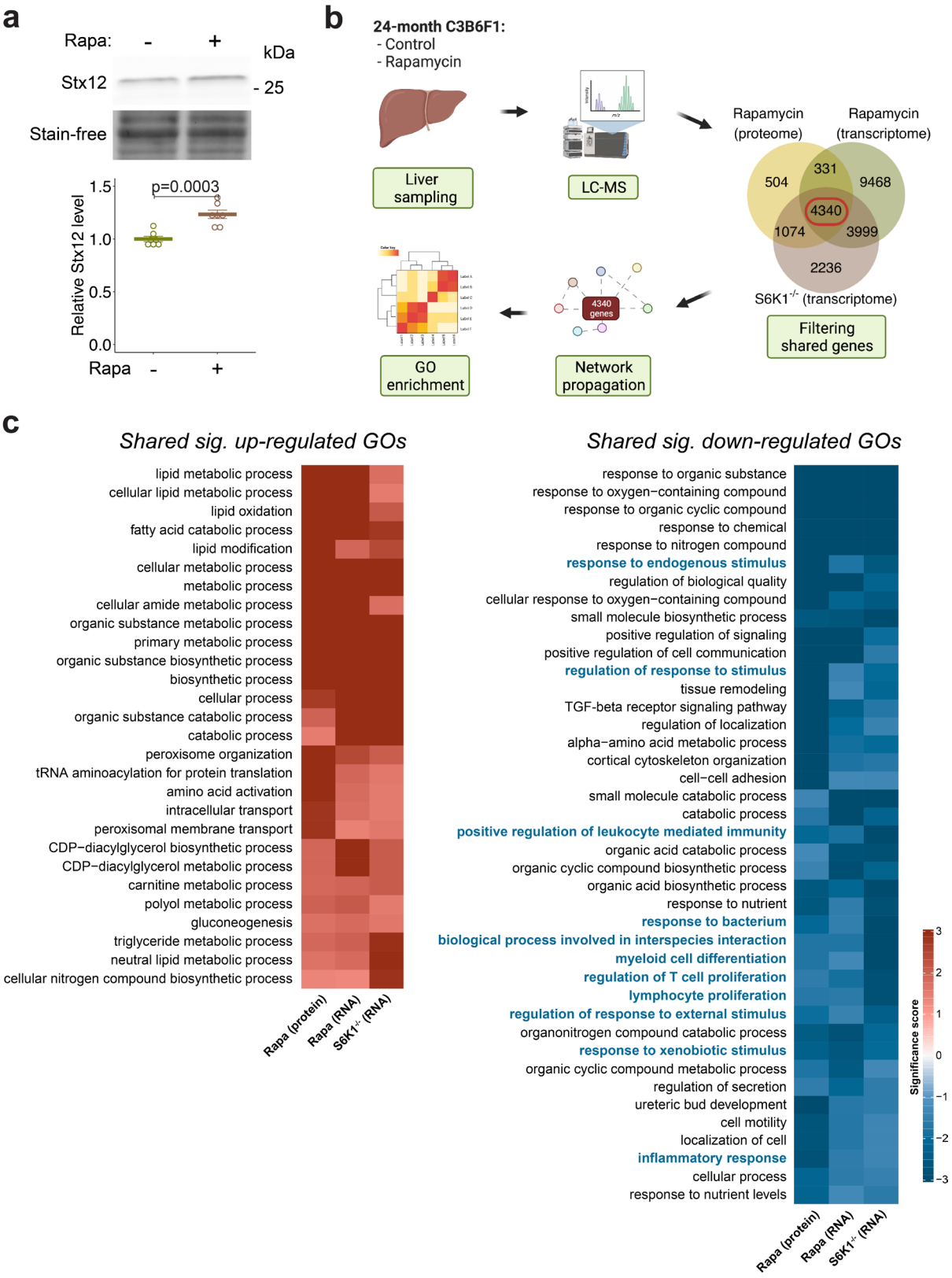

Figure 3-17: TORC1-S6K signalling regulates Syntaxin 12/13 expression and immune-related processes in mouse liver.

a, Rapamycin treatment increased Stx12 level in the liver of 12-month-old mice (n=7, two-sided Student's t-test). **b,** Schematic for the analysis workflow. Common genes present in all three

datasets were used for network propagation and GO analysis. **c,** Only GO terms significantly regulated ($p<0.05$) in the same direction in all three datasets are shown. Immune-related annotations are shown in blue. Cells are coloured by their log10 significance score.

4 DISCUSSION

4.1 The role of S6K in ageing and age-associated diseases

Although S6K is a key downstream effector of mTOR signalling and has been implicated in determination of lifespan in invertebrates and mammals, the molecular and cellular mechanisms underlying S6K-dependent longevity are still elusive. In this thesis we showed that, in *Drosophila*, lowered activity of S6K in the fat body is essential for mTOR-dependent longevity, and that it regulates endolysosomal morphology, inflammageing and immunosenescence in the ageing fat body. Modifying endosome formation, but not autophagy, affected age-related inflammageing, suggesting a causal link between endosome formation and inflammageing. We identified the SNARE-like protein Syntaxin 13 (Syx13) as a molecular link that regulates both endosome formation and inflammageing downstream of TORC1-S6K signalling. Furthermore, repression of the NF-κB like IMD pathway in the fly fat body enhanced clearance of bacteria and extended lifespan. Importantly, long-term treatment with rapamycin increased Syntaxin 12/13 (Stx12) levels in mouse liver, and alleviation of immune processes was a common denominator of TORC1-S6K inhibition in RNA and proteomics profiles from the liver of old rapamycin-treated and S6K1 null mice, indicating that the effects of TORC1-S6K-Stx12 on immunoageing may be evolutionarily conserved from flies to mice. In summary, our findings highlight an important role for the TORC1-S6K-Syx13 signalling axis in inflammageing, immunosenescence and longevity (Figure 4-1).

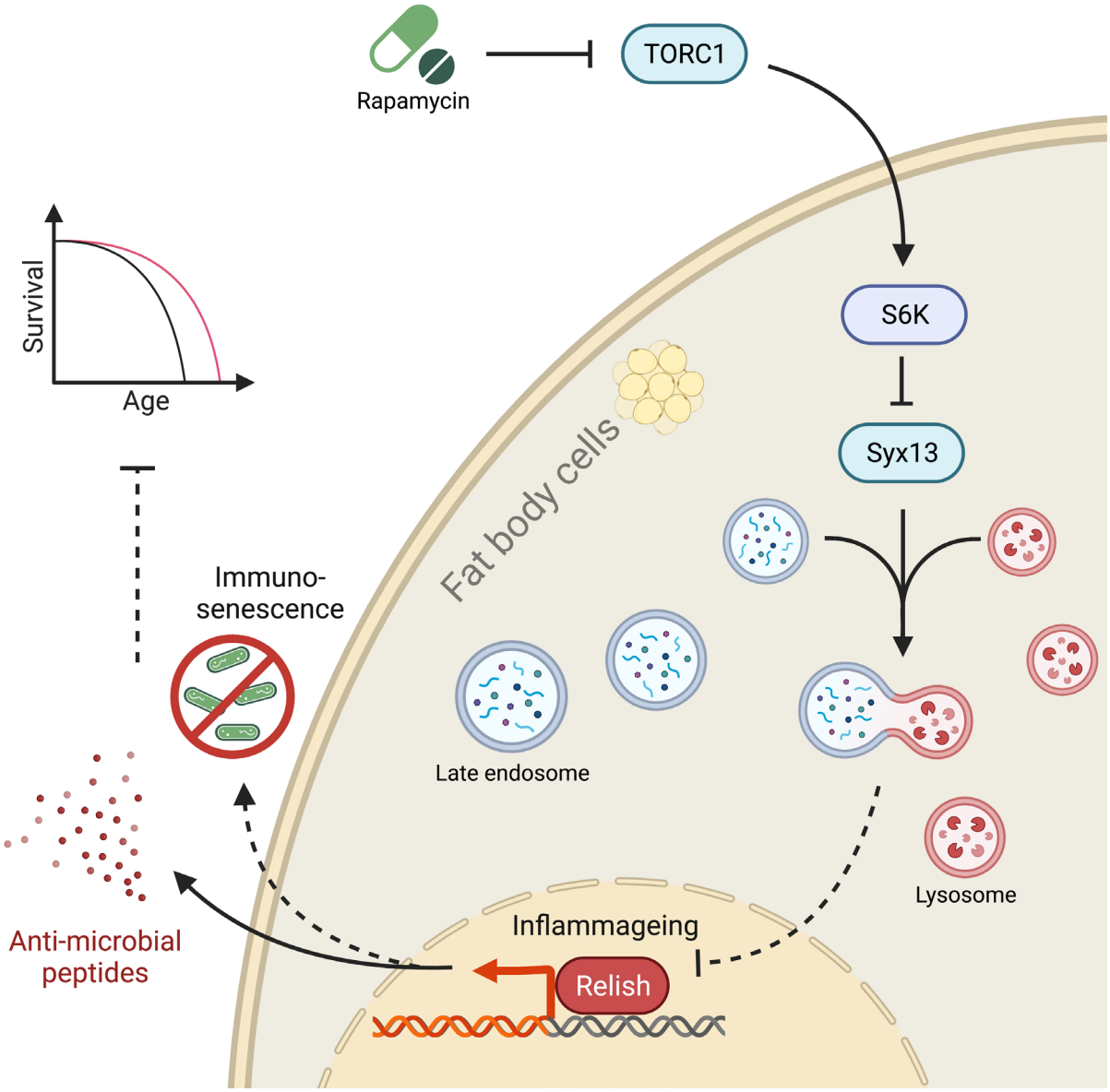

Figure 4-1: Schematic of the findings.

4.2 S6K acts in a tissue specific manner to regulate longevity

Deletion of S6K increases lifespan in worms and of S6K1 does so in mice, but these long-lived S6K deficient animals typically have reduced body size, which may indicate a trade-off between development and ageing (McQuary et al., 2016; Selman et al., 2009). In *Drosophila*, deletion of S6K is developmental lethal, but over-expression of a kinase-dead version of S6K has also been shown to extend adult lifespan (Kapahi et al., 2004). However, whether this lifespan extension was caused by a loss in S6K activity or by titration of the Raptor protein, a key component of the TORC1 complex that directly interacts with S6K via the Raptor-binding TOS motif, was not clear (Nojima et al., 2003). Our study utilized RNAi-mediated down-regulation of S6K specifically in the adulthood and indicated that the activity of S6K during the adult stage plays a critical role in promoting longevity downstream of TORC1.

Mice deficient of S6K1 function are not only long-lived, but are also resistant to age-related pathologies, such as insulin resistance, immune and motor dysfunction, with a gene expression pattern that is similar to caloric restriction or pharmacological activation of AMPK in multiple tissues (Selman et al., 2009). However, in which tissues S6K function is essential to mediate longevity is currently unknown. Here, we found that repression of S6K in the fat body, but not in the intestine, brain, muscle, or heart extended lifespan to a similar extent than ubiquitous knocking down, establishing the fat body as the key tissue for S6K mediated longevity in *Drosophila*. Consistently, fat-body-specific over-expression of a constitutively active S6K protein blocked the effect of rapamycin on longevity, demonstrating that lowered S6K activity in the fat body is essential for TORC1-mediated lifespan extension. S6K and autophagy are both essential for lifespan extension upon TORC1 inhibition (Bjedov et al., 2010). However, while S6K function is required in the fat body but not in the gut for TORC1 dependent longevity, autophagy is essential in the gut and not the fat tissue (Bjedov et al., 2020). Thus, effector mechanisms downstream of TORC1 act in a tissue specific manner to regulate longevity, and the different, tissue-specific, health-enhancing effects of reduced TORC1 signalling collectively contribute to the powerful geroprotective effects of rapamycin.

4.3 TORC1-S6K signalling does not regulate longevity via reduced reproduction, translation or lipid metabolism

4.3.1 Reproduction

In flies, reduced reproduction is often associated with increased longevity, and the fat body plays an important role in oogenesis and reproduction (Flatt, 2011; Flatt & Partridge, 2018). Inhibition of TORC1 by rapamycin treatment has been shown to extend lifespan and inhibit reproduction (Bjedov et al., 2010); however, we noticed that silencing S6K, the downstream target of TORC1, extended lifespan but had no effect on fecundity. Given that reduced TORC1-S6K signalling also extends lifespan in sterile animals (Bjedov et al., 2010; Hansen et al., 2007), we concluded that the relation between lifespan and fecundity is uncoupled under TORC1-S6K manipulation conditions.

4.3.2 Translational regulation

Animals with suppressed translational activity are shown to be long-lived (Hansen et al., 2007; Smith et al., 2008). Since S6K modulates translation initiation and elongation (Magnuson et al., 2012), we reasoned that TORC1-S6K-mediated longevity might be due to reduced translational machinery. However, we noticed that neither rapamycin treatment nor S6K constitutive activation affects the general translational activity indicated by puromycin-labelled newly synthesised proteins. This is consistent with the previous finding in mice that chronic rapamycin treatment or loss of S6K1 fails to reduce ribosome activity (Garelick et al., 2013). While we cannot rule out the effect of TORC1-S6K signalling on the translation of specific proteins such as Syx13, our results suggest that TORC1-S6K-related lifespan benefits are independent of the regulation of global translation rates.

4.3.3 Lipid metabolism

Lipid metabolism is involved in the ageing process (Johnson & Stolzing, 2019). Although rapamycin treatment has been shown to increase lipid storage and provide protection against food deprivation (Bjedov et al., 2010); our results suggest that S6K is not the downstream mediator of the TORC1-related effects on lipid metabolism. Furthermore, the effects of S6K1 deletion on lifespan and metabolism in mice have been linked to S6K1-dependent phosphorylation of glutamyl-prolyl-tRNA synthetase (EPRS) (Arif et al., 2017); however, we noticed that the S6K1-targeted phosphorylation site of mammalian EPRS is missing in

Drosophila EPRS. Although EPRS phosphorylation contributes to lipid accumulation (Arif et al., 2017), we did not observe any interaction between rapamycin treatment and S6K activation on triglyceride storage. Therefore, the EPRS-related mechanisms are probably independent of the mechanisms we found in flies.

4.4 S6K-related health benefits are independent of the gut

The gut has recently been identified as a key organ in determining health and longevity (Funk et al., 2020; Juricic et al., 2022; Y. X. Lu et al., 2021). Thus, we explored the crosstalk between the fat body and the gut. Interestingly, despite the existing connection between fat body and gut during ageing (Chen et al., 2014), we found that manipulating S6K activity in the fat body does not affect intestinal health parameters including age-related changes in intestinal autophagy, ISCs proliferation and intestinal barrier function. We also tested for causality between age-related intestinal permeability and inflammageing in the fat body. Our results suggest that the S6K-dependent immune benefits in the ageing fat body are not induced by microbes leaking from the gut. Consistent with this, eliminating bacteria from flies by antibiotic treatment did not disturb the TORC1-S6K-related immune regulation in old fat bodies. Although the involvement of other pathogens such as fungi and viruses in this regulation is still unclear, our results suggest that the S6K-dependent health benefits we observed are independent of the gut.

4.5 Lysosomes are affected by TORC1-S6K signalling via Syx13

Lysosomes play essential roles in macromolecule degradation, catabolite recycling, and cellular signalling. They serve as centres for integrating nutritional, energy, and growth factor information through regulatory modules on their surface (Lawrence & Zoncu, 2019). Lysosomes are critical for maintaining cell and tissue homeostasis, and their dysfunction is linked to age-related diseases, indicating their importance in the ageing process (Carmona-Gutierrez et al., 2016). Using lysotracker staining and electron microscopy we observed a strong increase in lysosomal size associated with multilamellar structures in fat bodies expressing activated S6K and treated with rapamycin. The morphological changes in the lysosome were reminiscent of lysosomal storage diseases (LSDs) (Platt et al., 2012), and probably suggest impaired function of the lysosome caused by defects in lysosome or membrane fusion. This was further supported by the finding that TORC1-S6K dependent morphological changes in the lysosome were rescued by overexpression of Syntaxin 13 (Syx13), a SNARE family protein that regulates autophagosome maturation and vesicle fusion (Lu et al., 2013; Tang et al., 1998). Knock down of Syx13 in human cells also induces multilamellar lysosome-like organelles (Vanunu et al., 2010), suggesting that the function of Syx13 in regulating lysosomal morphology is evolutionarily conserved between flies and humans. Furthermore, Syx13 levels were up-regulated in long-lived S6K mutant worms (McQuary et al., 2016) and also in various rapamycin-treated animals, including yeast (Paulo & Gygi, 2015), flies and mice, suggesting that the regulation of Syx13 by TORC1 via S6K is evolutionarily conserved among these species.

In contrast to our results from chronically rapamycin-treated mice, protein expression of Syx13 has been shown to be positively regulated by short-term TORC1 inhibition upon neuronal injury or in mammalian cell culture (Cho et al., 2014; Philippe et al., 2020; Thoreen et al., 2012), indicating a difference between acute stress and chronic inhibition of the mTOR pathway. Syx13 transcription was only mildly increased by rapamycin treatment in the fly fat body, suggesting that TORC1-S6K probably regulate Syx13 expression via a post-transcriptional mechanism. However, the exact mechanism by which TORC1-S6K regulates Syx13 protein levels needs further investigation.

4.6 TORC1-S6K-Syx13 signalling regulates immune ageing

4.6.1 TORC1-S6K axis mediates inflammageing

Chronic inflammation, known as 'inflammageing', and a decline in immune function, known as 'immunosenescence', are age-related changes in the immune system, that contribute to a variety of age-associated diseases, including cancer, diabetes, and cardiovascular diseases (Ferrucci & Fabbri, 2018; Fulop et al., 2017; Lopez-Otin et al., 2013; Muller et al., 2013). The NF-κB pathway plays a crucial role in innate immunity and inflammation, and rapamycin treatment ameliorates senescence-associated NF-κB activation (Laberge et al., 2015). Studies in mice have shown that the effects of rapamycin on lifespan and inflammation are reduced when NF-κB activity is enhanced (Correia-Melo et al., 2019), suggesting the anti-ageing effects of rapamycin are partially mediated by its ability to limit NF-κB signalling. However, the mechanisms remain largely unknown. Here, we found that TORC1-S6K-Syx13 signalling regulates age-associated activity of the NF-κB like transcription factor Relish and AMP expression in the fly fat body, the main immune-responsive tissue of *Drosophila*. Importantly, S6K had no effect on Relish activity in young flies upon an acute bacterial challenge, suggesting that the effects of TORC1-S6K on the IMD pathway are indirect via regulation of other processes that change with age and then affect inflammation.

4.6.2 Potential causes of inflammageing

Age-associated inflammation could be caused by external factors like pathogen stimulation due to increased gut leakiness with age and/or intrinsic deterioration of fat body function (Funk et al., 2020; Garschall & Flatt, 2018). The finding that rapamycin repressed the number of Relish positive nuclei and AMP expression even when bacteria growth was prevented by antibiotic treatment suggests that TORC1-S6K-signalling regulates inflammageing via suppression of pathogen-independent sterile inflammation, which is in line with the findings in mammals (Laberge et al., 2015; Majumder et al., 2012).

Cellular senescence is a main driver of age-related sterile inflammation and can be caused by several factors (Ferrucci & Fabbri, 2018). Loss of nuclear Lamin has been associated with age-related senescence in the *Drosophila* fat body (Chen et al., 2014). While we could confirm the age-related decline in Lamin levels in our proteomics dataset, neither rapamycin treatment nor S6K repression prevented the age-related loss of Lamin, suggesting that TORC1-S6K regulates

age-related inflammation independent of Lamin. DNA damage is another driver of cellular senescence that increases with age in the fly fat body and amelioration of DNA damage specifically in the fat body increases lifespan in flies (Tain et al., 2021). Furthermore, rapamycin treatment reduces senescence and improves immune function in DNA repair-deficient progeroid mice (Yousefzadeh et al., 2021). Thus, reduced DNA damage by inhibition of mTOR in the fly fat body is a potential candidate mechanism that should be explored in the future to address how TORC1-S6K signalling regulates inflammageing.

One of the most common triggers of inflammageing in mammals is the accumulation of adipose tissue during ageing (Zamboni et al., 2021). Adipose tissue from old mice experiences endoplasmic reticulum (ER) stress and inflammation (Ghosh et al., 2015); however, TORC1-S6K signalling did not affect the age-associated increase of ER stress indicator Hsc70-3, the ortholog of mammalian Grp78 (78-kDa glucose-regulated protein), in the fly fat body. Given that rapamycin suppresses ER-stress and its associated apoptosis in vitro (Kato et al., 2012), the involvement of TORC1-S6K-Syx13 signalling in ER-stressed-dependent inflammageing needs further investigation.

4.6.3 The endolysosomal system is involved in inflammageing

Maintaining lysosomal function with age improves healthspan and lifespan in animals as diverse as yeast, worms, flies, and mice (Bjedov et al., 2020; Hughes & Gottschling, 2012; Pyo et al., 2013; Sun et al., 2020; Xiao et al., 2015). However, the effects have been mostly attributed to improvements in autophagy (Aman et al., 2021; Carmona-Gutierrez et al., 2016) and not in endolysosomal function. Our results indicate that positive effects of reduced TORC1 signalling in the fly fat body do not rely on increased autophagy but on changes in the endolysosomal system. But how does the endolysosomal system affect age-related inflammation? One possibility is that the endolysosomal system is involved in the elimination of internal stimuli like cell debris and damage-associated molecular patterns (DAMPs), which accumulate during ageing and initiate pro-inflammatory immune signals (Feldman et al., 2015; Huang et al., 2015). Loss of phagocytosis or endocytosis capacity is a general age-associated alteration across various innate immune cells in mice and humans (Butcher et al., 2001; Chougnet et al., 2015; Wong et al., 2017), and may cause defects in tissue homeostasis, leading to chronic inflammation at advanced age. As Syx13 mediates endosome-lysosome fusion, overexpressing Syx13 may accelerate the internalisation and degradation of the internal stimulus and thereby suppress the age-associated immune hyperactivation. Alternatively, the

endolysosomal system may directly affect the intracellular transport and degradation of immune regulators. Supporting this hypothesis, disrupting endosomal trafficking induces the accumulation of cytokine receptors and subsequently activates the IMD/NF-κB pathway in fly fat bodies (Neyen et al., 2016) and in human cells (Maminska et al., 2016). Thus, TORC1-S6K-Syx13 signalling may regulate inflammageing via regulating endosomal trafficking and the turnover of immune factors, a hypothesis that should be addressed experimentally in the future.

4.6.4 TORC1-S6K-Syx13 axis controls immunosenescence

Another common feature of the aged immune system is immunosenescence (Kim et al., 2001; Ramsden et al., 2008). Inhibition of the TORC1 pathway in older individuals using a rapamycin derivative improved immune function following influenza vaccination (Mannick et al., 2018). In addition, studies in flies have shown that TORC1 activity is associated with bacterial load during ageing (Felix et al., 2012; Schinaman et al., 2019), indicating that TORC1 suppression may lead to enhanced immune function in a variety of organisms. Here we found that manipulation of TORC1-S6K-Syx13 signalling in the fly fat body alleviated pathogen clearance, suggesting suppression of immunosenescence in old flies. Inhibition of TORC1 in elderly humans elevates type 1 interferon signalling which mediates anti-virus immune response and improves immune function (Mannick et al., 2021). Interestingly, these interferon-induced genes include IFITM3 (interferon-induced transmembrane protein 3) (Feeley et al., 2011) and Mx1 (Ringer et al., 2018), which are associated with endocytic trafficking. Thus, the endolysosomal system might also play a role in improved immune function in humans in response to TORC1 inhibition.

4.6.5 Inflammageing may induce immunosenescence

Immunosenescence has long been considered to drive inflammageing (Yousefzadeh et al., 2021). However, ameliorating inflammageing by repressing the key inflammatory regulator Relish in the fly fat body is sufficient to ameliorate immunosenescence, suggesting that the inflammageing in the fly fat body acts upstream of immunosenescence. Immunosenescence in flies is often caused by the decreased phagocytic activity of haemocytes at advanced age (Garschall & Flatt, 2018; Horn et al., 2014). Since the immune response in the fat body controls haemocyte activation (Schmid et al., 2014), one potential hypothesis would be that TORC1-S6K-Syx13 signalling in the fat body regulates haemocyte ageing by inter-tissue

communication, thereby ameliorating immunosenescence. In addition to the haemocyte-related cellular immune response, the fly fat body secrets AMPs into the haemolymph to kill invading pathogens; however, the functional capacity of this humoral immune response declines with ageing (Zerofsky et al., 2005). Regulation of TORC1-S6K-Syx13 signalling in the fat body hence may be important for mediating pathogen-induced AMP expression in old flies.

In summary, our results establish that TORC1-S6K-Syx13 signalling regulates inflammageing in hepatic tissues via the endolysosomal system, thereby alleviating immunosenescence and enhancing longevity.

5 LIST OF FIGURES

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