Investigating the metabolic effects of RAS/MAPK pathway inhibition during ageing

Mihails Laskovs Doctor of Philosophy

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THESIS ABSTRACT

Ageing associated pathophysiology as obesity is on the rise in the last 100 years, coupled with increased lifespan, at the detriment of healthspan, led to economic and social burdens on a population scale. Therefore, improving the study of obesity and associated symptoms is essential to healthy ageing.

RAS/MAPK pathway is a nutrient sensing pathway involved in cell survival and differentiation in *Drosophila*. Previous work focused on RAS/MAPK pathway involvement in cancer. Recently, studies have highlighted the importance of RAS/MAPK pathway, specifically genetic inhibition, in the process of ageing and lipid metabolism. However, little is known regarding the pharmacological effects of RAS/MAPK pathway inhibition and its effects on obesity.

The aim of this thesis was to utilize *Drosophila* as a model organism to gain knowledge of pharmacological inhibition of RAS/MAPK pathway in response to obesity. In this thesis, unpublished data showed a lifespan extending and glyceride lowering effect of RAS/MAPK pathway using a MEK specific inhibitor, trametinib, through an unknown mechanism of action. Phenotypic analysis of trametinib treated flies in response to diet intervention showed glyceride reducing effect of trametinib through a lipid metabolism independent mechanism, additionally uric acid and Malpighian tubule deposits were reduced. Subsequent RNA-sequencing analysis identified subsets of genes that show interaction as potential mechanisms through which trametinib elicits its effects. Analysis of publicly available RNA-sequencing dataset found significant overlap of sugar-responsive genes, a subset of which were also altered by trametinib. Lipidomics analysis revealed changes in glyceride and phospholipid lipid profile of trametinib treated flies.

Summarily, novel molecular mechanisms of action were discovered of the beneficial phenotypic effects of trametinib in response to diet- and age-dependent obesity. Ultimately, a more in-depth 'multi'-omics approach of effects of trametinib will allow for identification of potential pharmaceutical targets to treat diet- and age-dependent obesity.

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ABBRIVIATIONS

ACC	Acetyl-CoA carboxylase	ISC	Intestinal step cell
AICAR	5′-phosphoribosyl-4-carboxamide-5- aminoimidazole	itp	lon transport peptides
AIR	5'-phosphoribosyl-5-aminoimidazole	KEGG	Kyoto encyclopedia of genes and genomes
akh	Adipokinetic hormone	KIM-1	Kidney injury molecular-1
amon	Amontillado	KSR	MEK-kinase suppressor of RAS
AMP	Antimicrobial peptide	LC	Liquid chromatography
AMP	Adenosine monophosphate	LC-MS	Liquid chromatography coupled mass spectrometry
AMPK	AMP-activated protein kinase	LD	Lipid droplet
ANOVA	Analysis of variance	LDL	Low-density lipoprotein
AOP	Anterior open	Leu-Enk	Leucine enkephalin
APRT	Adenine phosphoribosyltransferase	LMSD	LIPID MAPS
AQP	Aquaporin	MALDI	Matrix-assisted laser desorption/ionisation
BDSC	Indiana University Bloomington Drosophila Stock Centre	mhc	Myosin heavy chain
BMI	Body mass index	mip	Myoinhibiting peptide
BMM	Brummer	MLST8	Mammalian lethal with SEC13 protein 8

BSA	Bovine serum albumin	MS	Mass spectrometry
CAIR	5'-phosphoribosyl-5-aminoimidazole	MSI	Laser-assisted desorption/ionisation
CaMKK2	Calcium/calmodulin-dependent	MSV	Murine Sarcoma Virus
сс	Corpora cardiaca	MTBE	Mehyl tert-butyl ether
cDNA	Complementary DNA	mTOR	Mechanistic target of rapamycin
Chol	Cholesterol	mTORC1	mTOR complex 1
CL	Cardiolipin	mTORC2	mTOR complex 2
clk	Clock	NKCC	Cation-chloride cotransporter
CLS	Chronological lifespan	NO	Nitric oxide
cra	Croquemont	nos	Nitric oxide synthase
CRTC-1	CREB-regulated transctiptional coactivator-1	O ₂ -	Superoxide radical
Cu/ZnSOD	Superoxide dismutase	OH-	Hydroxyl radicals
DAG	Diglyceride	ONS	Office for National Statistics
DDA	Data dependent acquisition	ох	Oxen
DEPTOR	DEP domain-containing mTOR- interaction protein	PA	Phosphatidic acid
DG	Diacylglycerol	PBS	Phosphate buffered saline
DGAT	DAG acetyltransferase	PC	Phosphatidylcholine
dh31	Diuretic hormone 31	PCA	Principal component analysis
DHM	Dihydromyricetin	pdf	Pigment dispercing factor
DIA	Data independent acquisition	PDGFR	Platelet-derived growth factor receptor
Dilps	Drosophila insulin-like peptides	PE	Proboscis extension
DR	Dietary restriction	PE	Phosphatidylethanolamine
DTT	Dithiothreitol	PG	Phosphatidylglycerol
EBM	ETS-binding motifs	PI	Phosphatidylinositol
EGF	Epidermal growth factor	PI3K	Phosphoinositide 3-kinase
EGFR	Epidermal growth factor receptor	ΡΡΑRγ	Peroxisome-proliferator-activated receptor gamma
ESI	Electrospray ionisation	PRA	5'-phosphoribosyl-1-amine
ETC	Electron transport chain	PRAS40	Proline-rich Akt substrate of 40kDa
ETS	E-twenty-six	PRPP	5'-phosphoribosyl-1-pyrophosphate
FA	Fatty acid	PS	Phosphatidylserine
FAICAR	5'-phosphoribosyl-4-carboxamide-5- formamidoimidazole	Pss	Phosphatidylserine synthase
FASN	Fatty acid synthase	qRT-PCR	Quantitative reverse transcription PCR
FFA	Free fatty acid	R-RAS	RAS-related protein
FGAM	5'-phosphoribosyl-N- formylglycinamidine	Raptor	Regulatory-associated protein of mTOR
FGAR	5'-phosphoribosyl-N- formvlglvcinamide	RAS	Rat sarcoma virus
FIRKO	Fat-specific insulin receptor knockout	rdl	Resistant to dieldrin
FKBP	FK506-binding protein	Rictor	Rapamycin-insensitive companion of mTOR
FLIC	Fly-to-Liquid-Food-Interaction Counter	RLS	Replicative lifespan
FOXO	Forkhead box O	ROS	Reactive oxygen species
GABA	Aminobutyric acid	RP	Reverse phase
GAP	GTPase activating protein	RTK	Receptor tyrosine kinases
GAR	5-phosphoribosylglycinamide	RU486	Mifepristone

GEF	Guanine nucleotide exchange factors	S1/2P	Site-specific proteases 1/2
glucalpha	Glutamate-gated chloride channel subunit alpha	S6K	Ribosomal S6 kinase
glurIID	Glutamate receptor IID	SAICAR	5'-phosphoribosyl-4-(N- succinocarboxamide)-5- aminoimidazole
GMP	Guaninosine monophosphate	SCAP	SREBP cleavage-activating protein
GO	Gene ontology	SDS	Sodium dodecyl sulfate
GPAT	Glycerol phosphate acyltransferases	SH2	Sequence homology 2
GS	Gene-switch	Sik3	salt-inducible kinase 3
H_2O_2	Hydrogen peroxide	SIRT	Sirtuin
HDAC	Histone deacetylase	snpf	short neuropeptide F
hk	Hypokinetic	spr	sex peptide receptor
HMGCR	3-hydroxyl-methylglutaryl CoA reductase	SREBP	Sterol regulatory element binding protein
HPLC	High performance liquid chromatography	STAC	SIRT-1 activating compounds
HPRT	Hypoxanthine-guanine phosphoribosyltransferase	stj	Straightjacket
HRP	Horseradish peroxidase	STRE	Stress-response element
HSD	High sugar diet	SYA	Standard sugar, yeast, agar
Htt	Huntingtin	T2D	Type 2 diabetes
ICDR	lonic compatibility detergent reagent	TAG	Triglyceride
IGF	Insulin-growth factor	TLC	Thin layer chromatography
IIS	Insulin/IGF-1 signalling	tn	Thin
IMP	Inosine monophosphate	ToF	Time of flight
IMS	Ion mobility system	TRX	Thioredoxon-1
InR	Insulin receptor	Upd2	Unpaired 2
IRS	Insulin receptor substrate	Up	Upheld
		vdup1	Vitamin D3 up-regulated protein 1
		VGCC	Voltage-gated calcium channel
		wDah	White DAHOMEY
		wupa	Wings up A

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Chapter 1

1.1 AGEING

The population worldwide is living longer. Since the 1950s the proportion of people aged 65 and over has been rising steadily and this rise in ageing population is expected to increase dramatically to around 38 percent by 2050 (World Population Ageing 1950–2050. United Nations Population Division.)

Ageing is an intricate, private, and inevitable biological phenomenon that encompasses a myriad of deleterious physical, psychological, and social changes over time. Furthermore, in the developed world, ageing has become the main risk factor for age-related diseases such as cancer, diabetes, cardiovascular disorders, and neurodegeneration such as Alzheimer's and Parkinson's disease (Niccoli, Partridge 2012). As the global population continues to grow older, understanding the mechanisms and implications of ageing becomes increasingly critical. The multifaceted process of ageing impacts individual well-being but also exerts societal and economical effects, consequently leading to shrinking in the workforce and higher demand for health and social care. In the face of an ageing world, comprehensive research into the biology of ageing and development of innovative strategies for healthy ageing are pivotal for addressing the challenges associated with this demographic shift (Beard and Bloom 2015).

Interestingly, ageing is not only the major risk factor for age-related diseases, but also conditions such as obesity which exacerbates the ageing phenotype (Salvestrini et al 2019). In fact, obesity has been shown to reduce life expectancy by 5.8 years in men and 7.1 years in women after the age of 40. Ageing and obesity share a similar profile of symptoms such as weakened immunity, shift in body composition and enhanced systemic inflammation. Therefore, obesity and ageing can be considered as two different manifestations of the same underlying process, the accumulation of damage and inflammation, (Tam et al 2020).

Additionally, most genetic, and dietary manipulations that affect obesity in rodents and primates also have an impact on ageing phenotype and age progression, (Tam et al., 2020). For instance, diet-induced obesity in female mice has shown to decrease physiological performance associated with ageing (activity, gait, rotarod) as well as lifespan, (Zhang et al 2015). However, it remains to be clarified how obesity-associated changes contribute to ageing and age-associated diseases. Obesity seems to drive ageing at multiple levels; by increasing inflammation, apoptosis, genome instability among others, acting in synergy to promote the ageing phenotype, (Tam et al., 2020).

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Gerontology is the multidisciplinary study of the ageing process, encompassing the biological, psychological, and social aspects of growing old. It is a field of scientific research and practice dedicated to understanding the various ways of ageing. Gerontologists study how and why people age, examining factors that contribute to both healthy and pathological ageing. The field addresses a wide range of topics, such as age-related diseases, the impact of nutrition on longevity, cognitive decline, and psychological and emotional aspects of ageing. The final goal of biogerontology is to promote healthspan during ageing, by preventing or reducing the period of agerelated disease that occurs during ageing, termed morbidity. Healthspan refers to the period of individual's life during which they enjoy health, free from chronic or debilitating diseases and disabilities. Lifespan refers to the maximum duration of life that is biologically possible for a given species or organism. Lifespan can vary significantly among different species and is influenced by genetics, environmental factors, and evolutionary processes. However, interventions that extend healthspan are likely to increase lifespan, as interventions in model organisms have suggested (Lopez-Otin et al. 2013, Fontana et al., 2010, Mattison et al., 2017).

Ageing was seen as a random process, until the isolation of the first long-lived strain of *Caenorhabditis elegans* (Klass 1983). Nowadays, it is well recognized that ageing is a flexible process, which means that the rate of ageing is controlled, at least to some extent, by genetic and environmental factors and biochemical processes that underpin life, which are evolutionary conserved (Kenyon, 2010). Moreover, the vast advances of the ageing field over recentyears is largely due to the study of ageing in genetically tractable model organisms such as *Saccharomyces cerevisiae, Caenorhabditis elegans, Drosophila melanogaster* and *Mus musculus*. In fact, studies in model organisms have shown that mutations and interventions can extend the healthspan and lifespan of these animals, and that these mutations are evolutionary conserved from yeast to mammals (Kenyon 2010, Gonzalez-Freire et al., 2020).

1.2 THEORIES OF AGEING

Evolutionarily, survival and reproduction are the only features subject to natural selection, and traits that increase survival and reproduction are passed onto the next generation. However, ageing still occurs despite being deleterious to fitness and reproduction, as though a side effect. Ageing, otherwise known as senescence, occurs almost universally across all organisms, with some exceptions. For instance, small freshwater hydrozoans in the genus *Hydra* seem to avoid senescence and are biologically immortal, when asexual. As reported by Martinez, 1998, mortality and reproductive rates of a three-hydra cohort over a period of four years, provides no evidence of ageing in hydra. Furthermore, the transcription factor forkhead box O (FoxO), was shown to be a critical driver of continuous self-renewal responsible for the unlimited life span of *Hydra* (Boehm *et al.*, 2012). Specifically, overexpression of *foxO* transcription factor in *Hydra*'s long life, (Boehm *et al.*, 2012). Multiple theories have been proposed to try to answer the phenomenon of ageing.

1.2.1 MUTATION ACCUMULATION THEORY

The first modern conceptualisation of ageing was proposed by Peter Medawar in 1952. Medawar used the term 'senescence' to describe the process of ageing. Meaning, harmful mutations manifest later in life when reproduction has ceased. These harmful mutations would be already passed onto the next generation before the negative late-life effects became apparent, thereby affecting reproductive success. Medawar's mutation accumulation theory, since proposition, was supported by several experimental studies in Drosophila, (Hughes and Reynolds, 2005). Hughes and Reynolds used an example of oxidative stress resistance. Consequences of cellular oxidative damage caused by reactive oxygen species (ROS), superoxide radical (O^{2-}) and hydrogen peroxide (H_2O_2) can cause production of hydroxyl radicals (OH) which damage molecules such as lipids, proteins, and DNA (Raha and Robinson 2000; Martin, 2011). However, overexpression of specific antioxidant enzymes, namely superoxide dismutase (Cu/ZnSOD) in Drosophila was shown to produce an increase in life span of around 40% without affecting the metabolic rate, (Sun and Tower, 1999). This suggested that prevention of accumulation of mutations, due to oxidative stress, prevented some of the late life molecular damage, thus extending lifespan of Drosophila.

1.2.2 ANTAGONISTIC PLEIOTROPY

A highly popular article published by George C. Williams in 1957, made some advancements on Medawar's theory of ageing. The theory states that one gene controls more than one phenotype in an organism, and has a positive benefit early in life but detrimental effects in late life. Natural selection retains these alleles in individuals as these genes have early life benefits, increasing the chances of reproductive success. However, post reproduction these genes have negative effects due to their detrimental expression (Gladyshev, 2016). A positive example of antagonistic pleiotropy was shown in C. elegans age-1 mutants. Age-1 encodes the C. elegans orthologue of phosphoinositide 3-kinase (PI3K) involved in the Insulin/IGF-1 signaling (IIS) pathway. Age-1 mutants are long lived while maintaining their fecundity based on lifespan analysis of age-1 mutants compared to control C.elegans and egg counting respectively, (Johnson, 1990). Interestingly, when age-1 hypomorphic mutants compete against wild-type worms in the laboratory with abundant food, neither have an advantage. However, when food is scarce and only periodically available, a likely pattern to occur in nature, age-1 mutants quickly go extinct, suggesting why the wild-type allele persists in nature, (Walker et al., 2000).

1.2.3 HYPERFUNCTION THEORY OF AGEING

Blagosklonny, proposes that ageing is caused mostly by a programmatic, development-style mechanism. In essence, Blagosklonny states that higher than optimal activity of signal-transduction pathways (maintained by feedback loops) drives ageing, (Blagosklonny, 2008). An example of this would be genes involved in cellular processes fundamental to early life development, but which are hyper-activated post development. The nutrient-sensing mechanistic target of rapamycin (mTOR) promotes growth and cellular senescence, (Wullschleger, et al., 2006). Hyperfunction theory of ageing predicts that inhibition of mTOR by a drug called rapamycin can slow down ageing, and such was done in rapamycin fed mice which lived longer compared to the control, (Harrison et al., 2009). Furthermore, rapamycin extended lifespan of *Drosophila*, as well as increased starvation and oxidative stress resistance, (Bjedov et al., 2010).

1.2.4 DISPOSABLE SOMA THEORY OF AGEING

Disposable soma theory of ageing proposes that organisms age due to an evolutionary trade-off between growth, reproduction, and maintenance, (Kirkland, 1977). The theory states that an organism has limited energy resources therefore, resources must be shared between maintenance and reproduction. Low maintenance investment would be evolutionarily faulty as the organism would probably die before reaching reproductive age. However, high maintenance investment would probably lead to an offspring that would die before reaching reproductive age, leading to the balancing act of resources between maintenance and reproduction. For example, stimulation of the growth mTOR pathway, activates protein synthesis for growth, which consequently inhibits autophagy, a maintenance process (Johnson et al., 2013). Decline in autophagy, by mutation of key protein, ATG7, involved in autophagy in mice, has been shown to decrease lifespan but also cause neurodegeneration, (Komatsu et al., 2006). The theory suggests that ageing is not a programmed process, but rather a consequence of natural selection favouring reproductive success over long-term high maintenance investment, resulting in an age-related decline in physiological function and increased susceptibility to agerelated diseases. Generally, this theory holds up only in few studies in humans; comparison of castrated Eunuch men to intact men showed that castrated men lived longer than their comparison, (Hamilton and Mestler 1969). Furthermore, increased number of birthed children was associated with a decrease in lifespan; fertility analysis negatively correlated longevity with number of progeny and positively correlated with age at first childbirth, meaning females who lived longer, had their first child later in life compared to the female population, (Westendorp and Kirkwood, 1998). However, according to resource allocation, females should have a shorter lifespan compared to males due to energy spread to reproduction rather than maintenance and repair. According to the Office for National Statistics (ONS) in UK, average life expectancy was 79.0 years for males and 82.9 years for females in 2022, (Coombs et al., 2019). A surprising example which was thought to works against the disposable soma theory of ageing, is caloric restriction. Yet, caloric restriction without malnourishment, has been shown to increase lifespan in yeast (Lin et al., 2002), C. elegans (Klass, 1977), Drosophila (Partridge et al., 2005), and mice (Weindruch et al., 1986).

1.3 THE HALLMARKS OF AGEING

The hallmarks of ageing is a concept in the field of gerontology that refers to the underlying biological mechanisms and processes that contribute to the ageing of cells and organisms. Hallmarks represent a range of cellular and molecular changes that occur during ageing, and can be categorised as such, (López-Otín et al.2023). These hallmarks are: genomic instability, telomere shortening, epigenetic alterations, loss proteostasis, disabled macroautophagy, deregulated of nutrient sensina. mitochondrial dysfunction, cellular senescence, stem cell exhaustion, altered intracellular communication, chronic inflammation and dysbiosis, (López-Otín et al. 2023). The proposed twelve hallmarks of ageing are grouped into three categories: primary, antagonistic, and integrative hallmarks (López-Otín et al. 2023). Hallmarks considered to be the primary causes of ageing are genomic instability, telomere attrition, epigenetic alterations, loss of proteostasis and disabled macroautophagy. Hallmarks which reflect responses to damage or antagonistic responses to the damage are deregulated nutrient sensing, mitochondrial dysfunction, and cellular senescence. Antagonistic responses initially prevent/delay the damage, but eventually, if chronic or exacerbated, become deleterious themselves. Lastly, integrative hallmarks, such as stem cell exhaustion, altered intracellular communication, chronic inflammation and dysbiosis, are ultimately responsible for the functional decline of the organism as a response to accumulated damage inflicted by the primary and antagonistic hallmarks of ageing, (López-Otín et al. 2023). Moreover, the authors propose that each hallmark should fulfil the following criteria: (1) it should show during normal ageing; (2) its experimental intensification should accelerate ageing and (3) its experimental easing should retard the normal ageing process and hence increase lifespan. The hallmarks of ageing are interconnected to the eight hallmarks of failing health and may explain why amelioration of one hallmark may impinge on others, (López-Otín et al. 2023.) (Figure 1.1).



Figure 1.1: Hallmarks of ageing.

A scheme compiled of 12 recognised hallmarks of ageing, proposed in the field 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. The hallmarks are categorised into three categories: primary, antagonistic, and integrative. Adapted from López-Otín et al. 2023.

1.3.1 DEREGULATED NUTRIENT SENSING

Deregulated nutrient sensing is one of the hallmarks of ageing, representing a shift in the body's ability to sense and respond to nutrient availability with age. The nutrient-sensing networks are highly conserved in evolution and include extracellular ligands, such as insulins and insulin-growth factors (IGF), the receptor tyrosine kinases with which they interact, and an intracellular signalling cascade that carries out the nutrient response. The cascades involved are PI3K-AKT and Ras-MEK-ERK pathways as well as the transcription factors, FOXOs and E26 family factors, (López-Otín et al. 2023). The mechanistic target of rapamycin (mTOR) complex-1 (mTORC1) responds to nutrients, such as glucose and amino acids as well as stressors such as hypoxia and

low energy in turn to modulate activity of transcription factors such as SREBP and TFEB, responsible for lipid homeostasis and starvation response, respectively, (Eberle *et al* 2004, Soukas and Zhou 2019). The network responds to nutrition and stress grade by activating anabolism if nutrients are plentiful and stress is low, or by inducing cellular defences and catabolism if nutrients are poor and stress is high. There is extensive crosstalk between the different nutrient sensing pathways and genetic reduction in the activity of components of the nutrient-sensing pathways has been shown to increase lifespan and healthspan in a range of model organisms, (Slack et al., 2015, Singh et al., 2019).

1.4 INSULIN/INSULIN-LIKE GROWTH FACTOR-1 (IGF1) SIGNALLING (IIS)

Insulin/IGF-1 signalling pathway is a highly conserved nutrient sensing pathway, being conserved from C.elegans to humans (Figure 1.2), and was the first pathway, reduction of which, was shown to extend lifespan (Figure). In 1988, Friedman and Johnson have used three age-1 mutants (MK7, MK31 and MK546), previously isolated by EMS mutagenesis by Klass, 1983. Age-1 is a C, elegans catalytic orthologue to phosphoinositide-3-kinase (PI3K), and mutations in age-1 have been shown to extend mean lifespan in *C.elegans* by 30-50% and maximal lifespan by 10-60% compared to wild-type, N2 controls. (Friedman and Johnson, 1988). However, the lifespan extension of age-1 mutation came at a cost; age-1 mutant worms had reduced fertility of around 10-20%, (Friedman and Johnson, 1988). Later a study by the Kenyon lab in 1993 showed that mutations in the gene daf-2, encoding IGF1 receptor in C. elegans, caused active, adult worms to live more than double compared to wild type controls. Increasing lifespan from reduced IIS required the Forkhead FoxO transcription factor, encoded by *daf-16*, whereby double mutation of daf-2 and daf-16 did not extend lifespan of C. elegans suggesting that the lifespan extending properties of reduced daf-2 are dependent on presence of daf-16, (Kenyon et al., 1993). This early discovery led to a plethora of research into the role of IIS in ageing. In the fruit fly, Drosophila melanogaster, the insulin-like receptor (InR) is the homologue to C. elegans daf-2. Based on molecular similarity between fly InR and worm *daf-2* proteins due to both being members of the IIS receptor family and sharing amino acid similarity to mammalian insulin receptor, (Ruan et al 1995, Kimura et al., 1997), it was suggested that mutation of *InR* should lead to lifespan extension in adult flies. Tatar et al., 2001 has shown that mutation in insulin receptor (InR) is sufficient to extend median lifespan of adult flies by up to 85%, albeit causing a dwarf phenotype of both male and female flies, (Tatar et al., 2001). Upstream of InR, Gronke and colleagues have shown that reduced expression of Drosophila insulinlike peptides (*dilps*) involved in activation of the IIS specifically *dilp2* was sufficient to extend lifespan of both male and female flies, (Gronke et al., 2010). Additionally, ablation of DILP producing median neurosecretory cells in Drosophila led to an increase in median lifespan by 19.5%, (Broughton et al., 2010). Similarly, mutation of the insulin receptor substrate (IRS) homolog chico has been reported to extend lifespan of Drosophila. Clancy and colleagues show that null mutation of the chico, increased median lifespan of female fruit fly by 48%. Conversely, homozygous chico mutant male flies were short lived whereas heterozygous chico mutants were long lived, both male and females, (Clancy et al., 2001). Follow up work on chico mutant flies by Yamamoto and Tatar show that transcription factor dfoxo was essential for chico associated lifespan extension as chico and dfoxo double mutants were no longer long-lived, (Yamamoto and Tatar, 2011). Furthermore, overexpression of dfoxo in adult fat body has been shown to increase mean lifespan of female fruit flies by 20 to 50%, showing that tissue specific dfoxo overexpression is sufficient to elicit lifespan extension, (Giannakou et al., 2004). Similar work has been carried out in mice, concurrently, two laboratories published articles relating to mutation of IGF-1 receptor in mice, (Nakae et al., 2002; Holzenberger et al., 2003). Holzenberger and colleagues inactivated the IGF1 receptor in mice by creating heterozygous knockout mice, as null mutants were not viable. In their study, they reported that both female and male IGF1 receptor mutant mice lived on average 26% longer than their wildtype controls, with female mutant mice living 33% longer and male mice living 16% longer, albeit male lifespan extension was not statistically significant. Fascinatingly, *IGF1* receptor mutant mice did not develop dwarfism as did *InR* mutant flies, but also mice were physically and physiologically normal. Furthermore, IGF1 receptor mutant mice did not show a significant decrease in fertility, unlike *daf-2* mutant worms which became sterile and InR mutant Drosophila which had reduced fecundity, (Chen et al., 1996; Gems et al., 1998, Clancy et al., 2001). Holzenberger has attributed increased lifespan of IGF1 receptor mutant mice due to their increased oxidative stress resistance, (Holzenberger et al., 2003). Nakae and colleagues decided to take a step further and explore the role of FOXO1 in IGF1 receptor mutant mice. IGF1 receptor mutant mice were insulin resistant due to their inability to respond to insulin nor IGF1. Due to several embryonic defects of null FOXO1 mutants, Nakae decided to carry out all subsequent experiment with double-heterozygous mutants of IGF1 receptor and FOXO1. Gene expression analysis in adipose tissues of double-heterozygous mutants indicate that Foxo1 is involved in expression of key adipocyte differentiation markers (PPARy, LEP and SLC2A4), crucial for insulin sensitivity, suggesting that FOXO1 heterozygous mutation can improve insulin sensitivity of IGF1 receptor mutant mice via regulation of adipocyte differentiation, (Nakae et al., 2002). Likewise, fat-specific insulin receptor knockout (FIRKO) has been shown to increase mean lifespan by 18% and brain specific knockout mice live on average up to 18% longer,

(Bluher et al., 2003, Taguchi et al., 2007). Downstream of Insulin/IGF-1 receptor is phosphoinositide 3-kinase (PI3K) enzyme, reduction of which has also been implicated in longevity. In C. elegans, PI3K is encoded by a gene age-1, mutation of which was first identified by Michael Klass in 1983, (Klass, 1983). Later it was reported that mutation in age-1 extended mean lifespan by over 50% at 25°C compared to wild-type worm (Friedman and Johnson, 1988). Additionally, Dorman and colleagues have shown that age-1 and daf-2 genes are part of the same pathway, as recessive mutations in two genes, age-1, and daf-2, do not live significantly longer than *daf-2* single mutants, suggesting the two genes act in the same lifespan promoting pathway. Furthermore, *daf-16* and *age-1* double mutants did not extend worm lifespan suggesting that age-1 lifespan extension depended on presence of *daf-16*, (Dorman *et al.*, 1995). A similar phenomenon is observed in Drosophila, whereby pharmaceutical inhibition of PI3K using a specific inhibitor of PI3K, LY-294002 results in lifespan extension. Supplementation of 5 µM LY-294002 directly into the fly food, increased median lifespan of male and female flies by 14%, showing a direct geroprotective effect of pharmacological PI3K inhibition, (Moskalev and Shaposhnikov, 2010). In rodents, long term inactivation of principal mammalian IIS-responsive isoform of PI3K, p110a, has been shown to not only have beneficial effects on age-related reduction in insulin sensitivity and fat accumulation, but also extended lifespan of male mice, (Foukas et al., 2013). Partial inactivation of p110a via heterozygous mutation showed a modest increase in male lifespan of approximately 6% compared to wild-type controls, however this was only seen in mice that survived to middle age of around 500 days, as heterozygous mutant mice suffered increased mortality early in life (50-500 days) due to a pathologic effect of p110α inactivation, (Foukas et al., 2013).



Figure 1.2: Conservation of insulin signalling pathway.

A simplified schematic depiction of the glucose and insulin/IGF-1 pathways in yeast, worm, flies, and mice. The signalling pathways starts when the ligands bind an appropriate receptor. For yeast, the ligand is mainly glucose and amino acids. Worms have 38 ligands, discovered so far, INS1-38, flies seven (Dilps1-7), nice three (insulin, IGF11, and IGF12). The yeast receptor is mainly represented as Gpr1p, worm and flies present only one receptor, DAF-2 and InR, respectively., whereas mice present three (IGF1R,IRA,B). The activation signal is then transduced from the receptor via adaptor proteins: the GTPase RAS in yeast, the worm IST-1, and the fly Chico, the homologs to vertebrates are insulin receptor substrates 1-4 and RAS in mice. The respective downstream signalling homologues are pictures in blue and are horizontal to each respective organism with the respective signalling effects, transcription factors, pictures in purple. Many other genes involved in the pathway are not shown here, have been implicated in lifespan regulation.





A simplified schematic depiction of mTOR complex 1 and mTOR complex 2 components. mTORC1 and mTORC2 share the core proteins mTOR, mammalian lethal with SEC13 protein 8 (mLST8) and the inhibitory protein DEP domain-containing mTOR-interacting protein (DEPTOR). Additionally, mTORC1 contains a regulatory-associated protein of mTOR (Raptor) and inhibitory subunit proline-rich Akt substrate of 40kDa (PRAS40), whereas mTORC2 contains rapamycin-insensitive companion of mTOR (Rictor) and the regulator protein Protor. Figure was adapted from Sanguesa *et al.*, 2019.

In addition to the IIS pathway that participates in nutrient and growth factor sensing, another nutrient-sensing system is a focus of intense investigation, mechanistic target of rapamycin (mTOR). mTOR is a serine/threonine protein kinase which is an amino acid concentration sensor and is involved in regulating protein synthesis, cellular growth, and proliferation, (Saxton and Sabatini, 2017). mTOR receives and integrates hormonal stimuli coming from IIS as well as specific nutrients, particularly amino acids like leucine, (Kamei *et al.*, 2020). The mTOR kinase is part of two multiprotein complexes (Figure 1.3), mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2), that both regulates most aspects of anabolic metabolism, (Figure 1.3) (Ragupathi *et al.*, 2024). mTORC1 is shown to play a role In most of the actions exerted by mTOR kinase. Particularly, activation of mTORC1 transduces M. Laskovs PhD Thesis, Aston University, September 2024

anabolic signals, the main function of which are the biogenesis of ribosomes and synthesis of proteins and nucleotides according to the cell's needs, (Ragupathi et al., 2024). Initially, studies in budding yeast S. cerevisiae identified the target of rapamycin genes tor1 and tor2 as genetic mediators of rapamycin's inhibitory effects. Early in the 90s, Heitman and colleagues discovered that rapamycin, in vitro, binds and inhibits FK506-binding protein (FKBP) proline rotamase, (Heitman et al., 1991). It was shown that yeast strains that lack FKBP proline rotamase were viable yet fully resistant to rapamycin's growth inhibitory effects. In addition to growth inhibitory effects of rapamycin, the role of mTOR has been investigated for its role in longevity. Initial studies in S. cerevisiae by Powers et al., 2006 and Medvedik et al., 2007 have shown clear lifespan extending properties of mTOR inhibition by rapamycin. In yeast, lifespan can be measured in two ways, chronological lifespan (CLS), defined as the time that cells remain viable in stationary phase, and replicative lifespan (RLS) measured by the number of mitotic events an individual mother cell can undergo before senescence, (Mortimer and Johnston, 1959, Fabrizio et al., 2003). Powers and colleagues carried out a genome-wide deletion screen for longevity inducing genes, using a collection of ~4800 unique yeast strains, each with single homozygous gene deletion, (Winzeler et al., 1999). The screen generated a list of TOR regulated genes, shown to increase CLS, such as *gln3*, coding for TOR-regulated transcription of nitrogen acquisition genes, as well as gln3-regulated genes such as lys12, mep3, agp1, and mep2 involved in metabolism of amino acids containing nitrogen in their side chain, (Powers et al., 2006). Previous work has shown that TOR signalling in yeast is regulated by environmental amino acids, particularly intracellular glutamine levels, (Martin and Hall 2005). Additionally, it was shown that TOR is preferentially activated by nitrogen containing amino acids such as asparagine and glutamate, (Cunningham et al., 2002; Crespo and Hall, 2002; Oliveira et al., 2005). Therefore, Powers and colleagues hypothesised that removal of such preferred nitrogen containing amino acids should elicit a CLS extension. To test this, a three way mTOR inhibition was tested to investigate CLS extension: (1) Firstly, removal of mTOR activating amino acids from the media, asparagine, and glutamine, extended yeast's CLS even though total nitrogen content was held constant. (2) Low dose direct inhibition of mTOR by rapamycin treatment (300pg/ml – 1ng/ml) was shown to extend yeast CLS in a dose-dependent manner. (3) Inhibition of glutamine synthetase, involved in production of glutamine via condensation of glutamate and ammonia by a drug, MSX, also extended CLS in yeast by reducing intracellular glutamine which reduced TOR signalling, (Powers et al., 2006). Additionally, Medvedik and colleagues, independently, have shown that rapamycin extends yeast lifespan, (Medvedik et al., 2007).

Similar experiments using rapamycin were carried out in *C. elegans, Drosophila* and M. Laskovs PhD Thesis, Aston University, September 2024

mice. In C. elegans, the role of TOR signalling in longevity seems to act independently of IIS, due to not needing DAF-16, the FOXO orthologue in *C.elegans*, for the lifespan extension of reduced TOR kinase, (Bishop and Guarente, 2007, Sheaffer et al., 2008). Regardless, it has been shown that rapamycin treatment in C. elegans extends median lifespan of wild-type worms by 19%, (Robida-Stubbs et al., 2012). Furthermore, lifespan extension by rapamycin acts independently of DAF-16 (FoxO) but instead is dependent upon the transcription factor SKN-1/Nrf, that resembles mammalian NRF2 and is responsible for the detoxification response, (Robida-Stubbs et al., 2013). It was shown that rapamycin in C. elegans seems to affect both TOR complexes, with SKN-1/Nrf being essential for lifespan extension, as rapamycin did not extend lifespan in skn-1 mutant worms, (Robida-Stubbs et al., 2012). In Drosophila, feeding rapamycin to adult flies produces significant lifespan extension in both male and female wild-type flies, however rapamycin did not show a dose-dependent response in lifespan extension and a low dose of 1µM did not extend lifespan, (Bjedov et al., 2010). Furthermore, rapamycin treated flies showed increased survival under starvation, partly due to higher triglyceride levels as well as increased oxidative stress resistance against paraquat, (Bjedov et al., 2010). Also, rapamycin treatment can extend lifespan of some long-lived IIS mutants such as chico mutants, showing that rapamycin extends lifespan by a mechanism separate to that affected by downregulated IIS, (Bjedov et al., 2010). Lastly, rapamycin extends lifespan in mice, both inbred as well as in genetically heterogeneous mice, (Harrison et al., 2010, Miller et al., 2011, Anisimov et al., 2011). Miller and colleagues have shown that rapamycin administration within the food at a dose of 14mg/kg of young mice (9 months) produced a significant extension of lifespan (both median and maxiumal) with an increase of average median lifespan of male mice by 10% and 18% in females, (Miller et al., 2011). In addition to inhibition of TOR by rapamycin, genetic manipulation of genes involved in the TOR signalling pathway have also been associated with lifespan extension across a range of model organisms. In yeast, deletion of tor1 and removal of two proteins transcriptionally regulated by TOR, rpl31a or rpl6b, has been shown to extend replicative lifespan, (Kaeberlein et al., 2005). Likewise, TOR knockdown by RNAi in C. elegans from adulthood led to increased lifespan compared to wild-type, but also *daf-16* mutation did not suppress the longlived phenotype of TOR RNAi in worms, again showing that lifespan extension by TOR inhibition acts independently of DAF-16, (Vellai et al., 2003). It was also shown by Jia and colleagues that TORC2 heterozygous mutation (*daf-15*) led to an increase in lifespan, (Jia et al., 2004). In Drosophila, Kapahi and colleagues have shown that ubiquitous overexpression of a dominant negative form of *dTOR* led to a mean lifespan increase of 24% at 29°C, (Kapahi et al., 2004). In the same study, ubiquitous expression of two key proteins that physically interact with dTOR, dTsc1 and dTsc2,

also extended mean lifespan at 29°C by 14% and 12%, respectively. Furthermore, these effects on lifespan were shown to be tissue specific as overexpression of dTsc1 and dTsc2 in the muscle and fat tissue resulted in lifespan extension but not in the eve nor nervous system (Kapahi et al., 2009). One of the downstream effectors of translation initiation by TOR in flies and mammals is S6 kinase, which is activated upon phosphorylation by TOR, (Marygold and Leevers et al., 2002). Kapahi and colleagues showed that ubiguitous overexpression of a dominant negative form of S6 kinase extended lifespan by 22% at 29°C whereas ubiquitous overexpression of constitutively active form of S6 kinase decreased mean lifespan by 34%, showing that inhibition of TOR effectors is also sufficient to extend lifespan in Drosophila, (Kapahi et al., 2009). More recently, S6 kinase knockdown by RNAi in the fat body of Drosophila was shown to extend median lifespan, (Zhang et al., 2024). The effect of mutagenesis of mTOR in mice on lifespan extension is less obvious. Lamming and colleagues have shown no increase in lifespan in females nor males in heterozygous mutants of MTOR nor RAPTOR, nor double heterozygous MTOR:RAPTOR mutants . However, they did see a lifespan extension in female heterozygous MTOR, MLST8 double mutant mice, MLST8 being a subunit shared between the two mTOR complexes, (Lamming et al., 2012). Furthermore, deletion of S6 kinase in mice, does extend lifespan by 19% in female mice with no significant lifespan extension observed in male mice, (Selman et al., 2009). Due to mTOR's role in regulating protein synthesis and translation, ribosomal protein mutations as well as translation initiation factor mutations were studied for their effects in extending the lifespan of yeast and C. elegans. In Saccharomyces cerevisiae deletion of the ribosomal protein genes rpl6b, rpl9a and rpl19A as well as deletion of genes encoding the translation initiation factors tif4631 and tif1/2 have all been shown to extend replicative lifespan, (Smith et al., 2008). Likewise, reduction of ribosomal proteins and initiation factors in C. elegans has also been shown to extend lifespan, (Hansen et al., 2006).

Sirtuins are conserved nutrient sensors studied largely in the last decade due to their pleiotropic functions affecting lifespan. The term sirtuin was coined from its founding member Sir2 in Saccharomyces cerevisiae, discovered by Guarente in 1999, (Guarente, 1999). SIR is abbreviated from Silent Information Regulator and four SIR genes were identified through a genetic screen for mutations that suppressed transcription silencing, (Guarente, 1999). The gene product Sir2p is an enzyme called histone deacetylase (HDAC, Class III), that removes acetyl groups from acetylated lysine on histones. In mammals, SIRT family includes seven members (SIRT 1-7), located in different cell compartments with a range of roles. SIRTs play a key role in regulating metabolism, oxidative stress, cell survival, autophagy and consequently ageing, (Li and Kazgan, 2011). SIRT activity is dependent on NAD⁺, and since this molecule accumulates during fasting or exercise, SIRT become activated in situations of low energy levels, (Li and Kazgan, 2011). Research interest in the role of sirtuins in ageing started after a report showed that extra copies of sir2 in Saccharomyces cerevisiae, extended replicative lifespan by 30% by preventing the formation of age-associated extrachromosomal DNA circles, (Kaeberlein et al., 1999). Subsequently, sirtuins were shown to regulate longevity in a range of lower organisms. In *C. elegans* there are four sirtuin paralogues that code for three human homologues: SIR-2.1 is a human SIRT1 homologue, SIR-2.2 and SIR-2.3 are human SIRT4 homologue and SIR-2.4 is a human SIRT6 homologue, (Naseer et al., 2021). Tissenbaum and Guarente published the first report to show that *sir-2.1* plays a role in lifespan extension of *C. elegans*, (Tissenbaum and Guarente, 2001). By generating strains with duplications of many chromosomal regions, specifically those of sir-2.1, increased sirtuin activity was shown to extend mean lifespan by 20-27% compared to control animals, (Tissenbaum and Guarente, 2001). Furthermore, these effects on lifespan were shown to depend on Daf-16 as there was no lifespan extension by sir-2.1 overexpression in daf-16 mutants, but also no further lifespan extension in daf-2 mutants, indicating that sir-2.1 extends lifespan through modulation of the IIS pathway, (Tissenbaum and Guarente, 2001). The role of sir-2.2-4 paralogues in C. elegans longevity is more complicated. Both sir-2.2 and sir-2.3 are localised in the mitochondria, and play a role in mitochondrial functioning and metabolism regulation. Unlike sir-2.1 overexpression which led to lifespan extension, sir-2.2 and sir-2.3 deletion mutations have been shown to increase lifespan in C. elegans, the underlying mechanism is thought to be hermetic, but more investigation is required, (Chang et al., 2017). The knockdown of sir-2.4 expression via RNAi, has been shown to decrease lifespan and to be dispensable for Daf-16 function in response to reduced IIS, suggesting that *sir-2.4* does not play a role in longevity of *C. elegans*, (Chiang et

al., 2012). The *Drosophila* genome contains five sirtuin paralogues named *Sirt1*, M. Laskovs PhD Thesis, Aston University, September 2024

Sirt2, Sirt4, Sirt6 and Sirt7, after their closest mammalian orthologs, yet only Sirt2 and Sirt4 have been shown to play a role in longevity in Drosophila. Ubiquitous overexpression of Sir2 increased mean lifespan by 57% both in males and females, furthermore Sir2 overexpression using a neural-specific promoter also led to an increase in mean lifespan, (Rogina and Helfand, 2004). Additionally, caloric restriction did not further extend lifespan of long-lived Sir2 overexpressing flies, showing that Sir2 was necessary for lifespan extension of caloric restriction, (Rogina and Helfand, 2004). More recently, Wood and colleagues showed that Sirt4 knockout led to a decrease in lifespan, whereas ubiquitous and fat-body tissue-specific overexpression of Sirt4 led to an increase in lifespan of Drosophila, (Wood et al., 2018). Lastly, members of sirtuin family of proteins have been shown to extend lifespan in mammals. Kanfi and colleagues, showed that transgenic mice overexpressing SIRT6 have a significantly longer median lifespan than wild-type mice by around 15%, however this was only observed in male mice, not female mice, (Kanfi et al., 2012). Additionally, male SIRT6 overexpressing mice were shown to have lower serum IGF1 levels compared to wild-type control, which remained lower in male mice from 6 months up to 19 months, a difference which was not observed in female SIRT6 transgenic mice. Due to a key role of IIS in lifespan regulation, lower IGF1 levels in SIRT6 transgenic male mice could explain the lifespan extension associated with SIRT6 overexpression, (Kanfi et al., 2012). The role of other mammalian sirtuin paralogs in lifespan extension is more controversial. Roichman and colleagues, have shown that SIRT6 overexpression increases median lifespan both in male and female mice, by 27% and 15%, respectively, (Roichman et al., 2021), which contradicts the observations made by Kani et al., 2012, despite using the same genotypic strain. Furthermore, Roichman and colleagues have shown that SIRT1 overexpression does not regulate lifespan in mice, of both sexes, yet tissue specific *SIRT1* overexpression in the brain, significantly extended mean lifespan in both male and female mice, by 9% and 16% respectively, (Satoh et al., 2013). Additionally, to lifespan extension, SIRT6 overexpression led to improvement in health span, as mice of both sexes, had lower incidence of gastrointestinal adenomas, and did not experience age-dependent decline in physical activity, shown by distance ran and voluntary time spent on the running wheel, (Roichman et al., 2021). The SIRT6 overexpression has also been shown to prevent age-dependent impairment of normoglycemia as mice were able to maintain a youth-like blood glucose levels even during ageing. The activity of sirtuins are dependent on NAD⁺ levels, which have been shown to decrease with age, yet SIRT6 overexpression prevented the age-related decline in NAD⁺ showing that SIRT6 overexpression prevents age-related metabolic decline in mice, (Roichman et al., 2021). The role of SIRT2 in overall lifespan in less clear as overexpression of SIRT2 in models of

progeria can rescue aspects of health and increase overall lifespan, however the effects are context based, (North et al., 2014). Furthermore, SIRT2 deficiency has been shown to progress the ageing-induced vascular changes, associated with increased medial thickness and breakage of elastin fibres leading to increased arterial stiffness and vascular ageing, (Zhang et al., 2023). Yet, there is no impact of SIRT2 overexpression on lifespan in mice in a nonprogeroid, wild-type background, (Wu et al., 2023). This suggests that the role of sirtuins in ageing is subject to context as well as tissue specific expression. Several studies have been published exploring the role of sirtuin activators and inhibitors to try to target sirtuins pharmacologically to improve health and extend lifespan. SIRT1-activating compounds (STAC), such as resveratrol, have been extensively tested for their potential to extend lifespan. For example, Drosophila fed food containing 200 µM resveratrol showed an increase in average lifespan of 17% and 10 % for female and male flies, respectively, and increase maximum lifespan, (Bauer et al., 2004). Likewise, a dosage of 100 µM of resveratrol has been shown to extend replicative lifespan in Saccharomyces cerevisiae and extend both median and maximal lifespan of C. elegans, (Jarolim et al., 2004, Wood et al., 2004). However, the positive effects of resveratrol on lifespan came with controversy. An independent study by Bass and colleagues in 2007 tested the effects of 100 µM of resveratrol on lifespan extension in Drosophila. Out of four lifespan trials tested, resveratrol showed lifespan extension in one trial in wild-type and Sir2.1 mutant flies, despite no loss of activity in the resveratrol samples, suggesting lack of lifespan extension by resveratrol in *Drosophila*, (Bass et al., 2007). Additionally, an independent study by Kaeberlein and colleagues, testing three yeast strain backgrounds, showed no effect of resveratrol on replicative lifespan on yeast, (Kaeberlein et al., 2005). Lastly, the effects of sirtuin overexpression on lifespan have yet to be solidified in *C.elegans* and *Drosophila*. Burnett and colleagues have shown that in C.elegans, outcrossing of a line with high level sir-2.1 over-expression abrogated the longevity increase but not in sir-2.1 over-expression which still extended lifespan, also outcrossing of a line with low level sir-2.1 over-expression, abrogated longevity, (Burnett et al., 2012). Moreover, ubiquitous over-expression of dSir2 using a UAS-GAL4 system was longer lived compared to wild-type control, as previously reported by Rogina and Helfand, 2004, however the flies were no longer lived compared to the appropriate transgenic control and nor was a new line showing stronger over-expression of dSir2, (Burnett et al., 2012). The report by Burnett and colleagues, shows the importance of controlling for genetic background and the mutagenic effects of transgene insertions in studies related to genetic effects on lifespan, (Burnett et al., 2012). This further solidifies that the role of sirtuins in lifespan is yet to be fully defined and requires additional investigation.

AMP-activated protein kinase (AMPK) is a conserved serine/threonine kinase which is activated in case of low cellular energy resulting in increased levels of AMP and ADP originating from ATP depletion, (Hardie, 2011). AMPK is comprised of three subunits, α catalytic subunit and two regulatory β and γ subunits which respond to AMP and ADP via direct interaction, as well as the upstream kinases, liver kinase B1 (LKB1) or calcium/calmodulin-dependent protein kinase kinase 2 (CaMKK2), (Hardie, 2011). AMPK is involved in a variety of catabolic processes that produce energy such as uptake and utilisation of glucose and fatty acids, mitochondrial biogenesis, and autophagy, (Hardie et al., 2012). Opposite of activation, AMPK is inhibited by phosphorylation by acetyl-CoA carboxylases (ACC), glycerol phosphate acyltransferases (GPAT), 3-hydroxyl-3-methylglutaryl CoA reductase (HMGCR) and glycogen synthase, thereby repressing anabolic processes like fatty acid, triglyceride, cholesterol, and glycogen synthesis, (Hardie et al., 2012). The role of metabolic rate of animals in regulation of ageing was already associated previously, (Hulbert et al., 2007). Energy metabolism maintains homeostasis whereas excessive consumption of energy enhances the ageing phenotype, and processes such as dietary restriction (DR) that preserve energy expenditure have been lengthily observed to extend lifespan in yeast, Saccharomyces cerevisiae (Anderson et al., 2003, Kaeberlein et al., 2004), in C. elegans, (Klass, 1977, Houthoofd et al., 2003), in Drosophila melanogaster, (Partridge et al., 1987, Chapman and Partridge, 1996), as well as in mice and rats, (Masoro, 2000, Masoro, 2002). Furthermore, lifespan extension by DR has been linked to signalling pathways controlled by AMPK. In Saccharomyces cerevisiae, AMPK is encoded by the Snf1 heterotrimer complex. Snf1 is composed of a catalytic α subunit (Snf1p), and regulatory subunits β (Sip1p, Sip2p or Gal83p) and γ (Snf4p) each subunit with distinct substrate specificity (Schmidt and McCartney, 2000). Ashrafi and colleagues have shown that forced expression of Snf1p or loss of Sip1p but not other β subunits caused accelerated ageing, while removal of Snf4p extended lifespan (Ashrafi et al., 2000). Additionally, Sip2p acetylation has been shown to play a crucial role in mediating Sip2p-Snf1p interaction as well as lifespan extension by rapamycin. Lu and colleagues have shown that rapamycin only mildly increases lifespan in Sip2p acetylation mutants, and also that sch9p serves as a common downstream effector of Sip2p, (Lu et al., 2011). In C. *elegans*, the catalytic α subunit is encoded by the *aak-2* gene, which has been implicated in regulating C. elegans lifespan, whereby null mutation leads to 12% reduction in lifespan compared to wild-type worms and higher aak-2 gene dose increased average lifespan by 13% compared to control, showing that aak-2 regulates lifespan in C. elegans, (Apfeld et al., 2004). Furthermore, Apfeld and

colleagues showed that *daf-16* and *aak-2* are both required for maximal lifespan extension of *daf-2* mutants, as *daf-2,aak-2* double mutants had lifespan indistinguishable from those of aak-2 single mutants, and daf-16,aak-2 double mutants had 15% shorter lifespan than either single mutant, indicating that aak-2 is able to influence lifespan in a *daf-16* independent matter and acts in parallel to *daf-2* to influence lifespan, (Apfeld et al., 2004). Studies investigating DR, have shown that lifespan extension by DR is dependent on *aak-2*, whereby DR showed no lifespan extension in aak-2 mutants, (Greer et al., 2007). In addition, Greer and colleagues have shown that *daf-16* is directly phosphorylated by *aak-2*, which could partly explain the lifespan phenotype observed by Apfeld et al., 2004, (Greer et al., 2007). Moreover, AMPK's role in lifespan extension is not limited to DR-dependent lifespan extension, AMPK has been shown to modulate longevity exclusively through posttranslational modifications of CREB-regulated transcriptional coactivator-1 (CRTC-1) together with calcineurin, (Mair et al., 2011). Mair and colleagues show that aak-2 overexpression leads to lifespan extension in C. elegans, previously shown by Apfeld et al., 2004, but also aak-2-dependent lifespan extension seemed to depend on CRTC-1 as there was no lifespan extension in CRTC-1 mutants (mutated at the phosphorylation sites of AMPK/calcineurin, S76, S179), overexpressing aak-2, (Mair et al., 2011). Lastly, AMPK has been shown to mediate formation of stress granules in response to DR, but not heat-stress independent of IIS-mediated lifespan extension, (Kuo et al., 2020). Altogether, this shows that AMPK is responsible for DRmediated lifespan extension in C. elegans independent of IIS. The role of AMPK in longevity has been shown to be conserved in Drosophila. Funakoshi and colleagues have shown that overexpression of *lkb1*, a serine/threonine kinase that plays a master role for activating AMPK, increases mean lifespan by 6.5% in female Drosophila relative to controls, but they did not see a lifespan extension in male Drosophila. This sex difference may be due to metabolic differences between the two sexes, and it was suggested that overexpression of *lkb1* extends lifespan of females by AMPK activation and, in turn, inhibition of TOR signalling, (Funakoshi et al., 2011). Additional investigation by Stenesen et al., 2013, have definitively shown that AMPK plays a role in modulating lifespan in Drosophila. Stenesen and colleagues overexpressed and knocked down AMPK using tissue specific promoters in the adult fat body (S106) and the muscle (myosin heavy chain). The overexpression of AMPK in the adult fat body of female *Drosophila* increased mean lifespan by 19% and 32% respectively, whereas knockdown of AMPK in the adult fat body and muscle decreased mean lifespan by 16% and 23% respectively, (Stenesen et al., 2013). Considering AMPK is crucial for energy sensing and energy homeostasis, it is logical that genetic modulation of AMPK expression would have a huge impact in muscle tissue. Additionally, Stenesen and colleagues have shown that enzymes involved in

inosine monophosphate (IMP) can also regulate lifespan of Drosophila where heterozygous insertional mutations of enzymes involved in synthesis of IMP increased lifespan of both female and male Drosophila by altering adenosine nucleotide ratios (AMP:ATP), (Stenesen et al., 2013). The heterozygous insertional mutants of IMP synthesis enzymes were shown to have lower levels of IMP, a crude surrogate of acute DR, leading to increased AMPK activity and lifespan extension, whereas dietary adenine supplementation (0.05 or 0.1 % (w/v), completely rescued the lifespan extension of heterozygous mutants of IMP synthesis enzymes as well as restored the lifespan extension of flies on DR, indicating that lower IMP levels are crucial for AMPK activity and subsequent lifespan extension, (Stenesen et al., 2013). The results of AMPK overexpression in muscle of Stenesen et al., 2013 has been replicated in a study by Ulgherait and colleagues that also report increase in lifespan of female Drosophila that overexpress AMPK, (Ulgherait et al., 2014). Furthermore, Ulgherait and colleagues have also shown that tissue-specific overexpression of AMPK in neurones and in intestine increase lifespan in *Drosophila*. Additionally, expression of canonical autophagy genes (Atg1, Atg8a, Atg8b) were significantly increased in Drosophila overexpressing AMPK, in the neurones, muscle and the intestines, (Ulgherait et al., 2014). Interestingly, lifespan extension by AMPK overexpression, depended on presence of Atg1. Atg1 knockdown by RNAi in neurones prevented the lifespan extension by AMPK overexpression, yet neuronal knockdown of Atg1 alone did not affect lifespan of Drosophila, (Ulgherait et al., 2014). Together this indicates that AMPK relies on autophagy to extend lifespan in neurones. Lastly, it has been shown that neuronal overexpression of AMPK leads to decreased expression of *dilp2* and *dilp5*, the *Drosophila* insulin-like peptides involved in activation of IIS, and also increase expression of 4E-BP a direct transcriptional target of Drosophila FOXO, (Ulgherait et al., 2014). A similar phenomenon was observed in the heads of Drosophila overexpressing AMPK in the muscles, showing an inter-tissue effect of AMPK. Together, the findings suggest that the whole-body lifespan extending effect associated with localised overexpression of AMPK and Atg1 may be mediated by global alteration in IIS via reduction of Dilps, (Ulgherait et al., 2014).

The direct role of AMPK protein in mammalian ageing is not very well established, however there are many studies looking at the role of AMPK in age-related diseases such as neurodegeneration and atherosclerosis. For instance, in a mouse model of Huntington's disease, AMPK has been shown to play a protective role against mutant huntingtin (Htt) protein load, the causative protein responsible for Huntington's disease, (Vazquez-Manrique *et al.*, 2016). Overexpression of the over-activated form of $AMPK\gamma1$ has been shown to induce clearance of misfolded Htt, whereas

knockdown of AMPK led to increased cell mortality suggesting that AMPK activity is responsible for protecting cells against cell vulnerability by reducing soluble Htt levels, (Vazquez-Manrique et al., 2016). Also, overexpression of the over-activated form of AMPKy1 lowered the average volume of lesions by 27% compared to control, showing that AMPK activation can slow-down the neurodegenerative progression induced by Htt expression in the brain, (Vazquez-Manrique et al., 2016). Following on from this work. Yan and colleagues have shown that AMPK overexpression can improve cognitive dysfunction in aged rats, through AMPK-SIRT1 and autophagy signalling, (Yan et al., 2019). Cognitive dysfunction is a common complication in the elderly after surgery involving anaesthesia (POCD), and rats with POCD show decreased expression of AMPK, (Yan et al., 2019). AMPKa1 overexpression has been shown to improve several cognitive abilities of rats with POCD such as decreased maze escape latencies, increased swimming time, distance travelled as well as platform crossings in a target quadrant as compared to control, (Yan et al., 2019). Furthermore, blocking of autophagy by 3-MA has shown partial attenuation of AMPKa1-mediated cognitive improvements, suggesting that positive cognitive effects of AMPK overexpression are partly dependent on autophagy, (Yan et al., 2019). On the contrary to overexpression, genetic loss of the AMPKa subunit in the neural retina of mice, accelerates ageing in the retina, suggesting that AMPK plays an important role in maintenance of retinal function and survival in normal ageing, (Xu et al., 2017). Lastly, even though the direct effect of genetic manipulation of AMPK is not extensively studied in mammals for its role in ageing, several studies have investigated the effect of indirect AMPK activators, such as metformin on ageing. Many classical agonists activate AMPK indirectly by altering AMP/ADP:ATP balance in the cells, thereby activating AMPK. An example of such drugs is metformin, which disrupts mitochondrial function by inhibiting electron transport chain complex 1, (Fontaine, 2018). The beneficial effects of metformin on health are more robust than its effect on ageing per se, as metformin being a widely prescribed as a treatment for type 2 diabetes as well as cancer, (Gallagher and Leroith, 2011). Metformin has been shown to potentially bind AMPK directly, (Zhang et al., 2012), yet it is considered mainly that metformin elicits its positive effects by perturbing mitochondrial electron transport chain function, (Hardie, 2006). The use of metformin has been shown to extend CLS of Schizosaccharomyces pombe, with a dose of 25 mM of metformin shown to extend CLS of fission yeast by around 33%, (Seylan and Tarhan, 2023). In fission yeast, metformin has been shown to reduce a range of markers associated with ageing such as reactive oxygen species and protein carbonylation, which were reduced upon metformin treatment, Seylan and Tarhan, 2023). Additionally, metformin has protective effects against environmental stressors such as hydrogen peroxide and heat stress, suggested as one of the

mechanisms by which metformin extends CLS in fission yeast, (Seylan and Tarhan, 2023). In C. elegans, a metformin dose of 50mM has been shown to extend median lifespan by 40% compared to control, yet a lower dose of 1mM or 10mM did not extend lifespan, (Onken and Driscoll, 2010). Intriguingly, metformin extended lifespan in *C. elegans* independent of DAF-16 and AGE-1 contrary to lifespan extension seen by overexpression of *aak-2* which does depend on DAF-16. This shows that metformin extends lifespan independently of IIS. Yet, metformin does not seem to extend lifespan in aak-2 mutants showing that lifespan extension elicited by metformin depended on AMPK without requiring IIS, (Onken and Driscoll, 2010). Onken and Driscoll showed that lifespan extension by metformin depended on the transcription factor SNK-1 similarly to rapamycin. However, the lifespan extension observed by metformin in C. elegans are not lifelong. Espada and colleagues have shown that the ability of metformin to extend lifespan during ageing diminishes, and becomes toxic, linked to decrease in mitochondrial activity and lower ATP levels in worms resulting in toxicity to metformin. However, mutants resistant to metformin were shown to have higher mitochondria content and higher expression of electron transport chain complex 1, (Espada et al., 2020). A common husbandry procedure for C. elegans is to co-culture them with live E.coli that act as a food source for worms. Work from Cabreire and colleagues showed that metformin extends C. elegans lifespan via AMPK but does so indirectly by altering folate metabolism in the live E.coli co-culture, whereas metformin fails to extend lifespan of worms cultured on dead bacteria, (Cabreiro et al., 2013). Unlike C. elegans, in Drosophila metformin does not extend lifespan. Slack and colleagues have shown that metformin increases phosphorylation of AMPK in a dose dependent manner, yet is insufficient to extend lifespan in both male and female flies, (Slack et al., 2012). Metformin doses of 10 nM and 100 mM decreased triglyceride storage, and at a dose of 100 mM led to a greater excretion of concentrated oblong deposits, suggesting that treatment with high concentration of metformin leads to a decrease in lipid storage as well as fluid imbalance in the fly intestine, an effect that is evolutionarily conserved, (Slack et al. 2012). The effect of metformin on lifespan in rodents seems to be similar to that observed in C. elegans where metformin seems to extend lifespan but has reduced effectiveness in older mice, (Anisimov et al., 2008). Anisimov and colleagues showed that chronic treatment of female mice with metformin (100mg/kg in drinking water), increased mean lifespan by 38%, but also extended maximal lifespan by around 10%, compared to control mice, (Anisimov et al., 2008). However, metformin did not lower incidence of spontaneous tumours, and the effectiveness of metformin decreased in older mice suggesting that metformin effects on lifespan extension, in mice, are agedependent, (Anisimov et al., 2008). A further investigation by Anisimov and colleagues tested the effectiveness of metformin to extend lifespan in mice starting
the treatment at a range of ages (3,9 and 15 months), and showed that metformin treatment of 100mg/kg of body weight, from the age of 3 months extended mean lifespan by 14%, yet treatment from the age of 9 or 15 months proved to be insignificant, showing a clear age-dependent effect of metformin on lifespan, (Anisimov et al., 2011). In addition to time-specific treatment of metformin studied by Anisimov, long-term treatment of metformin (0.1% w/w in diet) from middle age extended mean lifespan by 6% and health span in male mice, whereas a higher dose of 1% w/w in diet was toxic, causing a reduction in mean lifespan by 14%, (Martin-Montalvo et al., 2014). Martin-Montalvo and colleagues have also shown that metformin treatment of 0.1% w/w, improved physical performance of mice such as distance ran on a treadmill, and decreased cataracts index compared to control mice, additionally, a long term low dose metformin treatment activated AMPK without altering the electron transport chain activity but also enhanced antioxidant defence together with lowering of inflammation, both of which contributed to increased lifespan of male mice, (Martin-Montalvo et al., 2014). Further publications from Smith, Jr et al., 2010 and Strong et al., 2016, have shown that metformin supplementation in male mice did not significantly extend mean lifespan, however, Strong and colleagues did show that metformin in combination with rapamycin, did extend lifespan and the authors suggested that metformin's benefit was offsetting the negative effects of rapamycin on metabolism, yet metformin alone did not extend lifespan, (Smith, Jr et al., 2010, Strong et al., 2016). Put together, the literature on the effects of AMPK on lifespan extension is not fully established yet. Studies in lower organisms such as yeast, worms and flies suggest that elevated AMPK is sufficient to extend lifespan, yet in higher organisms such as rodents the conclusion of the role of AMPK on lifespan extension is still controversial, but does show promise in mitigating the deleterious effects of ageing, without extending lifespan.

1.8 CROSSTALK BETWEEN NUTRIENT SENSING PATHWAYS

A great attention during the last few decades has been focused on studying the involvement of nutrient sensing pathways, such as IIS, mTOR, AMPK and SIRTs, in longevity, yet unsurprisingly, complex crosstalk exists between the different nutrient sensing components in most model organisms. Evidence shows that a balanced diet together with an active lifestyle, poses its effects on health span and ageing through critical nutrient sensors of AMPK, SIRTs, IIS and mTOR, as well as by modulating the interaction of those pathways, (Simpson and Raubenheimer 2009). Generally, in model organisms, calorie limiting diets are life extending via activation of AMPK-SIRT pathway as these diets primarily influence the energy state of the organisms. On the other hand, diets abundant in nutrients, shorten lifespan in model organisms via

activation of mTOR signalling, (Lushchak et al., 2017). In fact, current literature suggests that anabolic signalling pathways accelerate ageing and catabolic signalling pathways extend lifespan in model organisms, (Fontana et al., 2010). Under well fed conditions, the pathways IIS and mTOR are activated, participating in sensing glucose and amino acids, converging towards anabolism, cell growth and ageing, whereas during fasting or under caloric restriction, interconnected nutrient sensing pathways, AMPK, SIRT and FOXO are induced. These act in the opposing direction to IIS and mTOR, driving oxidative catabolism, improving metabolic efficiency, increasing preservation and stress resistance, and counteracting the anabolic effects of IIS and mTOR. Steady activation of nutrient sensing pathways may play a crucial role in the development in age-related diseases whereas in conditions of nutrient depletion, changes in intracellular AMP/ADP/ATP ratio and NAD+/NADH ratios represent activation signals that modulate AMPK and SIRT pathways, respectively. For instance, when energy levels are low, a positive feedback loop between AMPK-SIRT is activated, which activates cellular energy generating systems and inhibits anabolic processes that consume energy, to achieve homeostasis. Processes activated by AMPK-SIRT during low energy states, counteract signals dependent on IIS and mTOR. Furthermore, a citric acid cycle intermediate, α-ketoglutarate, has been proposed as a key metabolite mediating lifespan extension by calorie restriction through inhibition of key components of the ATP synthesis machinery downstream of mTOR, (Chin et al., 2014). As both AMPK and SIRT are in a feedback loop, inhibition of one can affect the expression of the other, (Canto et al., 2009, Palacios et al., 2009). Moreover, AMPK has been shown to promote cytosolic SIRT activity by increasing NAD⁺ synthesis, and activation of SIRT leads to deacetylation and activation of LKB1, a key upstream activator of AMPK, (Canto et al., 2009, Palacios et al., 2009). The main targets of SIRT are also targets of the transcription factor, FOXO, and during nutrient depletion, FOXO is activated through phosphorylation or/and deacetylation by AMPK and SIRT respectively, (Greer et al., 2007). AMPK phosphorylation of FOXO does not alter its localisation, but seems to promote interaction of cofactors and FOXO at specific target gene sites, (Brown and Webb, 2018). The transcriptional efficiency of FOXO needs cofactors such as PGC1a, which plays an important role during energy depletion, (Jiang et al., 2020). Additionally, FOXO can bind to the PGC1a promoter, representing an auto-regulatory feedback loop, (Jiang et al., 2020). For instance, under caloric restriction, expression of PGC1a is induced promoting the expression of gluconeogenic and mitochondrial fatty acid oxidation genes, enhancing the trans-activating potential of FOXO, (Oh et al., 2013). Taken together, the AMPK-SIRT-PGC1α signalling loop improves stress resistance and metabolic fitness, under caloric depletion contributing to health span.

On the other hand, the IIS pathway, and its downstream target mTOR are activated in fed conditions as well as by specific nutrients such as sugars, and amino acids. Activation of IIS leads to activation of mTOR via activation of AKT and inhibition of FOXO. This in turn, inhibits AKT and can negatively regulate IIS. This negative feedback loop between mTOR and insulin signalling has been causally linked to conditions of insulin resistance such as type 2 diabetes, (Khalid *et al.*, 2021). Both IIS and mTOR are central to anabolism and may play a crucial role in ageing.

1.9 A BRIEF INTORUDUCTION TO RAS SIGNALING

The RAS (Rat sarcoma virus) signalling pathway is a crucial cellular signalling pathway that plays a central role in regulating cell growth, differentiation, and survival among other processes, (Laskovs *et al.*, 2022). RAS proteins are binary switches, cycling between active and inactive states (Figure 1.4). RAS proteins and their downstream signalling pathway are tightly controlled and coordinated as aberrant activation of RAS pathway in case of RAS gene mutations can lead to RAS-related diseases such as cancer, (Prior *et al.*, 2012).

RAS genes were initially discovered in the late 1960s, JJ Harvey isolated a retrovirus from leukemic rats, capable of producing sarcomas in infected rodents. The virus was named Harvey-MSV (Murine Sarcoma Virus) and shown to be the result of the recombination between the retroviral genome and a cellular rat gene that was responsible for the transforming ability of the virus, (Harvey 1964). Shortly after, another isolation of a mouse retrovirus that carried the ability to induce erythroblastosis and sarcomas, demonstrated a recombination mechanism of origin for this new retrovirus, named Kirsten-RAS, (Kirsten and Mayer 1967). From a discovery in rodents, research on RAS moved onto humans, where is the early 1980s the three RAS oncogenes were isolated, *HRAS*, *KRAS* and *NRAS*, (Krontiris and Cooper 1982, Parada et al., 1982, Saton et al., 1982).

After the initial isolation and characterisation of human RAS oncogenes in the first half of 1980s, the use of specific antibodies and nucleic acid probes gave rise to the identification of a wide superfamily of related small GTPases that share phylogenetic similarities with the original H- ,K- and NRAS oncogenes, acting as signalling molecular switches that control the activation of a range of cellular functions. The related small GTPases formed the subfamilies RHO/RAC, AFR and RAB, (Fernandez-Medarde et al., 2021).



Figure 1.4: RAS activation feedback loop.

A simplified schematic depiction the activation feedback loop of RAS activation by SOS. RASmediated signalling commonly occurs through the receptor tyrosine kinase (RTK)-SOS-Ras. Dimerisation and autophosphorylation of receptor tyrosine kinase results from binding of growth factor ligands. SOS constitutively interacts with an SOS adaptor GRB2/Drk. The SOS/GRB2 complex is recruited to phosphorylated RTK and bind to the RTK. The RTK/GRB2/SOS complex positions SOS to bind to inactive (GDP-bound) Ras located at the plasma membrane. Binding of SOS to Ras activates Ras by facilitating the exchange of GDP to GTP. Active GTP-bound Ras is returned to its inactive GDP-bound state through the activity of GTPase activating proteins (GAP).

The Ras/Raf/MAPK pathway is a well characterised pathway in cell biology. Ras/Raf/MAPK signalling is involved in a range of processes such as cell cycle regulation, wound healing, and tissue repair, as well as the ability to stimulate angiogenesis, all of which are important for tumorigenesis, (Boonstra et al., 1995, Cary et al., 1999, Stacey 2003). Activation of the pathway begins when an extracellular ligand (growth factors, hormones, and cytokines) binds to a protein tyrosine kinase receptor, (McKay and Morrison 2007). The best-known receptors in the pathway are the epidermal growth factor receptor (EGFR) and platelet-derived growth factor receptor (PDGFR). Binding of a ligand to the EGFR induces oligomerisation of the receptor, resulting in the activation of its kinase activity and autophosphorylation generates binding sites for adaptor proteins such as Grb2 which recognizes sequence homology 2 (SH2) domains of proteins such as Shc which

recruits guanine nucleotide exchange factors (GEF) like SOS and CDC25 to the cell membrane, (Schlessinger 2000). In turn, SOS interacts with Ras proteins at the cell membrane to promote a conformational change and exchange of GDP to GTP thereby activating Ras, (Figure 1.4). Following Ras activation, Raf is recruited to the cell membrane. Raf is the best characterised Ras effector and is a member of family of serine/threonine kinases. Raf activation initiates a phosphorylation signalling cascade downstream, via MEK, by phosphorylation of MAPKK which successively phosphorylates and activates downstream proteins such as ERK. Activated ERK phosphorylates multiple cytoplasmic and cytoskeletal proteins including ribosomal S6 kinase (S6K), (Mendoza et al., 2011). Activated ERK also translocates to the nucleus where it phosphorylates and activates members of the E-twenty-six (ETS) transcription factor family, (Slack 2017), (Figure 1.5). The ETS transcription factors are conserved across animals. They contain an ETS domain that is ~85 amino acids in length and folds into a winged helix-turn-helix DNA-binding motif that binds DNA at 5'-GGA(A/T)-3' ETS-binding motifs (EBM), (Dobson et al., 2019). ETS transcription factors commonly function as activators of transcription, but a few repress transcription, and different ETS factors can be differentially regulated by activated ERK. The ETS transcription factors Pointed (Pnt) and Anterior open (Aop) are key transcriptional effectors of RAS/MAPK signalling in Drosophila and play a key role in development (Boisclair Lachance et al., 2014). Both Pnt and Aop regulate the same set of genes but in opposite directions, (Dobson et al., 2019). In Drosophila, Pnt is activated in response to RAS signalling and functions as a transcriptional activator, whereas Aop is activated by RAS signalling inhibition and represses gene expression, (Dobson et al., 2019). Despite the well-known role of RAS signalling in cancer development, there is increasing evidence suggesting RAS signalling also plays an important role in animal ageing.

1.9.2.1 YEAST

Budding yeast, Saccharomyces cerevisiae, is commonly assessed by measurement of replicative and/or chronological lifespan. Replicative lifespan corresponds to the number of daughter cells produced by an individual mother yeast cell, whereas chronological lifespan measures the survival time of non-diving yeast cells in the stationary phase (Mirisola et al., 2014). S.cerevisiae have two RAS homologues, RAS1 and RAS2. RAS signalling in yeast plays a role in relaying nutrient signals into cell growth and division (Fontana et al., 2010). In the presence of nutrients, activation of RAS-cAMP-PKA signalling pathway mediates the activation of a growth program, which partially depends on the inhibition of stress-responsive transcription factors MSN2/4 (Conrad et al., 2014), (Figure 1.5). On the other hand, inhibition of RAScAMP-PKA pathway activates MSN2/4 transcription factors leading to enhanced cellular protection by activation of stress responsive genes such as heat-shock proteins, superoxide dismutase, catalase, and autophagy-related factors (Martínez-Pastor et al., 1996; Fabrizio et al., 2004; Mizuno and Irie, 2021). Martinez-Pastor and colleagues have shown that disruption of both MSN2 and MSN4 genes resulted in higher sensitivity to different stresses such as heat shock, osmotic and oxidative stress, and showed that MSN2 and MSN4 are required for activation of stress-resistance genes such as CTT1, DDR2 and HSP12, induction of which are mediated by stress-response elements (STRE), (Martinez-Pastor et al., 1996). Additionally, Mizuno and Irie have shown that MSN2 or MSN4 overactivation led to an increase in expression of autophagy gene, ATG39, whereas deletion of MSN2 or MSN4 led to a decrease in ATG39 expression under nitrogen depletion stress and ER stress, showing clearly that autophagy gene expression depends on MSN2 and MSN4 under stress, (Mizuno and Irie, 2021). On the other hand, activation of MSN2/4 is required for the effects of reduced RAS-cAMP-PKA signalling on extension of chronological lifespan (Fabrizio et al., 2004) and may also extend replicative lifespan in response to RAS-cAMP-PKA pathway inhibition (Medvedik et al., 2007). Sun and colleagues have carried out lifespan experiments on yeast strains which overexpressed both RAS1 and RAS2, and possessed either a partial deletion of RAS1 in its coding region or a disruption of RAS2 in its open reading frame, (Sun et al., 1994). It was shown that RAS1 deletion led to increased replicative lifespan whereas the strain with the disrupted RAS2 had a significantly shorter replicative lifespan than the control, (Sun et al., 1994). It was thought that lifespan extension of the RAS1 deletion mutant was due to compensatory increases in expression of RAS2, however northern blot analysis showed no increase in RAS2 mRNA in RAS1 mutants, (Sun et al., 1994). Furthermore, RAS1 overexpression did not lead to lifespan extension nor shortening whereas *RAS2* overexpression led to a 20-30% increase in mean lifespan compared to control, suggesting a divergent role of RAS in yeast longevity, (Sun *et al.*, 1994). Even though *RAS2* deletion shortened replicative lifespan, it was shown to extend chronological lifespan of yeast, (Fabrizio *et al.*, 2003). Additionally, genetic inhibition of *CDC25*, a yeast homologue of mammalian SOS, which stimulates the GDP to GTP nucleotide exchange of RAS proteins required for their activation, led to ~50% increase in chronological lifespan (Lin *et al.*, 2000). It was further shown that the lifespan extension associated with *CDC25* genetic inhibition was not dependent on heat stress resistance as the lifespan extension of the *CDC25* mutant strain was not mediated by *MSN2* nor *MSN4*, two transcription factors involved in stress-induced gene expression, repressed by cAMP-PKA pathways, (Lin *et al.*, 2000).

The transcriptional activity of MSN2/4 is influenced by phosphorylation by serine/threonine kinase effector of PKA, rim15 (Lee et al., 2013). RIM15 is activated in response to a low level of RAS-cAMP-PKA signalling and is crucial for the chronological lifespan extension of RAS2 deletion (Wei et al., 2008). Wei and colleagues have shown that RIM15 deletion in RAS2 mutant yeast, prevents the lifespan extension mediated by RAS2 mutation, showing that lifespan extension of RAS2 mutation is dependent on RIM15, (Wei et al., 2008). Additionally, RIM15 prevented lifespan extension of SCH9 mutant yeast, a yeast homologue of AKT/S6K, (Wei et al., 2008). Rim15 activity is also regulated by sch9, and upon activation rim15 translocates to the nucleus and is required for rim15-dependent msn2/4 activation. whereas sch9 inhibits rim15 nuclear translocation, yet the mechanism is not known (Pedruzzi et al., 2003). Lastly, genetic deletion of SCH9 extends replicative lifespan in yeast, in a RIM15 and MSN2/4 dependent manner (Kaeberlein et al., 2005; Wei et al., 2008). Both Kaeberlein and Wei, separately, have shown that SCH9 genetic deletion extended lifespan in yeast, dependent on RIM15 and MSN2/4, (Kaeberlein et al., 2005; Wei et al., 2008). RAS proteins in yeast can be activated by adenylate cyclase cyr1, genetic deletion of which extends chronological lifespan (Fabrizio et al., 2001). Additionally, Fabrizio and colleagues have shown that mutation of CYR1 in the absence of both MSN2/4 abolished the lifespan extension of CYR1 mutation. Also, CYR1 genetic deletion showed increased heat-stress and oxidative stress resistance, (Fabrizio et al., 2001).

1.9.2.2 *C.ELEGANS*

The canonical RAS signalling pathway is also conserved in C. elegans, however its function differs to that in yeast and flies, (Sternberg and Han, 1998). In worms, RAS GTPase, encoded by the *let-60* gene, plays a vital role in vulval development and excretory systems (Nanji et al., 2005), (Figure 1.5). Contrasting to yeast and flies, reduction-of-function mutations in *let-60* alone do not extend lifespan and partially suppress daf-2-dependent lifespan extension showing that the lifespan effect of RAS pathway inhibition is not conserved in *C. elegans*, (Nanji *et al.*, 2005). On the contrary, gain-of-function mutations in let-60 reinforce the longevity effects of daf-2 loss of function, thereby increasing lifespan in *daf-2* mutant worms (Nanji *et al.*, 2005). An important difference between the RAS pathway in C. elegans and that in other model organisms is the absence of aop orthologues in C. elegans. In its place, C. elegans may potentiate downstream signalling of let-60 via the ETS transcription factor lin-1,(Tiensuu et al., 2005), also implicated in transduction of IIS downstream of daf-2 receptor during ageing, (Dobson et al., 2019). This difference in RAS signalling in C. elegans may account for the differential effects of RAS inhibition on lifespan in C. elegans. Regardless of the absence of a direct orthologue of pnt in C. elegans, the transcription factor ETS-4 may represent a functional homologue, (Thyagarajan et al., 2010). A loss-of-function allele for ets-4 suggests that it acts in parallel pathway to daf-2 during ageing, (Thyagarajan et al., 2010). Thyagarajan and colleagues have shown that a deletion mutant of ets-4 extends the mean lifespan of C. elegans by around 25%, and additionally ets-4 knockdown using RNAi, also extended mean lifespan by around 20%, (Thyagarajan et al., 2010). Moreover, ets-4 was shown to act in parallel to IIS converging onto DAF-16 in respect to longevity. The double knockdown of ets-4 and akt-1/2, an effector of IIS, extended lifespan compared to sole knockdown of ets-4, showing that ets-4 and akt-1/2 play a parallel role in C. elegans lifespan extension. Furthermore, daf-16 knockdown eliminated lifespan extension of ets-4 knockdown, showing that the lifespan effects of ets-4 knockdown depend on *daf-16*, (Thyagarajan *et al.*, 2010). ETS-4 activity is a limiting factor in worm lifespan as restoring ets-4 expression within the intestine of ets-4 mutant worm is sufficient to rescue lifespan defects (Thyagarajan et al., 2010). Similarly, to aop protein and *dFOXO* protein in *Drosophila*, ETS-4 shares transcriptional targets with the worm FOXO orthologue, DAF-16 (Thyagarajan et al., 2010). Therefore, even though RAS signalling has different roles during ageing in *C. elegans* and flies, ETS transcription factors in these two model organisms may regulate the expression of common target genes that affect lifespan. Therefore, even though, direct inhibition of let-60 does not extend lifespan in C. elegans, inhibition of downstream ets-4 target genes,

involved in RAS/MAPK signalling in worms, may extend lifespan.

LET-60 signalling is crucial for vulval integrity which might also affect the ability of *let-60* loss-of-function to increase lifespan in *C. elegans.* Loss of vulval integrity decreases lifespan, whereas preventing vulval defects correlates with longevity (Leiser et al., 2016). The ETS transcription factor, LIN-31, is a vulval-specific effector of LET-60 signalling in worms, and forms a complex with LIN-1 which inhibits vulval induction. Direct phosphorylation of LIN-31 by MPK-1, the worm orthologue of ERK, which disrupts the LIN-1/LIN-31 complex, promotes vulval cell fate (Tan et al., 1998). It is suggestive that ubiquitous loss of LET-60 signalling in reduction-of-function mutants may decrease worm lifespan through the loss of vulval integrity via disruption to LIN-1/LIN-31 complex formation and activity. Preventing the reduction of LET-60 reduction of function in vulva. Also, the spatial activation of different transcriptional mediators of RAS/MAPK signalling may influence its extent of lifespan extension in *C. elegans* (Dobson et al., 2019).

1.9.2.3 DROSOPHILA

RAS signalling in Drosophila functions downstream of Insulin/insulin-like growth factor signalling (IIS) in modulating lifespan. Insulin receptor substrate (IRS) proteins couple downstream molecule activation during Insulin/IGF-1 receptor stimulation by recruiting downstream signalling molecules to the intracellular domain of the insulin receptor where they are activated by phosphorylation by the kinase activity of the receptor and then act as docking sites for other proteins for example, by recruiting SOS to the activated receptor via association with GRB2 adaptor protein (Dreadlocks/Drk protein) (Figure 1.5). Drosophila express a single IRS protein called Chico. Mutation of *chico* within the Drk binding sites disrupt the Chico-Drk protein interaction in vitro and prevents the lifespan extension associated with chico null mutation (Slack et al., 2015). Additionally, mutation of chico in flies expressing a constitutively active Ras was unable to extend lifespan showing that Chicodependent lifespan extension at least partly depends on downregulation of Ras protein, (Slack et al., 2015). Also, adult-onset ubiquitous and fat body specific expression of a dominant negative form of Ras, has been shown to extend median lifespan by 8% and 7%, respectively, (Slack et al., 2015). Moreover, ubiquitous and fat body specific, adult-onset knockdown of both ras and rl, Drosophila ERK ortholog, also extended median lifespan of Drosophila compared to control, (Slack et al., 2015). The key outputs of RAS-ERK signalling pathway during Drosophila development are mediated by two transcription factors: Pointed (Pnt), a transcriptional activator stimulated by RAS-ERK pathway, and Anterior open (Aop),

a transcriptional repressor inhibited by RAS-ERK pathway. The two transcription factors bind the same regulatory elements but with opposing outcomes, (Brunner *et al.*, 1994, Halfon *et al.*, 2000). The inhibition of Ras activity by fat body-specific expression of dominant negative Ras lead to an increase in the translocation of Aop into the nucleus, but also *aop* knockdown prevented the lifespan extension associated with *ras* inhibition, showing that *ras* inhibition depends on the function of Aop for its lifespan extending effects. On the contrary, overexpression of an activated form of *aop* together with *ras* inhibition did not lead to an enhanced lifespan extension, showing that Aop is both necessary and sufficient to mediate the lifespan extension effects of *ras* inhibition, (Slack *et al.*, 2015). Also, inhibition of RAS signalling alleviates the effects of neuronal mitochondrial dysfunction in a *Drosophila* model of familial Parkinson's disease, in which loss-of-function mutation in *ras85D* rescued the locomotive defects of *park*²⁵ homozygous mutant flies (Duncan et al., 2018). This suggests that RAS signalling inhibition may play a role in alleviating at least some of the deleterious symptoms of age-related diseases.

Unexpectedly, the effect of RAS/MAPK suppression on neuronal mitochondrial dysfunction phenotype were reiterated with RNAi-mediated knockdown of *aop* or *pnt*, which, generally, are considered to act antagonistically in the canonical RAS/MAPK signalling pathway. Whereas, in *Drosophila* motor neurons, *aop* and *pnt* proteins seem to act in a positive manner (Duncan *et al.*, 2018). Duncan and colleagues have shown that either genetic mutation of *pnt* or *ras* improves climbing function of *Drosophila* in models of mitochondrial disease and Parkinson's, (Duncan *et al.*, 2018). Therefore, even though direct inhibition of RAS/MAPK signalling offers beneficial outcomes in models of neuronal mitochondrial dysfunction and during normal ageing, *aop* protein may function differently based on context (Peláez *et al.*, 2015). For instance, *aop* stability in neurons is differently regulated by the RAS/MAPK pathway depending on the differentiation state of neuronal cells, (Peláez *et al.*, 2015).

RAS/MAPK signalling also has additional upstream inputs from other receptor tyrosine kinases (RTK) in addition to insulin receptor (IR) activation, which may contribute to the differential regulation of RAS/MAPK transcriptional effectors such as *aop* and *pnt* proteins. For instance, expression of dominant-negative isoform of the EGFR (epidermal growth factor receptor), but not IR, in the intestinal enterocytes of adult flies extends lifespan, similarly to the expression of activated *aop* in the same cells (Dobson *et al.,* 2019). Also, overexpression of *cic,* an inhibitor of *pnt* protein, in the gut and fat body of *Drosophila* extends lifespan, (Dobson *et al.,* 2019). Additionally, RAS/MAPK signalling has differential transcriptional outputs and tissue-specific expression, and regulation of other RAS-responsive transcription factors may contribute to lifespan extension associated with reduced RAS/MAPK signalling. Nonetheless, genetic inhibition of

RAS/MAPK signalling in different tissues by targeting specific upstream components of the pathway, especially RAS itself, is sufficient to extend lifespan, regardless of the downstream mediators (Slack et al., 2015, Duncan et al., 2018).

1.9.2.4 MAMMALS

The effects of RAS pathway inhibition on mammalian ageing have not yet been well defined. Mammals carry four members of the RAS protein family, N-, H-, KRAS4A and KRAS4B, which are expressed from three genes (Figure 1.5). KRAS4A and KRAS4B are both isoforms of the same Kras gene (Prior et al., 2012). The knockouts of Nras, Hras and Kras4A in mice are adult viable, however knockout of the entire Kras locus is embryonically lethal (Johnson et al., 1997). Furthermore, Kras4A deletion has been shown to have no significant effect on survival as Kras4a homozygous mutant mice were fertile and showed no histopathological abnormalities compared to inbred and crossbred genetic backgrounds, (Umanoff et al., 1995; Ise et al., 2000; Plowman et al., 2003). Inhibition of *Hras* has also been achieved indirectly via genetic knockout of Rasgrf1, which encodes the guanine nucleotide exchange factor, RASGRF1 that stimulates the GDP to GTP exchange and subsequently activates HRAS, as well as other members of RAS-related proteins (R-RAS) and RAC families of small GTPases (Fernández-Medarde and Santos, 2011). Homozygous knockout of *RasGrf1* has been shown to increase both average and maximal lifespan by ~20% compared to control animals (Borrás et al., 2011). Additionally, the increase in survival observed by knockout of Rasgrf1 was also seen in tumour-free animals suggesting that the lifespan extension was not a consequence of reduced tumour incidence (Borrás et al., 2011). Furthermore, metabolic analysis of RasGrf1^{-/-} mice has shown *RasGrf1^{-/-}* mice express higher levels of sirtuins in the liver and heart but also have lower IGF1 circulating levels, both sirtuins and IGF1 being associated with longevity, and showing an interplay between RAS, IIS and sirtuin pathways, (Borrás et al., 2011). Metabolomics analysis also revealed that $RasGrf1^{-/-}$ mice show a metabolic shift towards a calorie restricted phenotype, suggesting one of the potential reasons for why RasGrf1^{-/-} mice are longer lived, (Borrás et al., 2011).

In humans, mutations in *Hras* are associated with Costello syndrome, a rare genetic condition characterised by short stature, developmental delay, and distinctive facial features (Gripp et al., 2019). Costello syndrome patients show multiple physiological disorders normally associated with ageing, including osteoporosis and osteopenia (Gripp et al., 2019). *Hras1* allele variants consisting of a variable number of 28 bp tandem repeats downstream of the coding region are found at a reduced frequency in centenarians compared to young controls (Bonafè et al., 2002). These alleles of

Hras1 contain proposed binding sites for NF-kB, a key stress-responsive transcription M. Laskovs PhD Thesis, Aston University, September 2024

factor and inflammatory mediator (Bonafè et al., 2002). Variant analysis of copy-numbers associated with mortality at old age across three different populations, identified a deletion that covers Hras1 was consistently associated with higher mortality at older age (Kuningas et al., 2011). However, the *Hras1* region deletion is in a gene-rich region, thus other genes located in or near this region might also be relevant, as being potentially coregulated by a common transcription factor, (Kuningas et al., 2011). In the search for longevity-associated genes in humans, genome-wide association studies have not found an association between allelic variants of RAS genes and longer lifespan, to date. However, variants in *Hras1* are associated with exceptional longevity when occurring in combination with certain allelic variants of two other genes, APOE and LASS1 (known as CERS1) (Jazwinski et al., 2010). Analyses by Jazwinski and colleagues, have shown increased frequency of APOE, HRAS1, and LASS1 haplotypes as a function of increasing age, seen in both European cohorts that were analysed, (Jazwinski et al., 2010). Furthermore, allelic combinations of the three genes were also associated with reduced incidence of self-reported functional deficiencies in study participants (Jazwinski et al., 2010). Interaction between allelic variants of HRAS1 and longevity-associated genes such as APOE may therefore play a role in genetic effects in human ageing with LASS1 playing a regulatory role depending on its haplotype expression, as more moderate expression of LASS1 (approximately one-half of maximal expression) was associated with healthier ageing, (Jazwinski et al., 2010).



Figure 1.5: Evolutionary conservation of the RAS/MAPK signalling pathway from yeast to mammals.

The figure represents a schematic of the RAS/MAPK signalling pathway across a range of organisms. The RAS/MAPK signalling pathway responds to extracellular stimuli such as growth factors and nutrients to control cell survival, proliferation and metabolism and is highly conserved from yeast to mammals. (A-D) In this schematic is showing yeast (A), *Drosophila* (B), *C. elegans* (C) and mouse (D), orthologous signalling components are positioned in analogous locations within the pathway and are colour coded. In multicellular organisms, RAS/MAPK signalling pathway is activated with the binding of growth factors and/or nutrients to the receptor tyrosine kinase (RTK). Among species, association of the GEF SOS with RAS promotes GDP to GTP exchange on the RAS small GTPase, initiating the signalling cascade. The key downstream effector of RAS/MAPK signalling pathway activation is ERK, which translocates into the nucleus upon activation to regulate the activity of RAS-responsive transcription factors, including members of the ETS family. Figure adapted from Laskovs *et al.*, 2022.

1.10 USING SMALL MOLECULAR INHIBITORS TO INHIBIT RAS SIGNALLING PATHWAY

To understand the genetic pathways that underlie the ageing process, molecular targets were identified for pharmacological intervention that might both extend lifespan and improve age-related health. Small molecule RAS pathway inhibitors have been initially developed as anti-cancer therapeutics to target hyperactivating mutations in key RAS/MAPK signalling cascade components that drive unrestricted cell proliferation and differentiation, some of which are in clinical use. A few selected small molecule inhibitors that have been shown, directly or indirectly, to inhibit the RAS/MAPK pathway in a range of model organisms and increase lifespan and/or

1.10.1 TRAMETINIB

Trametinib is a specific inhibitor of MEK and is currently used to treat some forms of metastatic melanomas, (Hoffner and Benchich, 2018). Trametinib administration, orally, to adult Drosophila has been shown to extend median lifespan by 8-12% when added directly to fly food at concentrations from $1.56 - 15.6 \mu M$, (Slack *et al.*, 2015). Also, feeding of 15.6 μ M of trametinib to flies later in life, from 30 days of adulthood, was sufficient to extend adult flies' lifespan ($\sim 4\%$), however to a smaller extent as compared to trametinib treatment from the start of adulthood, (Slack et al., 2015). Trametinib is not the only clinically available MEK inhibitor, however it has yet to be determined whether these effects of trametinib on lifespan extension in Drosophila are drug specific, (Han et al., 2021). Recently, structural, and biochemical studies have shown an important difference in the mechanism of action of trametinib compared to other allosteric inhibitors of MEK. Gonzalez-Del Pino and colleagues have shown that trametinib acts more potently on MEK when it is complexed with RAF rather than free MEK, (Gonzalez-Del Pino et al., 2021). However, allosteric inhibitors such as PD0325901 and CH5126766, seem to induce the formation of MEK/RAF complexes in KRAS-mutant lung cancer cell lines, which prevents durable inhibition of ERK, (Lito et al., 2014). Additionally, expression of a MEK mutant that has weaker interactions with RAF resulted in better inhibition of ERK signalling in response to the forementioned allosteric inhibitors, suggesting that the ability of MEK inhibitors to inhibit MAPK activity and inhibit KRAS mutant cancer cells, strongly depends on the specific biochemical properties of the drug bound MEK complex, (Lito et al., 2014). Furthermore, crystal structure analyses of trametinib bound to MEKkinase suppressor of RAS (KSR) complexes have identified binding sites for trametinib in the predicted allosteric binding pocket of MEK but also at the location where MEK interacts with KSR, an interaction not observed with other MEK inhibitors, such as PD0325901, cobimetinib and selumetinib, (Khan et al., 2020). This makes it fascinating to determine whether the lifespan-extending properties of trametinib can be recapitulated by other MEK inhibitors with a differing mechanism of action.

The mechanism by which trametinib extends lifespan In *Drosophila* in not fully understood, however trametinib treatment in *Drosophila* has been shown to ameliorate ageing-associated gut pathology, specifically in female flies, (Urena *et al.,* 2024). Trametinib treatment was shown to prevent age-associated intestinal barrier function decline in female flies but not males, (Urena *et al.,* 2024). It was also shown that gut and intestinal stem cell (ISC) tissue specific knockdown of *mek* extended

median lifespan of female flies. The lifespan extending effects of trametinib, were dependent on the function of Pol III specifically in the ISC, as ISC-specific knockdown of Pol III blocked lifespan extension of trametinib, (Urena et al., 2024). Additionally, trametinib treatment has been associated with positive metabolic effects, especially in lipid metabolism. As mentioned previously, age-related deregulation in nutrient sensing can lead to diseases such as obesity and Type 2 diabetes yet trametinib has been shown to alleviate some of the age-associated phenotypes of deregulated nutrient sensing. For instance, as early as 2002, Bost and colleagues have shown that treatment of mouse embryonic stem cells with a MEK inhibitor, PD98059, prevented adipocyte formation and expression of the adipogenic markers, adipocyte lipid-binding protein and peroxisome-proliferator-activated receptor y (PPARy), both shown to play a key role in adipocyte development, (Bost et al., 2002). Intriguingly, PD98059 did not interfere with the commitment of embryonic stem cells into other lineages such as neurogenesis, myogenesis and cardiomyogenesis, showing a specific role of MEK inhibition in adipocyte development, (Bost et al., 2002). The effects of MEK inhibition are not limited to cell culture, as mice lacking ERK have also been shown to have lower body lipid content, as well as a lower number of adipocytes relative to control mice, (Bost et al., 2005). Bost and colleagues generated an ERK knockout mice, which not only carries less fat, but also seems to be resistant to high fat diet-induced obesity, as ERK knockout mice did not gain as much weight as wildtype controls, (Bost et al., 2005). Additionally, ERK knockout mice showed lower transcript levels of the adipogenic markers, PPARy, adiponectin and leptin. Furthermore, ERK deficient mouse embryo fibroblasts did not differentiate upon stimulation with adipocyte differentiation media and a MEK inhibitor, U0126, had no effect on preventing mouse embryo fibroblast differentiation, showing that U0126 depends on the activity of ERK to prevent differentiation of mouse embryo fibroblasts into adipocytes, (Bost et al., 2005).

Additionally, pharmacological inhibition of MEK using trametinib has been shown to reduce insulin resistance in obese but genetically wild-type mice and in genetically obese (*ob/ob*) mice, suggesting that RAS inhibition may affect metabolic regulation during in the context of obesity, (Banks *et al.*, 2015). Banks and colleagues have shown that a MEK inhibitor, PD0325901, lowered the glucose response in a glucose tolerance test of high fat diet fed mice, but also have shown that PD0325901 increased glucose infusion rate, uptake and suppression of endogenous glucose production, showing a more efficient utilisation of glucose, (Banks *et al.*, 2015). Protein analysis of genetically obese mice has shown that obese mice have higher levels of activated phosphorylated ERK, whereas treatment of *ob/ob* mice with the MEK inhibitor, GSK1120212, had a lowered response in glucose tolerance tests, but also increased transcript expression of genes involved in the induction of M. Laskovs PhD Thesis, Aston University, September 2024

thermogenesis and 'browning' of white adipose tissue. Taken together, the study shows that MEK inhibitors in adipose tissue play a role in insulin-sensitisation, (Banks *et al.,* 20150).

1.10.2 ACARBOSE

Acarbose is a complex oligosaccharide that acts as an inhibitor of alpha-glucosidase and alpha-amylase, digestive enzymes that are involved in breakdown of complex carbohydrates in the intestine. By inhibiting these enzymes, acarbose limits the absorption of dietary carbohydrates thereby limiting associated increases in blood glucose levels and subsequent insulin release. Due to these properties of acarbose, it is routinely used together with metformin, exercise, and diet to treat type 2 diabetes, (Martin and Montgomery, 1996; Hsu et al., 2018). Direct effects of acarbose on RAS pathway inhibition in the context of obesity have not yet been studied, however, the effects of acarbose on RAS pathway inhibition and obesity, have been studied independently of each other. Yu and colleagues, have shown that treatment of vascular smooth muscle cells (A7r5) in culture with increased doses of acarbose for 24 and 48 hours, resulted in dose- and time-dependent inhibition of their proliferation, associated with decreased levels of RAS protein and reduction in phosphorylation of ERK, (Yu et al., 2018). Additionally, acarbose is thought to regulate RAS protein expression through microRNA (miRNA)-dependent regulation of gene expression as acarbose upregulates expression of miR-143, which specifically targets transcripts to regulate expression of RAS proteins, (Wang et al., 2014; Akao et al., 2018).

Acarbose also inhibits RAS in rabbits fed a high-cholesterol diet. It was shown that oral administration of acarbose at a dose of 2.5mg/kg and 5mg/kg resulted in a dose-dependent decrease in RAS protein levels, (Chan *et al.*, 2016b) and acarbose, increases lifespan in mice in a dose-dependent manner, (Harrison *et al.*, 2019). Harrison and colleagues fed mice with doses of 400, 1000, and 2500 mg per kg diet, from 8 months of age, and saw an increase in median lifespan of female mice between 0-6% and male mice between 10-20%, respective to the dose, (Harrison *et al.*, 2019). Additionally, acarbose supplementation had positive health effects, such as reduction of lung tumors, liver degeneration as well as better responses to glucose tolerance tests, (Harrison *et al.*, 2019).

Acarbose, has also been tested in humans for its potential to treat metabolic syndrome, referring to a cluster of risk factors for cardiovascular disease and type 2 diabetes. Analyses of three separate studies, have shown that acarbose intervention results in a reduction in body mass index (BMI), as well as reduction of triglyceride levels in obese and overweight patients, (Nakhaee and Sanjari, 2013; Khalili and

Safavipour, 2020; Yu *et al.*, 2021). Currently, acarbose is in phase 2 clinical trials to study its effect during ageing in individuals aged 60-100 years, (Garay, 2021).

There is therefore a clear connection that acarbose has an inhibitory effect on RAS by lowering RAS protein levels as well as reducing phosphorylation of ERK, but also that acarbose can extend lifespan in addition to having positive metabolic effects in obese and overweight individuals.

1.10.3 DIHYDROMYRICETIN

Dihydromyricetin (DHM) is a flavonoid, extracted from the Ampelopsis plant, that has has been used in traditional Chinese medicine to treat cough, pain, and jaundice, and has a range of anti-inflammatory, anti-carcinogenic and antioxidant effects, (Li et al., 2017). Administration of DHM at 2mg/kg of weight in rodent models of age-related neurodegeneration resulted in improvements in a range of phenotypes, including locomotor activity, and reduced seizure susceptibility in transgenic Alzheimer's disease mouse models, (Liang et al., 2014). Additionally, administration of 100-200mg/kg of DHM to transgenic Alzheimer's disease mouse models improved spatial learning and memory abilities in a dose-dependent manner, and was shown to increase expression of AMPK and SIRT1 proteins, which are associated with longevity, (Sun et al., 2019). In Drosophila, oral administration of 40 µM DHM extended median lifespan of female Drosophila by 16% and maximal lifespan by 6%. Additionally, later-life (30 days of adulthood) administration of 40 µM DHM also showed an increase in both median and maximal lifespan of female Drosophila, although to a lesser extent, comparable to trametinib-treated flies, (Fan et al., 2021). In addition to extending lifespan, DHM also increased starvation resistance which was associated with higher levels of triglycerides. DHM treatment also improved climbing ability during ageing from day 25-35 and decreased the number of 'smurf' flies, typically used as an indicator of gut health suggesting that DHM improves intestinal integrity in aged flies, (Fan et al., 2021). Fan and colleagues also demonstrated that DHM decreases phosphorylation of ERK, and increases protein levels of Aop and increased localisation of dFOXO in the nucleus, all associated with longevity in flies, (Fan et al., 2021).

One of the potential mechanisms for lifespan extension seen in DHM treated fruit flies is through autophagy. Autophagy is an evolutionarily conserved cellular recycling mechanism, the upregulation of which by overexpression of key regulatory factors has been shown to increase lifespan in worms and flies, (Barbosa *et al.*, 2019). The long-lived DHM treated flies, showed increased expression of the autophagy-related genes *Atg1*, *Atg5* and *Atg8b*, showing that DHM may promote longevity and improve

1.10.4 STATINS

Statins are a group of drugs that are used to lower the level of low-density lipoproteins (LDL) cholesterol in the blood to prevent dyslipidaemia, (Liao and Laufs, 2005). Statins are a class of highly specific inhibitor of HMG-CoA reductase, which is a ratelimiting enzyme of the mevalonate pathway, the metabolic pathway which produces cholesterol and other isoprenoids, such as farnesyl pyrophosphate and geranylgeranyl pyrophosphate, (Liao and Laufs, 2005). Such isoprenoids are required for the post-translational farnesylation and geranylgeranylation of proteins, especially for small signalling GTPases, including RAS itself, (Bonetti, 2003). These post-translational modifications are critical to RAS activation and membrane anchoring via prenylation, (Bonetti, 2003). Statins reduce the prenylation of both RAS and Rho in endothelial cell culture, leading to the accumulation of inactive forms of both proteins in the cytoplasm, (Liao and Laufs, 2005).

Oral administration of 240 µM of the statin, simvastatin, to adult male flies increased their mean lifespan by 25%, an effect which was associated with reduction of membrane bound Ras and Rab4 proteins, and an increase in cytoplasmic, inactive, form of Ras and Rab4, (Spindler et al., 2012). Additionally, direct inhibition of enzymes involved in protein prenylation using L744832, a specific farnesyl transferase inhibitor, and GGTI-298, a specific geranylgeranyl transferase inhibitor, have both been shown to increase median lifespan in Drosophila, in a dose dependent manner, (Spindler et al., 2012). Simvastatin, not only increases lifespan of Drosophila but also improves healthspan by decreasing arrhythmias in old male flies, thus showing that RAS inhibition by simvastatin may increase longevity by improving heart health, (Spindler et al., 2012). The effects of statins on lifespan extension are not simvastatin specific. A closely related analogue, lovastatin, also extends lifespan of C. elegans at doses of 25 μ M, 50 μ M and 100 μ M by 12, 23 and 24% respectively, but also post-reproductive treatment of lovastatin of equivalent doses extended lifespan by 18, 24, and 29% respectively, (Jahn et al., 2020). The lifespan extension observed from treatment of lovastatin were shown to be dependent on daf-16, C. elegans analogue of FOXO, as lovastatin did not extend lifespan in *daf-16* mutant worms, (Jahn *et al.*, 2020). Ageing in worms is associated with the accumulation of fluorescent pigments such as lipofuscin, formed from oxidised and cross-linked protein, lipids and carbohydrates, and advanced glycation end-products formed from non-enzymatic addition of sugar to free amino acid groups of proteins, (Son et al., 2019). Treatment of lovastatin significantly reduced the accumulation of lipofuscin during ageing, showing that statins can act on biomarkers of ageing in worms, in addition to lifespan, (Jahn *et al.,* 2020). Nevertheless, the direct effect of RAS prenylation in response to lovastatin treatment has yet to be established in worms.

In KRAS-mutant cancer cell lines and KRAS-mutant tumour mouse models, statins have been shown to inhibit RAS prenylation, (Nam et al., 2021). Additionally, treatment of simvastatin (20mg/kg) decreased tumour growth and survival in a KRASmutant mouse model of pancreatic cancer, (Nam et al., 2021). Furthermore, exposure of blood cancer cells to simvastatin has been shown to decrease phosphorylation of ERK and suppress RAS prenylation in human acute promyelocytic leukaemia (HL-60) cells and human histiocytic lymphoma (U937) cells in vitro. Similar results were observed with fluvastatin, a similar statin to simvastatin, (Fujiwara et al., 2017). Lastly, an epidemiological study found an association between statin use and reduced risk of cancer incidences and cancer-related morbidities in patients with heart failure, (Ren et al., 2021). Ren and colleagues analysed a territory-wide database developed by the Hong Kong Hospital Authority, of around 80,000 patients over a span of 10 years (1993-2002), and found that statin users had on average 16% lower risk of cancer than nonusers. Additionally, when looking at all-cause mortality, statin users had an 18% lower chance of all-cause mortality over a 10-year period than non-statin users, (Ren et al., 2021).

1.11 THE USE OF MODEL ORGANISMS IN AGEING RESEARCH

The ageing research field has enormously benefitted from the use of genetically tractable model organism such as *Saccharomyces cerevisiae, Caenorhabditis elegans*, *Drosophila melanogaster* and *Mus musculus* (Kenyon, 2010).

S. cerevisiae has been used to investigate both replicative lifespan, measured by the maximum number of mitotic divisions a cell can undergo, and chronological lifespan, measured by the length of time a cell can survive in a post-mitotic stage. However, the lack of complex interactions derived from multi-cellularity is a major disadvantage of using yeast as a model. In contrast, *C. elegans* possess complex organised tissues and yet has a very short lifespanof about 2 to 3 weeks at 25°C, making it one of the preferable organisms to study ageing. Worms are transparent so cells can be observed and recorded. Its disadvantage isa very low percentage of males (0.05%) because most worms are hermaphrodites, making it very complicated to study sex-specific effects on ageing. Vertebrate models such as *Mus musculus* possess the advantage of genetic proximity to humans and the availability of geneknockout and progeroid premature ageing models. However, as mice live on average 3

years in laboratory conditions, longevity studies are a lot more time consuming and expensive. Therefore, it has been recently proposed that an alternative short-lived vertebratemodel could be the African turquoise killifish, which lives for 4-6 months and recapitulates many of the age-related pathological changes found in humans, bridging the gap between yeast, worms, and flies on one side and mice on the other (Harel et al. 2016).

1.11.1 USING DROSOPHILA AS A MODEL

The first use of *Drosophila* as a model to study ageing date back from 1916, when Loeb and Northrop studied the effects of temperature and food on fly longevity (Loeb and Northrop 1916). Indeed, the use of *Drosophila* in ageing research has many advantages. First, flies have a relatively short lifespan of about 2 to 3 months. Second, flies are relatively easy and inexpensive to maintain making it practical to have hundreds of individuals in a single longevity experiment. Third, most cells in Drosophila are post-mitotic, except for distinct populations of stem cells that reside within the intestine, Malpighian tubules and the gonads, allowing the study of a continuously ageing organism. Additionally, another prominent advantage of using Drosophila as a model to study ageing is the rich genetic toolbox that exists for the fly. Of remarkable importance is the UAS-GAL4 system and its variation the Gene Switch system that allows for any gene of interest to be downregulated or overexpressed in a specific tissue at a specific time during adult life (Osterwalder et al. 2001; Poirier et al. 2008). The Drosophila genome is about 5% the size of the human genome containing approximately 13,600 genes, of which 60% are evolutionary conserved in humans. Moreover, more than 75% of known human disease genes, covering a broad range of disorders, have fly homologs, making Drosophila an attractive tool to model human diseases and ageing (Rogina 2011).

1.11.2 GAL4-UAS SYSTEM

In the early 90s, Brand and Perrimon designed and developed a system for targeting gene expression in a tissue- and cell-specific manner, which they called GAL4-UAS (upstream activating sequence), (Brand and Perrimon, 1993). The expression system is based on findings published by Kakidani and Ptashne, 1988 and Webster et al., 1988. Kakidani and Ptashne showed that GAL4 activates transcription in yeast which was dependent on a GAL4-binding sequence inserted upstream of the gene, (Kakidani and Ptashne, 1988). Moreover, a derivative of GAL4 that binds to DNA but cannot activate transcription in yeast, also failed to activate transcription in mammalian cells, (Kakidani and Ptashne, 1988). The same year, Webster and colleagues showed that GAL4 binds to a promoter binding site containing a 17-mer sequence, (Webster *et al.,* 1988).

GAL4 encodes a protein of 881 amino acids in length that was initially discovered in yeast *Saccharomyces cerevisiae*. In yeast, GAL4 recognises upstream activating sequences, primarily of genes involved in galactose metabolism, GAL1, GAL10, GAL7 by directly binding to the 17 base pairs (bp) sites located between these loci, (Duffy, 2002). These binding sites make up the UAS, which are analogous to enhancer elements in multicellular eukaryotes (Brand and Perrimon, 1993, Duffy, 2002).

Brand and Perrimon used a two-component system: the drive and the responder, providing a powerful technique for targeting gene expression in *Drosophila* (Brand and Perrimon, 1993). To elicit target gene expression, a parental line expressing GAL4, under the control of a gene specific promoter, known as the driver, is mated with a parental line carrying the gene of interest, the responder, the expression of which is controlled by the UAS element. The transcription of the responder requires the presence of GAL4 and in the absence of GAL4, the responder remains transcriptionally silent. To activate expression of the transgene, responder lines are mated with lines expressing GAL4, which results in progeny that expresses the transgene dictated by the driver (Brand and Perrimon 1993, Duffy 2002).

1.11.3 GENE-SWITCH SYSTEM

Spatial control of gene expression is straightforward using GAL4-UAS and relies on using a tissue specific promoter. However, temporal control of gene expression requires co-expression of GAL4 with a temperature sensitive repressor of GAL4, GAL80ts. GAL80ts has a couple of caveats: rearing temperature needs to be tightly controlled, and transgene expression can only be attained at high temperatures (> 29°C). Such conditions are not always suitable especially when studying ageing as

increases in temperature negatively impact on lifespan in *Drosophila*, (Suster., 2004, Molon et al.,2020). To combat these problems, a chemically inducible GAL4-UAS system was developed called Gene-Switch (GS), (Osterwalder et al.,2001). GS uses a modified GAL4 protein chimera which is fused to a progesterone steroid receptor, allowing for regulation of GAL4 activity in presence or absence of the synthetic progesterone analogue, mifepristone (RU-486). The progeny containing the UAS transgene and hormone responsive GAL4 chimera will therefore only express the transgene in the presence of RU486, (Osterwalder et al.,2001). These features of GS make it attractive to ageing studies as it allows for comparison of genetically identical flies with the only difference being whether they express the gene of interest or not depending on the presence of RU486 in the fly's food, (Figure 1.6), (Poirier et al., 2008).



Figure 1.6: Gene-Switch expression system.

The diagram illustrates the cross between a gene switch/Gal4 driver and a UAS-line to obtain a progeny that contains both UAS-GAL4 components. The UAS-GAL4 system is displayed on the left panel; UAS-Gene switch system is displayed on the right panel. The gene of interest is expressed constantly in the UAS-GAL4 system. The gene of interest is expression once the ligand is present in the UAS-Gene switch system. Flies with identical genotypes in the absence of RU486 serve as perfect experimental controls.

1.12.1 INTRODUCTION

One of the proposed hallmarks of ageing is deregulated nutrient-sensing, and it forms one element of the antagonistic hallmarks. The antagonistic hallmarks, are those which reflect responses to damage, playing a more nuanced role in the ageing process, (Lopez-Otin et al., 2023). A common cause of deregulated nutrient-sensing in the developed world is obesity, the prevalence of which rises steadily among older age groups. It is reported by WHO that in 2022, 1 in 8 people in the world were living with obesity, and that adult obesity has more than doubled since 1990 worldwide while adolescent obesity has quadrupled, (Obesity and overweight by WHO). It is suggested that changes in lifestyle of older adults, as they enter retirement, together with slowing of their metabolic rate, may cause chronic energy surplus, leading to excess fat tissue accumulation and accelerated development of age-related diseases, (Jura and Kozak, 2016). It is becoming more evident that obesity leads to reduced lifespan and increases the prevalence of certain age-related diseases. Altered metabolic regulation, insulin resistance and impaired immune function are all associated with obesity, and also significantly overlap with mechanisms that drive ageing, suggesting that if obesity can be prevented, certain ageassociated phenotypes may also be ameliorated, (Trim et al., 2018). As RAS/MAPK signalling is a key nutrient-sensing pathway with important roles in both metabolism and lifespan regulation, it was hypothesised that inhibition of RAS/MAPK signalling may extend lifespan through beneficial metabolic effects.

1.12.2.1 METABOLIC EFFECTS OF RAS/MAPK INHIBITION IN RESPONSE TO HIGH SUGAR FEEDING AND AGEING

RAS/MAPK inhibition by MEK inhibitors has been previously shown in mice to decrease lipid storage, however the effects had not been replicated in *Drosophila*. To assess the metabolic effects of MEK inhibition in *Drosophila*, the MEK-specific inhibitor, trametinib, was fed to adult flies and a lifespan assay was conducted in conjunction with the measurement of triglyceride levels using a biochemical assay. Survival in response to trametinib treatment was measured in flies fed both a normal (1x) and high sugar (8x) diet, with flies collected on days 10, 20 and 30 after drug exposure for triglyceride measurements (Figure 1.7 and Figure 1.8).



Figure 1.7: Survival of female *Drosophila* fed a normal and high sugar diet in response to trametinib.

(A) Survival analysis of wild type, *wDah*, female flies. Results are plotted as proportion of survivors as a function of time (in days). Median survival times of flies are shown in the table (B). Table showing median lifespan, Log rank test comparing absence (-) of trametinib to presence (+) of trametinib for normal (1x) and high sugar (8x) diets, *** p<0.001. Black arrows shows the points at which flies were taken for TAG analysis but only for the 1x sugar diet condition.

High sugar diet significantly decreased median lifespan in wild-type female flies (-56%, $p = 1x10^{-7}$) but trametinib treatment significantly extended median lifespan on both normal and high sugar diets (normal sugar: +10%, $p = 1x10^{-6}$, high sugar: +20%, $p = 1x10^{-7}$) (Figure 1.7).

High sugar diet also significantly increased triglyceride levels but not in the presence of trametinib (Figure 1.8A). Similarly, triglyceride levels increased during ageing from day 10 to day 30 in a time-dependent manner but not in trametinib-treated flies (Figure 1.8B).



Figure 1.8: Triglyceride (TAG) concentrations in *Drosophila* fed high sugar diet and during ageing in response to trametinib.

TAG concentration of *Drosophila* fed normal (1x) and high sugar (8x) diets in both the absence (-) and presence (+) of trametinib (A). TAG concentration of *Drosophila* at the indicated timepoints both the absence (-) and presence (+) of trametinib (B). Results are shown as μ g TAG per μ g of protein for each treatment condition. Floating bars show the minimum, median and maximum value (bars). Dots show individual data points (n=10 replicates per condition of 5 pooled flies in each). ** p <0.01, *** p<0.001, two-way ANOVA with *post-hoc* Tukey multiple comparison test).

1.12.3 CONCLUSION

The preliminary data described above indicated that feeding adult flies with the MEKinhibitor, trametinib, not only extends lifespan on normal and high sugar diets but imposed positive metabolic effects preventing lipid accumulation. This preliminary data has set the precedent for this project, to investigate the metabolic effects of trametinib in *Drosophila* and their contribution to trametinib-dependent lifespan extension.

1.13 PROJECT AIMS AND OBJECTIVES

The overall aim of this project was to characterise the metabolic effects of trametinib in *Drosophila* in response to both diet-induced obesity and ageing. This would identify the mechanisms of metabolic dysfunction potentially targeted by trametinib that contribute to trametinib-dependent lifespan extension.

The overall hypothesis was that RAS/MAPK pathway inhibition by trametinib leads to positive metabolic changes that prevent the deleterious effects of diet-induced obesity and ageing-related phenotypes thereby extending lifespan in *Drosophila*.

The first aim was to determine the impact of trametinib treatment on metabolic changes that arise from high sugar feeding and during ageing. The hypothesis was that

RAS/MAPK pathway inhibition by trametinib would prevent diet- and age-dependent M. Laskovs PhD Thesis, Aston University, September 2024

obesity in *Drosophila*. To investigate this, changes in storage molecule content in response to high sugar feeding and trametinib treatment were characterised using triglyceride and glycogen biochemical assays. To further analyse lipid content changes in response to trametinib treatment, fat body tissue was dissected from flies fed a high sugar diet and during ageing, to measure the lipid droplet size.

The second aim was to fully characterise the global lipid changes in flies fed with a high sugar diet in response to trametinib. This was used to identify candidate metabolic pathways that could be mediating the beneficial effects of RAS/MAPK inhibition. The hypothesis was that inhibiting RAS/MAPK signalling prevents diet-induced accumulation of deleterious lipid species. Liquid chromatography coupled mass spectrometry (LC-MS) was used to identify lipid species which were differentially abundant in *Drosophila* fed with a high sugar diet in response to trametinib.

The third aim was to fully characterise the global transcript changes of flies fed a high sugar diet in response to trametinib, to identify mechanisms for the observed metabolic effects of trametinib treatment. The hypothesis was that inhibiting RAS/MAPK signalling would prevent certain sugar-dependent transcriptional changes that drive the detrimental effects of high sugar diet. RNA-sequencing (RNA-seq) was used to analyse the transcriptional response of flies fed a high sugar diet in response to trametinib. This identified genes that were differentially expressed in response to high sugar feeding and reversed by trametinib treatment.

The fourth aim was to investigate the effects of trametinib on purine metabolism in flies fed with a high sugar diet. High sugar feeding is associated with elevated levels of purines leading to uric acid accumulation and dysfunction of the Malpighian tubules. The hypothesis was that trametinib would increase the survival of flies on a high sugar diet by reducing purine synthesis thereby maintaining Malpighian tubule function. Malpighian tubule physiology was characterised by measuring uric acid levels using a biochemical assay in flies fed high sugar diet in response to trametinib. This was coupled with live imaging of purine deposits within the Malpighian tubules. Similar experiments were carried out after purine supplementation to determine if the trametinib-associated response was dependent on purine synthesis. Lifespan assays were carried out on flies supplemented with dietary purines to investigate whether the lifespan extension of trametinib was dependent on purine clearance.

Chapter 2

2.1 DROSOPHILA STOCKS

The control strain of *Drosophila* used during this study was *white*^{Dahomey} (w^{Dah}). w^{Dah} was originally created by backcrossing the *white*¹¹¹⁸ (w^{1118}) mutation into the *Dahomey* background. *Dahomey* flies are a wild-type strain, collected originally in Dahomey, West Africa. Since their collection in 1970, they have been kept in large population cages at 25 °C with overlapping generations on a 12-hour light:12-hour dark cycle (Bass *et al.* 2007). All strains used in this study were backcrossed for six generations into the w^{Dah} background prior to experimental analysis.

A range of transgenic Drosophila stocks were used in this study, listed below:

The *bmm*¹ mutant fly strain was generated by Gronke *et al.*,2005. The deletion mutant is missing *bmm* DNA sequence from 5006-7651 causing lack of BMM as at positions 52-249.

2.2 GENE EXPRESSION SYSTEM

A range of transgenic lines using Gene-switch system were used in this study, listed below. To elicit expression of the gene of interest, a ubiquitous Actin GAL4 gene switch was used.

2.2.1 GENE-SWITCH SYSTEM

The acetyl-CoA carboxylase (ACC) overexpression strains used in this study were obtained from the Indiana University Bloomington *Drosophila* Stock Centre (BDSC). Two suitable strains were identified: BL-63224 and -63225. Both lines express ACC under the control of GAL4 upstream activating sequence on chromosome 2 and 3 respectively.

The sterol regulatory element binding protein (SREBP) strains used in this study were obtained from BDSC). Two strains were used in this study: #41017 and #38396. #41017 encodes a truncated, constitutively active form of SREBP under control of the GAL4 upstream activating sequence . #38396 expresses a wild type SREBP under control of the GAL4 upstream activating sequence.

2.3 FLY HUSBANDRY

Drosophila stocks were maintained at 25°C on a standard sugar, yeast, agar (SYA) medium consisting of 5% (w/v) granulated sugar (Tate & Lyle, UK), 10% (w/v) brewer's yeast (MP Biomedicals, Germany), 1.5% (w/v) agar (Merck, Germany), and preservatives 3% (v/v) nipagin (diluted from a 10% (w/v) stock solution of tegosept (Apex Bioresearch Products, USA) prepared in 95% ethanol (Thermo Fisher Scientific, USA)), and 0.3% (v/v) propionic acid (Thermo Fisher Scientific, USA) (Bass et al. 2007). The high sugar diet experiments used a high sugar diet which was identical to the standard SYA medium, except for granulated sugar which was increased to 40% (w/v). The experiments in which flies were treated with trametinib, stock trametinib solution (62.4mM, LC laboratories, USA) was diluted to 15.6 µM per 1 litre of fly food in DMSO, (Thermo Fisher Scientific, USA). A final DMSO concentration of 0.25% (v/v) was maintained to avoid toxicity. The experiments in which flies were treated with purines (Adenine Merck, Germany, Guanine Merck, Germany), purine powder was dissolved to 5mM concentration in 1:1 (v/v) dH2O: 1M Acetic acid (Thermo Fisher Scientific, USA). All lines were maintained on a 12-hour light-12-hour dark cycle at constant humidity (Bass et al. 2007). All experiments were conducted at 25°C. Flies used for downstream assay were flash frozen in liquid nitrogen at an appropriate time point and stored at -80oC.

All experimental flies were reared from synchronised egg collections. Adult parental flies were transferred to collection cages and eggs were collected on grape juice-agar plates (produced using 85% dH2O (v/v), 5% (w/v) agar (Merck, Germany), 5% (w/v) sugar (Tate & Lyle, UK), 10% (v/v) red grape juice (Ritchies, UK), 3% (v/v) nipagin) with yeast paste (made by mixing dH2O with active dried yeast (S.I. LeSaffre, France)). Flies were left in cages overnight to acclimatize, and grape-juice agar plates replaced after 24 hours. The eggs were collected from an overnight collection around 16 hours later and washed from the surface of the grape juice-agar plate using 1x phosphate buffered saline (PBS) (Thermo Fisher Scientific, USA). Eggs (20uL) were dispensed into bottles of fresh SYA food to maintain standard larval densities and incubated at 25 °C for 10 days until the adult flies emerged. Adult flies were transferred to fresh SYA food and allowed to mate for 24 hours. Adult flies were then separated by sex using a Leica M80 zoom stereomicroscope (Leica Microsystems, Germany) and CO2 anaesthesia and placed into experimental food vials, (Regina Industies, UK).

Catalogue numbers and manufacturers for all reagents used in this project can be found in Appendix 1.

2.4 MEASUREMENT OF FEEDING BEHAVIOUR

To measure feeding behaviour, a proboscis extension (PE) assay was performed. Adult female flies were tipped into vials containing standard SYA media at a density of 5 flies per vial, 15 vials per condition. To reduce observer bias, vials were randomized and numbered blind. Vials were lined up and left for 5 minutes for flies to acclimatise, the number of feeding events were noted by observing the number of flies extending their proboscis into the food over the course of 3 minutes intervals over 60 minutes.

2.5 MEASUREMENT OF LIFESPAN

For lifespan assays, female flies were reared as described in section 1.3 and housed at a density of 15 flies per vial and 10 vials per genotype. Flies were transferred, without CO2 anaesthesia, onto fresh SYA media 3 times per week. During transfer any deaths, transfers and censors were recorded. Flies were censored when the death is considered unnatural (e.g., stuck, squashing) or flies have escaped.

2.6 MEASUREMENT OF FECUNDITY

For measurements of female fecundity, fully fed adult flies used in the measurement of lifespan were transferred to fresh SYA vials. After 24 hours, the flies were removed, and the number of eggs laid on the surface of the food over the 24- hour period were counted.

2.7 MEASUREMENT OF ADULT BODY MASS

Individual flies were weighed using an analytic balance MH-124 (FB73651, Thermo Fisher Scientific, USA) to measure whole body mass. After weighing, each individual fly was placed into a numbered 1.5 mL tube (Eppendorf, UK) and incubated at 65°C in a heating block set for 48 hours to dry. After 48 hours, each individual fly was weighed again to measure dry body mass.

2.8.1 DROSOPHILA TISSUE DISSECTION

Adult female flies were dissected in ice cold PBS using a Leica M80 zoom stereomicroscope (Leica Microsystems, Germany). Dissected tissues were kept separate in ice cold PBS until all were dissected for a maximum of 1 hour. All dissected tissues were fixed using 4% (v/v) methanol-free formaldehyde (Thermo Fisher Scientific, USA), in PBST, at room temperature for 30 minutes under rotation. The fixative was removed, and tissues were rinsed 3x with PBS under rotation for 10 minutes at room temperature. Fat body tissue was then incubated with freshly prepared Nile Red solution made by diluting 0.5mg/ml Nile Red stock solution (Sigma Aldrich, USA) 1:2000 in PBS, for 30 minutes under rotation. Tat body tissue was then rinsed 2x with PBS for 10 minutes under rotation. Tissues were mounted on glass slides using VECTASHIELD mounting medium containing DAPI (Vector Laboratories, USA).

2.8.2 CONFOCAL MICROSCOPY

All confocal images were taken using a Leica SP8 lightning confocal microscope (Leica Microsystems, Germany) at 20x or 40x magnifications using LASX imaging software with representative images used for analysis. For Nile Red stained tissues, the following filters were selected: Alexa546 (562ex/573em) and DAPI (358ex/463em) . Image processing was performed using the lightning tool in the LASX software and Fiji ImageJ. To measure the lipid droplet size, using LASX Quantify function, a polyline was manually drawn around the lipid droplet. This would form a shape around the lipid droplet from which area of pixels could be summarized, giving an average lipid droplet size.

2.9 MALPHIGHIAN TUBULE DISSECTIONS

For imaging of the Malpighian tubules, live flies were anesthetised using CO2 gas, and the tubules dissected in ice-cold PBS before mounting onto poly-L-lysine coated slides. The slides were imaged on a Zeiss Axio Zoom V16 microscope with Axiocam 503 mon camera (Zeiss, Germany). To quantify the tubule stone phenotype, 50 fly tubules per treatment were scored blind according to a scale adapted from van Dam et al., 2020, see Figure 2 below.



Figure 2.1: Scoring of the tubule phenotype based on light microscopy imaging of Malpighian tubules.

Arranged from the ureter (left) to the tip (right). Scale bar = 500μ m. The scoring represents the percentage of blockage of the tubule: 0 = ~0%, 1 = <25%, 2 = 25-50%, 3 = 50-75%, 4 = >75%. Figure was adapted from van Dam et al., 2020.

2.10 LIPID EXTRACTIONS USING METHYL TERT-BUTYL ETHER (MTBE)

Lipid extraction method was adapted from Matyash *et al.*, 2008. 5 adult female *Drosophila* per sample replicate were homogenized in 300 µL of methanol (Thermo Fisher Scientific, USA) and 1 mL of Methyl tert-Butyl Ether (MTBE) (Thermo Fisher Scientific, USA) in a MP Biomedicals FastPrep-24 5G bead beating grinder and lysis system (MP Biomedicals, USA) at level 6.0 for 30 seconds. Samples were then incubated for 1 hour at room temperature with continuous shaking. After, 200 µL of H₂O (Thermo Fisher Scientific, USA) was added to each sample to give a final approximate volumetric ratio of 10:3:2.5 MTBE:methanol:water. Samples were vortexed for 20 seconds and centrifuged for 10 min at 1000 g using an Eppendorf 5415R refrigerated microcentrifuge (Eppendorf, UK) to aid in layer separation. Approximately 1 mL of upper lipid-containing phase was transferred into a glass collection vial (Thermo Fisher Scientific, USA), making sure none of the aqueous phase was transferred. The lipid extracts were then dried down under Nitrogen gas and stored in glass vials at -80°C. Before use, samples were resuspended in 50 µL Iso-propanol (Thermo Fisher Scientific, USA).

2.11 MEASUREMENT OF GLYCOGEN IN ADULT FLIES

Glycogen concentrations were measured in whole adult female Drosophila - 5 flies per replicate samples were homogenized in 50 µL 0.2 M Na₂CO₃ in a MP Biomedicals FastPrep-24 5G bead beating grinder and lysis system (MP Biomedicals, United States) at level 6.0 for 30 seconds, then centrifuged at 93g for 1 minute. All centrifugation steps were carried out using an Eppendorf 5415R refrigerated microcentrifuge (Eppendorf, UK) 2 µL of homogenate were transferred in a separate microcentrifuge tube in 18 µL of 0.2 M Na₂CO₃ for the measurement of protein concentration in the homogenate, according to 2.5. Glycogen standards were prepared in 0.2M Na₂CO₃ at concentrations of 0-2 µg/µL using glycogen from oyster (Merch, Germany). Standards and samples were incubated at 95 °C for 2 hours, then placed on ice for 2 minutes before centrifugation at 93 g for 1 min. 30 µL of 1M acetic acid (Fisher Bioreagents, UK) and 120 µL 0.2 M Na-acetate (pH 5.2) were added to each tube. To each glycogen standards and samples, 0.0096 units of amyloglucosidase from Aspergillus niger (Merch, Germany) were added to 50 µl of glycogen standard or sample homogenate and incubated at 57 °C overnight. Glucose was then measured by adding 5 µL of each standard or sample to 150 µL of Infinity[™] Glucose reagent (Thermo Fisher Scientific, USA) (linear range: 0-45 mmol/L) in a clear flat-bottom 96 well-plate (Corning, United States). Plates were incubated at room temperature for 15 minutes and absorbances read using Tecan Infinite M Nano + plate reader, (Tecan, Switzerland) at 340nm. Protein concentration was measured for all samples according to Section 2.14.

2.12 MEASUREMENT OF (TRI)GLYCERIDE (TAG) IN ADULT FLIES

TAG levels were measured in whole adult female Drosophila. 5 flies per replicate sample were homogenized in 150 µL 0.05% (v/v) Tween-20 (Thermo Fisher Scientific, USA) in dH2O in a MP Biomedicals FastPrep-24 5G bead beating grinder and lysis system (MP Biomedicals, USA) at level 6 for 30 seconds. Glycerol standard concentrations ranging from 0-2 µg/µL were prepared by serial dilution of 2.5mg/mL stock solution (Merch, Germany) in 0.05% (v/v) Tween-20 (Thermo Fisher Scientific, USA) in dH2O. Standards and samples were incubated at 70 oC for 5 minutes after which samples were centrifuged at 2,320 g for 1 min using an Eppendorf 5415R refrigerated microcentrifuge (Eppendorf, UK). Samples were diluted 1:4 in 0.05% (v/v) Tween-20 (Thermo Fisher Scientific, USA) in dH2O in fresh 1.5 mL microcentrifuge tubes (Thermo Fisher Scientific, USA). 5µL of each sample or standard were added to 150 µL of InfinityTM Triglycerides reagent (Thermo Fisher Scientific, USA) (linear range: up to 10mmol/L) in wells of a clear flatbottom 96-well plate (Corning, USA). The plate was then incubated for 5 minutes at 37 oC, then centrifuged at 164 g for 1 min using an Eppendorf 5804R centrifuge (Eppendorf, UK). Absorbance was read using Multiskan GO (Thermo Fisher Scientific, USA) at 574 nm. Protein concentration was measured for all samples according to Section 2.14. This assay is facilitated by lipase mediated hydrolysis of glycerides, indiscriminately. The assay therefore measures triglyceride, diglyceride and monoglyceride species, based on the glycerol generated from the hydrolysis of glycerides. In this study the results of this assay are presented as the amount of TAG per amount of protein in the sample.

2.13 URIC ACID ASSAY

Uric acid levels were quantified from extracts of whole adult flies using the QuantiChrom Uric Acid Assay kit (DIUA-250, BioAssay Systems) according to the manufacturer's protocol. 5 frozen flies per sample were homogenized in 100 μ L of 0.05% Tween-20 (v/v) using a microtube pestle. Standards of uric acid from 0-0.1 μ g/ μ L were prepared in 0.05% Tween-20 (v/v). 5 μ L of each individual sample or standard were loaded in duplicate into a 96-well plate with 200 μ L of working assay buffer and incubated for 30 minutes at 30°C. After, the absorbance was measured at 590 nm using a Multiskan GO (Thermo Fisher Scientific, USA) plate reader.

2.14 WESTERN BLOT ANALYSIS

Western blot analysis was carried out to quantify protein changes in whole fly protein samples. Protein extracts were produced by homogenising 5 adult female flies in 100 μ L of Laemmli Sodium Dodecyl Sulphate (SDS) sample buffer (Alfa Aesar, UK) containing

0.1 M dithiothreitol (DTT) (Thermo Fisher Scientific, USA). Flies were homogenised in a 1.5 mL tube (Eppendorf, UK) using a clean microtube pestle, after which the homogenate was incubated at 85 °C for 10 minutes and then centrifuged at 4 °C, 13,000g for 5 mins using an Eppendorf 5415R refrigerated microcentrifuge (Eppendorf, UK). Protein concentration was measured using the Pierce 660 nm Protein Assay Reagent (Thermo Fisher Scientific, USA) containing ionic compatibility detergent reagent (ICDR) (Thermo Fisher Scientific, USA) as per the manufacturer's instructions. Standards of Pierce bovine serum albumin (BSA) protein standard (Thermo Fisher Scientific, USA) from 0-1 µg/µL were also prepared in dilute Laemmli SDS sample buffer (1:10, Laemmli:Sterile water ratio) (Alfa Aesar, UK) containing 0.1 M DTT (Thermo Fisher Scientific, USA) Protein samples were diluted 1:10 in sterile dH2O and then 5 µL of the standards or diluted samples were added in duplicate to 150 µL of Pierce 660nm Protein Assay Reagent containing ICDR in a clear flat-bottomed 96-well plate (Corning, USA). The plate was then incubated for 5 mins at room temperature before absorbances were read using a Multiskan GO (Thermo Fisher Scientific, USA) plate reader at 660 nm. Protein samples were then flash frozen in liquid nitrogen and stored at -80 °C or placed on ice for use shortly. Proteins were resolved on 10% acrylamide:bis-acrylamide (33.5:0.3) (Thermo Fisher Scientific, USA) gels in 1x SDS-PAGE running buffer pH 8.3 (made as follows: 0.25 M Tris-base (Fisher Scientific, UK), 1.91 M glycine (Thermo Fisher Scientific, USA), and 0.03 M SDS (Thermo Fisher Scientific, USA)) at 120 V for 20 minutes, up to the resolving gel, then 150 V to end. 10-20 µg of protein were loaded into each well alongside 5 µL PageRuler Plus Prestained Protein Ladder (Thermo Fisher Scientific, USA). Before transfer of gels to a nitrocellulose membrane, the gels were incubated in Towbin buffer (made as follows: 25 mM Tris-base (Thermo Fisher Scientific, USA), 192 mM glycine (Thermo Fisher Scientific, USA), and 20% (v/v) methanol (Thermo Fisher Scientific, USA)) for 10 mins to equilibrate. Amersham Protran premium 0.45 µm nitrocellulose membrane (Cytiva, UK) and 2 pieces of heavy-duty blotting paper (BioRad, UK) per gel, were also soaked in Towbin buffer. Blots were then assembled on the electrode of a semidry transfer cassette, (TransBlot Turbo transfer system (BioRad, UK)) in the following order: heavy-duty blotting paper, membrane, gel, heavy-duty blotting paper. A plastic roller was used to remove air bubbles, after which the cell was run at a constant 25 V for 30 minutes. Once transferred, blots were incubated in blocking solution (5% (w/v) skimmed milk powder (Sigma Aldrich, USA)) in Tris-buffered saline (0.15M Tris-HCL (Thermo Fisher Scientific, USA), 0.05M Tris-base (Thermo Fisher Scientific, USA) and 1.5M NaCl (Thermo Fisher Scientific, USA) containing 0.1% (v/v) Tween-20 (Thermo Fisher Scientific, USA) (TBST) for 1 hour. All washes and incubations are carried out on a rocking platform. Blots were then washed 3x in TBST and incubated overnight at 4 °C in an appropriate primary antibody (Table 1). Antibodies were diluted in 5% (w/v) BSA (Merck, Germany) in TBST, in a ratio recommended by the retailer.

Primary antibody	Manufacturer	Dilution
Rabbit anti-PhosphoERK #4370	Cell Signalling, USA	1:1000
Rabbit anti-TotalERK #4695	Cell Signalling, USA	1:1000
Non-phospho-4E-BP1 #4923	Cell Signalling, USA	1:1000
Acetyl-CoA Carboxylase #YC3873824A	Invitrogen	1:1000
Mouse anti-beta Actin #ab8224	Abcam, UK	1:10000
Rabbit anti-beta Tubulin #ab108342	Abcam, UK	1:1000

Table 2.1 – Primary antibodies used for western blots in this study.

After incubation, blots were washed 5x in TBST for 5 minutes. Subsequently, blots were incubated in TBST for 1 hour at room temperature with Horseradish Peroxidase (HRP)-conjugated secondary antibody, either goat anti-rabbitHRP (diluted 1:10000, Abcam, UK) or goat anti-mouseHRP (diluted 1:10000, Abcam, UK), diluted in 5% (w/v) skimmed milk in TBST. Another 5x 5 minute washes in TBST were repeated after incubation with the secondary antibody. The blots were then incubated in Luminata Forte Western HRP substrate (Millipore, USA) for 5 mins at room temperature. Excess substrate was blotted from the back of the membrane with paper towels which was then inserted into a plastic wallet. Visualisation of blots was carried out using a G:Box Chemi HR 1.4 (Syngene, UK) imaging system. Image analysis and densitometry were performed using ImageJ (Schneider et al., 2012). Membranes were re-probed with either Anti-beta actin or Anti-beta tubulin antibodies as loading controls.
2.16 GLOBAL LIPID PROFILE ANALYSIS USING MASS SPECTROMETRY

2.16.1 LIPID EXTRACTION METHOD

Lipid extraction was carried out as specified in 2.10 with adjustments. The Avanti EquiSPLASH reference stock solution was prepared by diluting 10 μ L of Avanti EquiSPLASH in 990 μ L isopropanol, the mixture was vortexed for 20 seconds to ensure homogeneity. During extraction 5 μ L of Avanti EquiSPLASH (Waters, USA) mixture was added to each sample. Once lipid samples were lyophilised and ready to use, each sample was resuspended in 200 μ L of isopropanol (Thermo Fisher Scientific, USA).

2.16.2 LIPID ANALYSIS BY LC-MS/MS

Samples were analysed using an ACQUITY UPLC I-Class PLUS System (Waters, USA), coupled with a SELECT SERIES Cyclic ion mobility separation (IMS) mass spectrometry system (Waters, USA) by reverse phase (RPLC) liquid chromatography. Samples were injected directly into an ACQUITY Premier CSH C18 Column 1.7 µm, 2.1 x 100 mm (Waters, USA) kept at 55 oC, 2µL of samples in the positive ion mode, 4µL of samples in the negative ion mode. Samples were kept at 8 oC during the run to prevent solvent evaporation. A 10% (v/v) isopropanol solution was used for the seal wash, and a mixture of isopropanol:acetonitrile:water (Thermo Fisher Scientific, USA) at 45:35:20 ratio was used for the weak wash. Strong washes, needle washes and purges were performed using 95-100% isopropanol (Thermo Fisher Scientific, USA). Elution of lipid samples through the chromatography column was achieved with a solvent gradient. Solvent A (60:39:1 mixture of acetonitrile:water:1M ammonium formate with 0.1% (v/v) formic acid) and solvent B (90:9:1 mixture of isopropanol:acetonitrile:1M ammonium formate with 0.1% (v/v) formic acid) were used to elute the analytes at 0.4 mL/min at a gradient. The gradient started from equal proportion of solvent A and solvent B, and was changed to 47% (v/v) solvent A and 53% (v/v) solvent B by 0.5 minutes, to 45% (v/v) solvent A and 55% (v/v) solvent B by 4 minutes, to 35% (v/v) solvent A and 65% (v/v) solvent B by 7 min, to 20% (v/v) solvent A and 80% (v/v) solvent B by 7.5 minutes, to 1% (v/v) solvent A and 99% (v/v) solvent B by 10 minutes, then returned to the starting conditions by 12 minutes, for a total run time of 12 minutes per sample. As a lock mass standard, leucine enkephalin (Leu-Enk) (Waters, USA) was prepared at 200 pg/µL in a 1:1 mixture of acetonitrile:water and was infused as a Lockspray. Data was collected in ESI positive (+) and ESI negative (-) ionization modes separately. The capillary voltage was set to 2.8

kV, cone voltage was set to 40 V and the source offset was set to 30 V. The source temperature was set to 120oC and the desolvation gas temperature to 500oC. The gas flow for cone and desolvation was 150 L/h and 750 L/h, respectively. The nebulizer gas pressure was set to 6 bar with reference capillary set to 3 kV. The Lockspray flow rate was set to 20 μ L/min. The MS was operated in full scan V-mode with an acquisition mass range from 100 to 2000 m/z. The scan time of 0.1 seconds was set on the MS and MS/MS analyses. The same scan time was set for the acquirement of the lockspray and correction applications, with an interval of 30 seconds, average scans of 4 and a mass window of ±0.5. Data was collected using data independent acquisition (DIA). Initially, a full precursor and fragment ion scanning was carried out followed by tandem MS/MS fragmentation with collision energy of 20 eV up to 45 eV.

Prior to sample processing, checks of detector, lockmass, calibration, resolution and sensitivity were performed. Leu-Enk was infused at 20 μ L/minute with attenuation set to 5%, once the beam was stable, MS of 1 scan was acquired. A peak corresponding to the lockmass Leu-Enk ion was expected at 556.2771 m/z in the positive ion mode. The resolution (FWHM) was expected to be ≥ 30,000 and the sensitivity ≥ 8,000 cps.

For LC-MS/MS acquisition, an appropriate number of blank injections were incorporated to ensure the stability and reproducibility of the black injections. A minimum of 2 solvent blanks were injected for column conditioning. Once the column was conditioned, samples were injected with 2 solvent blanks being injected in-between treatment conditions. Blank samples were used to make sure there is no significant change in column condition/detected background ions throughout the experiment run.

2.16.3 DATA PROCESSING AND LIPID IDENTIFICATION

Lipid identification was performed by Progenesis QI software (Waters, United States) using a public lipid database LIPID MAPS® Structure Database (LMSD) to identify lipid species. Further ion peak verification was performed by manual MS and MS/MS spectral interpretation using MassLynx mass spectrometry software (Waters, United States). MassLynx was used to general total ion chromatograms and extracted ion chromatograms, while Progenesis QI allowed for identification of compounds, retention times, mass error and their abundances.

Leu-Enk lock mass of m/z 556.2771, in the positive ion mode, was used to calibrate the data in Progenesis QI. Sample ions were automatically aligned to compensate for drifts in the retention time between runs, and the ion composition was assigned by performing automatic peak picking. The adduct forms [M+H]+, [M+Na]+, [M+NH4]+ were included for lipid identification. To allow comparisons across different treatment conditions, total ion

abundance was used as a normalisation method. Alternatively, for lipid verification, internal standards Avanti EquiSPLASH were used for normalization. Compounds were identified by Progenesis MetaScope method using LIPID MAPS ® Structure Database (LMSD) with a precursor tolerance of 6 ppm and performing theoretical fragmentation with fragment tolerance of 6 ppm.

To filter the data for lipid identification, features with no identification were discarded. The data was sorted based on maximum abundance, and fragmentation spectra of all compounds were checked for presence of molecular ion peaks to accept their identification. The 3D-montage was checked for all compounds with accepted identification to determine clear observable peak separation corresponding to each compound. A list of identified lipids with high and low confidence were generated. High confidence compounds were compounds for which fragmentation data clearly matched individual fragments, the 3D-montage displayed clear peaks, clear separated isotopic and chromatographic peaks, whereas low confidence compounds were those for which characteristics were ambiguous. For data interpretation, identified lipids were grouped into phospholipid and glyceride classes, including phosphatidylcholine (PC), phosphatidylinositol (PI), phosphatidylethanolamine (PE) phosphatidylserine (PS) and phosphatidylglycerol (PG) for phospholipid classes and diglyceride (DAG) and triglyceride (TAG) for glyceride classes. Peak intensities of identified lipids were reported as 'normalised abundance' by Progenesis QI (Waters, USA), by combining peak intensities to generate total signal intensity. The relative percentage abundance of each lipid was calculated using Microsoft Excel (Microsoft Corporation, USA) and plotted using GraphPad Prism 8 (GraphPad Software, USA). Statistical differences between treatment conditions were calculated using GraphPad Prism 8 according to two-way ANOVA followed by Tukey's multiple comparison test.

Alternatively, variation between treatment conditions was displayed using Principal Component Analysis (PCA) and Heatmaps using MetaboAnalyst (Pang et al., 2022). Pareto scaling method of normalisation was applied, using the square root of the standard deviation as the scaling factor to emphasize the most variable lipid species in the dataset.

2.17.1 RNA EXTRACTION

RNA was isolated from 15 female flies. Flies were added to a 2 mL screw cap tube containing glass beads and 0.5 mL TRIzol (Thermo Fisher Scientific, USA) on ice. Flies were then homogenised using an MP Biomedicals FastPrep-24 5G bead beating grinder and lysis system (MP Biomedicals, USA) at speed 4.0 for 30 seconds. Samples were then incubated at room temperature for 5 minutes, before 100 µL of chloroform (Thermo Fisher Scientific, USA) were added. Tubes were vortexed for 15 s and incubated at room temperature for 2 mins. After incubation, tubes were centrifuged at 19,090 g for 5 minutes at 4 °C. All centrifugation steps were carried out using an Eppendorf 5415R refrigerated microcentrifuge (Eppendorf, UK). Subsequently, 250 µL of the upper colourless phase containing the RNA were transferred into a new 1.5 mL tube (Eppendorf, UK) and 250 µL of ethanol (Thermo Fisher Scientific, USA) were added. After mixing, all the sample (500 µL) was placed into a Rneasy spin column (Rneasy Kit, Qiagen, Germany) within a 2 mL collection tube. All centrifugation steps were carried out at 9,279 g at room temperature. Columns were centrifuged for 30 seconds, after which the flow through was discarded. 350 µL of Buffer RW1 were then added to the column and centrifuged for 15 seconds after which flow through was again discarded. For on-column Dnase-I digestions of gDNA, 10 µL of Dnase-I stock solution (Qiagen, Germany) were added to 70 µL of Buffer RDD (Qiagen, Germany) and gently mixed by inverting the tube. 80 µL of this solution were then added directly to the center of the membrane of each spin column. This was then incubated at room temperature for 15 minutes. 350 µL of Buffer RW1 were again added to the spin column, centrifuged for 15 seconds, and the flow through discarded. 500 µL of Buffer RPE were added to the column, centrifuged for 30 seconds, and the flow through discarded. This step was repeated with a 2 minute centrifugation. The column was centrifuged for an additional 1 minutes to dry. The column was then transferred to a new 1.5 mL Eppendorf tubes (Eppendorf, UK) and 30 µL of Rnase-free water (Rneasy Kit, Qiagen, Germany) were added directly to the column membrane to elute the RNA. Columns were incubated at room temperature for 5 minutes, before centrifugation for 1 minutes. The eluate was reapplied onto the column membrane, incubated for a further 5 minutes at room temperature and centrifuged for 1 minutes. RNA concentration and purity was assessed by UV absorbance using a Nanodrop (Thermo Fisher Scientific, USA).

2.17.2 RNA-SEQUENCING AND ANALYSIS

For RNA-sequencing, total RNA was prepared as described above. Library preparations and paired-end RNA sequencing were performed by Novogene Co. Ltd UK (Cambridge, UK) using 3 biological replicates per condition.

Raw data files were analysed using Linux/Unix commandline. Paired-end reads were first assessed for quality using FastQC (Andrews, 2010) and MultiQC (Ewels et al. 2016) tools. The quality of sequences was ensured by checking per sequence quality scores and over-representation of adapter content. Subsequently, reads were aligned to the Berkeley *Drosophila* Genome Project assembly release 6, dm6.32 (Hoskins et al. 2015) using HISAT2 (Kim et al. 2019). The parameters of the alignment script, using HISAT2, were adapted from Baruzzo et al., 2017 and tailored to the data used in the study. Furthermore, known splice sites were extracted from the dm6.32 genome using HISAT2 to improve alignment percentage. The quality of the mapping was then examined using FastQC (Andrews, 2010) as above, and the Integrated Genome Viewer (IGV) (Robinson et al. 2011) was used to check for appropriate alignment.

The aligned reads were then sorted using SAMtools (Li et al., 2009) to generate sorted .sam files. Subsequently, the number of reads per gene were counted using featureCounts (Liao et al. 2014) and the sorted .bam file outputs from SAMtools. Differential expression was then analysed using a generalised linear model using DESeq2 tool (Love et al. 2014), allowing for normalisation in library composition. Differential expression analysis was carried out using SegMonk (version 1.48.1, Babraham Bioinformatics). Fold changes in expression were calculated by the internal DESeq2 functions. Differentially expressed genes were extracted and annotated with the parameters p- adjusted value < 0.05. Differentially expressed genes were visualised using the Volcano Plot and the heatmap tools using Srplot (Tang et al., 2023). The overlap between the differentially expressed gene lists of different conditions were visualised using Venn diagrams generated in BioVenn (Hulsen et al. 2008) and analysed using hypergeometric distribution analysis in Excel (version 2401) (Microsoft Corporation, USA). Heatmaps were produced using Srplot (Tang et al., 2023). Gene ontology (GO) analysis and Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathway analysis were carried out using ShinyGO (version 0.80) (Ge et al. 2020). Transcription factor enrichment analysis on the differentially expressed gene lists was carried out using icisTarget (Herrmann et al. 2012; Imrichová et al. 2015), a web-based tools to predict regulatory and cis-regulatory features and modules.

2.17.3 COMPLEMENTARY DNA SYNTHESIS

Complementary DNA (cDNA) for quantitative reverse transcription PCR (qRT-PCR) was synthesised in a Rnase-free environment as follows: 1 μ g of total RNA taken from the same extracts used for RNA-seq (prepared as described above in Section 1.14) were mixed with 1 μ L of oligo(dT) at concentration of 0.5 μ g/ μ L (Thermo Fisher Scientific, USA), and 1 μ L of 10 mM dNTP mix (Thermo Fisher Scientific, USA), dH2O was also added to make a total reaction volume of 12 μ L. Samples were incubated at 65 °C for 5 mins and then immediately placed on ice for 5 mins. All incubation steps were carried out using a 5PrimeG Thermal Cycler (Prime Techne, UK). Subsequently, 4 μ L of 5x first-strand buffer (Thermo Fisher Scientific, USA), 2 μ L of 0.1 M DTT (Thermo Fisher Scientific, USA), and 1 μ L of Rnase-OUT Rnase inhibitor (Thermo Fisher Scientific, USA) were added. Samples were then mixed and centrifuged briefly. Samples were incubated at 42 °C for 1 min, before 1 μ L of Superscript-II RT enzyme (Thermo Fisher Scientific, USA) was added whilst keeping the tubes at 42 °C as much as possible. A further 50-minute incubation at 42 °C was carried out. The reaction was then inactivated by incubating at 70 °C for 15 mins. Samples were subsequently stored at -20 °C.

2.17.4 QUANTITATIVE REVERSE TRANSCRIPTION PCR (QRT-PCR)

gRT-PCR was carried out using the LightCycler® 480 instrument II (Roche Life Science, Penzberg, Germany) with bright-white real-time PCR 96-well plates (Primer Design, Eastleigh, UK) sealed using StarSeal Advanced Polyolefin film (Star Lab, Milton Keynes, UK). Each 20 µL reaction contained: 10 µL 2x PrecisionPLUS gPCR Master Mix with SYBRgreen (containing 2x reaction buffer, 0.025 U/µL Tag Polymerase, 5 mM MgCl2, 100 μ M dNTP mix (containing 200 μ M of each dNTP)) (Primer Design, UK), 1 μ L of each forward and reverse primer (diluted from a 6 µM stock to give a final concentration of 300 nM) (Eurofins Genomics, UK), 6 µL of sterile dH2O, and 2 µL of template. For downstream processing, cDNA samples were diluted 1:10 to 1:15 in sterile dH2O and 1-2µL were added to each reaction. All reactions were performed in duplicate. A negative control was also run for each primer set, where sterile dH2O was used instead of the template. Relative quantities of transcripts were determined using a relative standard curve made from a pool of cDNA. Standards were also used to check for non-specific amplification and the presence of primer-dimers. Relative expression of genes of interest were calculated relative to the expression of a suitable reference gene (β COP, RPL-23, β -tub, RPL-9 (Table 2)) identified via analysis of reference gene stability using Normfinder (Andersen et al. 2004). Primers used in this study summarised in Table 2.

Primer name	Sequence	Gene	
βCOP-F	CTCTCCGAAAATGGACTTGG	Coat Protein β	
βCOP-R	GACACCGAGTTCCGTCAAAT	Coat Protein β	
B-Tub-F	TGGGCCCGTCTGGACCACAA	β-tubulin	
B-Tub-R	TCGCCGTCACCGGAGTCCAT	β-tubulin	
RPL-32-F	ATATGCTAAGCTGTCGCACAAATGG	Ribosomal protein 23	
RPL-32-R	GATCCGTAACCGATGTTGGGCA	Ribosomal protein 23	
RPL-9-F	CATGATCAAGGGAGTCACGT	Ribosomal protein 9	
RPL-9-R	ATGTACTTCTCACCCAAGAAG	Ribosomal protein 9	
ACC-F	ATGAGCGAAACAAATGAGTCCA	Acetyl-CoA carboxylase	
ACC-R	GGAACTCGTCACATGCCTCG	Acetyl-CoA carboxylase	
Bmm-F	GTCTCCTCTGCGATTTGCCAT	Brummer	
Bmm-R	CTGAAGGGACCCAGGGAGTA	Brummer	
Akh-F	AAAGCGGGCTGAATCGAAGT	Adipokinetic hormone	
Akh-R	AGATTGCACGAAGCGGAAGA	Adipokinetic hormone	
AkhR-F	AGCATTCCACAGGCCTTTCT	Adipokinetic hormone receptor	
AkhR-R	TCGATGGAGTCGCCACATTT	Adipokinetic hormone receptor	

Table 2.2: Primers used for qPCR analysis.

2.18 STATISTICAL ANALYSES

Statistical analyses were carried out on the results obtained using R (version 4.3.1) (Rstudio, USA), GraphPad (version 9.3.0) (GraphPad Software, USA), and Excel (version 2401) (Microsoft Corporation, USA). Tests for normality (Shapiro-Wilk), variance (F-test for equality of variances), and outliers (Grubbs) were performed on various datasets using GraphPad.

On normal data, one-way analysis of variance (ANOVA) or general linear models were carried out using R and GraphPad depending on the number of treatment variables. Where significance was observed, Welch's t-tests were used in R for post-hoc analysis on datasets showing unequal variance. When equal variance was observed standard t-tests were carried out in Graphpad.

For non-normal data, generalised linear models in R were carried out depending on the number of treatment variables. Kruskal-Wallis test followed by post-hoc Dunn's test was carried out to identify significance.

All survival data was analysed using a Log Rank test in Excel.

Chapter 3

3. EFFECT OF RAS/MAPK PATHWAY INHIBITION ON LIPID METABOLISM

3.1 INTRODUCTION

3.1.1 LIPID DROPLETS

3.1.1.1 STRUCTURE

Triglycerides (TAG) are the most important caloric source of energy in almost all organisms from bacteria to humans, making TAG turnover crucial to the metabolism and signalling of lipids, (Murphy, 2012). All animals are faced with energy requirements and upon nonfeeding developmental or metabolically stressful stages, animals developed an ability to store a surplus of calories which serve as flexible energy depots that can be used on-demand. In invertebrates such as *Drosophila melanogaster*, surplus dietary macromolecules are stored in a range of ways: dietary carbohydrates are stored as glycogen and dietary fatty acids as neutral TAG lipids. TAGs represent the most concentrated form of chemically bound energy as oxidation of TAGs yield more chemical energy compared to carbohydrates, and due to their apolar nature can be stored in energy dense intracellular organelles called lipid droplets, (LD), (Thiam *et al.*, 2013, Zechner *et al.*, 2017). Most importantly, the metabolic function of TAGs are not limited to energy storage, TAGs also serve as building blocks for membrane lipids and act as signalling molecules, (Fujimoto and Parton, 2011, Heier and Kuhnlein, 2018).

Microscopically, LDs are observed as round structures. Their diameters generally range from 0.1-5 µm in nonadipocyte cells, however, can reach more than 100 µm in adipocyte cells and even bigger under certain conditions such as high sugar feeding, (Musselman *et al.*, 2011) The LD surface is delimited by a phospholipid monolayer which includes phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), lysoPC and lysoPE, (Tauchi-Sato *et al.*, 2002, Bartz *et al.*, 2007). Free cholesterol is also likely to exist within LD's phospholipid monolayer, (Prattes *et al.*, 2000). The unique LD structure of neutral lipid core surrounded by phospholipid monolayer membrane and peripheral proteins is a property which is conserved from bacteria to humans, (Martin and Parton, 2006; Ding *et al.*, 2012), (Figure 3.1). Additionally, the biological functions of TAGs have been highly conserved during evolution and are similar in *Drosophila* making the fly a popular model to study the general physiology of TAG metabolism and its pathology, (Musselman and Kuhnlein, 2018).

3.1.1.2 FORMATION OF LIPID DROPLETS

The process of biogenesis of LDs has not yet been fully established by the available experimental data, however several hypotheses have been proposed and discussed, (Fujimoto and Parton, 2011). The consensus classical model theorizes that lipid ester globules bud from the cytoplasmic leaflets of the endoplasmic reticulum (ER), which explains the conserved LD structure suitably, (Murphy *et al.*, 1999). Additionally, in eukaryotes LDs are formed *de novo* from the ER, (Choudhary *et al.*, 2015). This is a long-standing belief as enzymes for neutral lipid synthesis are localized in the ER, (Buhman *et al.*, 2011), where the enzymes generate neutral lipids for LD formation. However, the next step of formation of emulsion droplets from the synthesised neutral lipids and which proteins function in the formation process are still subject to investigation.

In the formation of LDs, initially, neutral lipids such as TAGs are synthesised predominantly in the ER, (Buhman *et al.*, 2001). Fatty acid (FA) moieties which are stockpiled in TAGs are either derived from the diet or *de novo* through a cascade of enzymatic reactions. Different enzymes are involved in synthesis of TAGs and sterol esters. TAGs are the products of condensation of DAG acyltransferase (DGAT1 and DGAT2), (Cases *et al.*, 1998b; Lardizabal *et al.*, 2001). At low concentrations, TAGs disperse between the leaflets of the ER bilayer but as their concentration increases, neutral lipids coalesce and form an oil lens in a process called demixing. Studies have suggested that lens formation is a simple principle of physical chemistry by which the demixing of neutral lipids being energetically favourable leads to reduced interactions with membrane components, (Thiam and Foret, 2016). The process of formation of the neutral lipid lens is not well studied, and whether the process randomly occurs throughout the ER or whether preferential sites exist is still up for debate.

Once the neutral lipid lens is formed, it expands resulting in the LD budding from the ER membrane. Recent *in vivo* studies suggested that the phospholipid composition of the ER membrane is critical for LD budding. Phospholipid composition affects budding efficiency through geometric effects where conically shaped molecules such as DAGs and phosphatidylethanolamine (PE) discourage budding whereas molecules such as lysophospholipids promote budding, (M'baker *et al.*, 2017, Choudhary *et al.*, 2018). Additionally, LD membrane protein and/or lipid composition can affect the tension of the membrane and determine budding directionality, leading to some LDs being detected in the ER lumen, yet in most cases budding of LDs occurs towards the cytosol, (Mishra *et al.*, 2016).

After budding occurs, the LDs expand which commonly occurs by droplet-droplet fusion through the transfer of TAGs to LDs or through TAG synthesis directly on the LDs surface, (Olzmann and Carvalho *et al.*, 2019). The localized synthesis of TAG occurs through the

localization of lipid synthesis enzymes from the ER to the LD surface, with newly synthesised TAGs accumulating in the hydrophobic core, (Wilfling *et al.*, 2013). However, the process of membrane fission of LDs from ER is not fully understood, (Figure 3.1).

3.1.1.3 GENERAL FUNCTION

One of the main functions of LDs is the storage of lipid esters and their utilization during nutrient deprivation or during cell growth, which requires membrane expansion and phospholipid biosynthesis. Fas stored as TAGs in the core of the LDs can be mobilized by lipolysis to drive metabolic processes and membrane synthesis, (Heier and Kuhnlein, 2018). A less studied and appreciated function of LDs is the protection against lipotoxicity by sequestration of Fas inside the LD core to prevent the deleterious effects of free Fas. Free Fas can act as detergents that disrupt the membrane integrity and can be incorporated into lipid species that are cytotoxic at high levels such as ceramides and DAGs, (Olzmann and Carvalho, 2018). Consequently, conditions in which FA storage is impaired or storage capacity is overcome can result in diseases related to lipotoxicity such as Type 2 diabetes, (Krahmer *et al.*, 2013).

LDs have also been shown to protect against ER stress, generally referred to as imbalances in ER protein folding capacity, calcium uptake and/or lipid composition, (Walter and Ron, 2011). Disruption of LD biogenesis or simple overwhelming of the LD storage capacity often results in upregulation of the unfolded protein response (UPR). For instance, UPR has been observed in yeast strains which lack the enzymes required for the biosynthesis of TAGs and sterol esters, and therefore lack LDs, (Olzmann and Carvalho, 2018). In response to starvation, these yeast strains exhibited altered ER morphologies that were rescued by inhibiting *de novo* FA synthesis or by inducing ER phospholipid synthesis, showing that phospholipid synthesis is a compensatory response to the inability to form LDs, (Velazquez et al., 2016). Interestingly, in the absence of LDs, ER phospholipid composition is altered to contain increased levels of phosphatidylinositol, shown to result in impaired autophagosome biogenesis, (Velazquez et al., 2016). This suggested that, in yeast, LDs are essential for the maintenance of ER homeostasis by buffering excess Fas and preventing UPR activation, (Figure 3.1).

The mechanisms of impaired LD biogenesis and UPR activation are not clear, however one plausible theory is that aberrant FA storage activates the UPR by altering ER homeostasis causing impairment in ER protein folding and/or ER calcium storage, (Fu *et al.*, 2011). Alternatively, impaired FA storage could result in aberrant ER membrane lipid composition which itself activates the UPR, independently of the alteration in the ER protein folding and recent findings indicate that the UPR can be directly activated by altering the lipid composition of the ER membrane, (Volmer *et al.*, 2013).

Lastly, LDs have been shown to protect against mitochondrial damage during autophagy, (Figure 3.1). Prolonged starvation induces autophagy, resulting in the release of amino acids and lipids from the breakdown of proteins and membranous organelles, under the control of the mTOR complex 1 (mTORC1), (Efeyan et al., 2015). Intriguingly, a portion of autophagy-related lipids are esterified to form TAGs which are packaged into new LDs, (Rambold et al., 2015). Consequently, inhibition of autophagy, genetically or pharmacologically, blocks the formation of LDs in response to starvation or mTORC1 inhibition, (Rambold et al., 2015). The observation that LDs increase during starvation is surprising as it begs the question of why cells would expend their energy during energy crisis to generate TAG-containing LDs. Recently, it was shown that LDs function as a buffer to prevent lipotoxic damage to mitochondria, (Nguyen et al., 2017). Nguyen and colleagues have shown that inhibition of LD biogenesis during starvation-induced autophagy impaired mitochondrial potential, increased cell death and led to increased incorporation of Fas into acylcarnitine, a FA which is generated at the mitochondrial outer membrane and is required for FA uptake for mitochondrial β -oxidation, (Nguyen et al., 2017). Inhibition of enzymes involved in acylcarnitine synthesis rescued the mitochondrial membrane potential, suggesting that high levels of acylcarnitine are toxic to the mitochondria, (Nguyen et al., 2017).

Even though LD biogenesis may prevent lipotoxicity, the precise phenotype depends on a combination of factors including the cell type, lipotoxic insult and the type of lipids that accumulate. In general, studies have shown that LDs are required to prevent lipotoxicity during ER stress and autophagy because it is advantageous to sequester Fas as TAGs in LDs and release the Fas gradually through controlled lipolysis to prevent the conversion and buildup of toxic lipid species such as acylcarnitine, (Olzmann and Carvalho, 2018).



Figure 3.1: Schematic showing general lipid droplet structure, biogenesis and function.

(A) General 2D tomogram of lipid droplet (LD) where the lipid droplet phospholipid monolayer, neutral core and LD-associated proteins are evident. The monolayer which has a composition reminiscent of the ER bilayer, phospholipid polar heads face the cytosol whereas the acyl chains contact the hydrophobic neutral core. Associated with the monolayer are a variety of proteins, which decorate the surface of the LD and are absent from the neutral core. (B) LDs emerge from the endoplasmic reticulum (ER). Firstly, TAG machinery deposits neutral lipids in between the leaflets of the ER bilayer leading to the formation of a lens leading to an expansion of a nascent LD. Secondly, increased TAG synthesis leads to the budding of the LD from the ER membrane. Thirdly, local TAG synthesis machinery encourages the growth of the LD, alternatively smaller LDs fuse together to form larger LDs. (C) Simplified diagram showing alternative functions of LDs, other than storage. Mature LDs can prevent lipotoxic stress in response to ER stress and mito-autophagy by soaking up free fatty acids (FFA).

3.1.2 TAG METABOLISM

3.1.2.1 LIPOGENESIS

One of the core signalling pathways involved in the hormonal control of *Drosophila* lipogenesis is insulin signalling. In *Drosophila* during feeding, insulin-like peptides (Dilps) serve as ligands for the single insulin receptor (InR), which leads to initial activation of the *Drosophila* Akt1 kinase, (Cho *et al.*, 2001). Akt1 phosphorylates and regulates a range of metabolic enzymes and transcription factors involved in lipogenesis, (Bolukbasi *et al.*, 2017). Akt1 promotes phosphorylation of Foxo and decreases its transcriptional output, (Puig *et al.*, 2003). In turn, Foxo promotes the expression of lipases and increases the enzymatic breakdown of TAG under nutrient deprivation, (Wang *et al.*, 2011). Subsequently, the transcriptional repression of lipolysis by the insulin/Foxo axis in the *Drosophila* fat body is a critical determinant of TAG homeostasis, (Wang *et al.*, 2011).

The stockpiles of TAG are made of FA moieties and are either derived from the diet or

synthesised *de novo* through a cascade of enzymatic reactions, (Figure 3.2). Firstly, acetyl-CoA carboxylase (ACC) catalyses the formation of malonyl-CoA from acetyl-CoA, Secondly, a multienzyme complex FA synthase condenses malonyl-CoA units with acetyl-CoA to produce long-chain Fas, (Barber *et al.*, 2005). The *Drosophila* genome encodes a single *ACC* gene and three distinct FA synthase (*FASN*) genes, (Parvy *et al.*, 2012). *ACC* is ubiquitously expressed with the highest levels in the oenocytes and fat body cells, (Parvy *et al.*, 2012). All three *FASN* (1,2,3,) show a distinct expression pattern in the fat body, oenocytes, midgut and muscles, (Garrido *et al.*, 2015, Wicker-Thomas *et al.*, 2015). Muscle specific knockdown of *ACC* has been shown to prevent lifespan extension by dietary restriction, (Katewa *et al.*, 2012). On the other hand, unrestricted overexpression of ACC has been shown to play a pathogenic role in cardiac dysfunction of flies with mutation in the *easily shocked* (*eas*) gene, encoding ethanolamine kinase, (Lim *et al.*, 2011).

Additionally, enzymatic control of *de novo* lipogenesis is governed by changes in the dietary status of the animals, which are sensed and transmitted by transcription factors that govern the response to distinct dietary and hormonal cues. A common group of transcription factors are sterol regulatory element-binding proteins (SREBP) which are involved in the regulation of cholesterol, FA and TAG biosynthesis, (Walker et al., 2011). SREBPs regulate the transcription of genes involved in *de novo* lipogenesis including ACC and FASN in response to hormonal and dietary cues. SREBPs are membrane bound bHLH leucine zipper transcription factors that are activated by proteolytic cleavage which in turn facilitates the release and nuclear translocation of the active bHLH domain. The activation of SREBPs is mainly controlled by a lipid-sensing chaperone termed SREBP cleavage-activating protein (SCAP) and specific proteases, site-specific proteases 1 and 2 (S1P and S2P), (Heier and Kuhnlein, 2018). Notably, SREBP control was shown to be dependent on the PI3K/Akt/Tor pathway, (Krycer et al., 2010), which is highly conserved in flies and mammals, (Porstmann et al., 2008). The Drosophila genome encodes a single ortholog of SREBP, and consistent with its function, SREBP is predominantly expressed in the midgut, fat body and oenocytes, (Kunte et al., 2006).



Figure 3.2: Schematic showing lipogenesis in Drosophila.

Fatty acids (FA) derived from the diet or synthesised *de novo* by Acetyl-CoA carboxylase (*ACC*) and the FA synthase complex (*FASN*) are consequently esterified into TAG in the LDs. The transcription factor sterol regulatory element-binding protein (SREBP) promotes FA-CoA synthesis through a positive control of *ACC*, *FASN* and other lipid synthesis expression. The canonical pathway of SREBP activation requires the interaction with the escort factor SREBP cleavage-activating protein (SCAP) and its proteolytic processing by Golgi-residing S1P and S2P. SREBP activation is promoted by target of rapamycin (Tor) kinase which is activated via insulin receptor activation under feeding.

3.1.2.2 LIPOLYSIS

On the other hand, the TAGs in LDs can also be mobilized by lipolysis. During nonfeeding developmental stages, periods of high energy demands or deprivation, insects mobilize their energy stores. The mobilization of energy stores requires the hydrolysis of TAGs within the LDs, by the action of lipases, (Figure 3.3). The main model of insect fat mobilization involves TAG hydrolysis directly into diacylglycerol (DAG), which is exported into the hemolymph and into other tissues, (Arrese and Wells 1997). One of the main initiators of LD lipolysis in *Drosophila* is the TAG hydrolase Brummer, (Bmm), a member of the patatin-like domain-containing family of proteins, (Gronke *et al.*, 2005). Similar patatin-like domain containing orthologues also exist in plants, yeast and mammals, (Eastmond, 2006; Kurat *et al.*, 2005; Zummermann *et al.*, 2004) which highlights the ancestral conservation of Bmm-related proteins in TAG mobilization. In *Drosophila, bmm* mutant flies have been shown to accumulate excessive TAG during adulthood and display a reduced rate of TAG breakdown upon starvation. Due to delayed TAG

consumption, *bmm* mutants outlive wild-type flies during nutrient starvation, and showed reduced, yet functional, TAG mobilization, (Gronke *et al.*, 2005). Additionally, it was shown that interaction of Bmm with LDs is essential for the lipolysis of LDs, (Gronke *et al.*, 2005).

Major physiological functions with respect to energy metabolism in Drosophila are regulated both on the transcriptional and post-translational levels. TAG mobilization being one of these functions. Bmm lipase is transcriptionally suppressed during feeding and induced upon starvation by the insulin-responsive transcription factor Foxo, (Wang et al., 2011). Nuclear translocation of Foxo promotes transcription of a range of fasting-induced genes involved in TAG mobilization including bmm. (Karpac et al., 2013). A canonical inhibitor of Foxo is Akt1, phosphorylation by which prevents nuclear phosphorylation of Foxo and consequently, decreased bmm expression, (Wang et al., 2011). Another inhibitory kinase of Foxo is the salt-inducible kinase 3 (Sik3) which phosphorylates histone deacetylase 4 (HDAC4) to sequester HDAC4 in the cytosol, thereby preventing it from interacting with and activating nuclear Foxo, (Choi et al., 2015). Under starvation, the starvation sensitive *sik3* mutant flies are unable to suppress *bmm* expression which causes reduced TAG levels, (Wang et al., 2011). It is likely that the insulin pathway directly activates Sik3 by Akt1-dependent phosphorylation suggesting that various pathways converge at Sik3 to control bmm expression via Foxo, (Choi et al., 2015). Additionally, the transcriptional control of *bmm* by Foxo is suggested to be highly conserved as the mammalian ortholog of Bmm is also subject to FoxO1 regulation, (Chakrabarti and Kandror, 2009). Bmm expression shows an antagonistic response to disturbed Akh, (Baumbach et al., 2014a). Under starvation, glucagon-like peptide, Akh, plays a stimulatory role in TAG mobilization and was first described in locusts, (Mayer and Candy, 1969). The peptide is produced in the neuroendocrine cells of the corpora cardiaca and is released on demand to the hemolymph to bind its G protein-coupled Akh receptor (AkhR) on fat body cells. The activation of AkhR leads to downstream activation of cAMP, an activator of protein kinase A (PKA), which promotes phosphorylation of a range of transcription factors, kinases and LD proteins involved in lipolysis, (Lee et al., 2018). Baumbach and colleagues, have shown that loss-of-function mutation of AkhR reduced *bmm* expression suggesting that Akh signaling promotes *bmm* expression, (Baumbach et al., 2014a). Recently, it has been shown that Akh signaling via Ca²⁺ and calcium/cam-dependent protein kinase II (CaMKII) inhibits secretion of the adipokine unpaired 2 (Upd2) from the fat body, a protein involved in triggering systemic insulin signaling from the central brain, (Rajan and Perrimon, 2012; Rajan et al., 2017). This was shown to impair TAG mobilization in the fat body, possibly by the repression of *bmm* expression in a Foxo-dependent manner, (Rajan and Perrimon 2012; Rajan et al., 2017). This model suggests that alterations in Upd2 and systemic insulin signaling regulate the Akh/Ca²⁺- dependent control of *bmm* expression, (Figure 3.3).



Figure 3.3: Schematic showing lipolysis in Drosophila.

Fatty acids (Fas) derived from the diet or synthesised *de novo* by Acetyl-CoA carboxylase (*ACC*) and the FA synthase complex (*FASN*) are consequently esterified into TAG in the LDs. The transcription factor sterol regulatory element-binding protein (SREBP) promotes FA-CoA synthesis through a positive control of *ACC*, *FASN* and other lipid synthesis expression. The canonical pathway of SREBP activation requires the interaction with the escort factor SREBP cleavage-activating protein (SCAP) and its proteolytic processing by Golgi-residing S1P and S2P. SREBP activation is promoted by target of rapamycin (Tor) kinase which is activated via insulin receptor activation under feeding.

3.2 AIMS AND OBJECTIVES

The preliminary data has shown that trametinib extended lifespan on normal and high sugar diets as well as prevented the accumulation of TAG in response to high sugar diet and during ageing. Therefore, it was essential to further investigate the effects of trametinib on lipid storage. In this section, to build on from the preliminary data, the main aim was to investigate the effects of RAS/MAPK pathway inhibition by trametinib on lipid metabolism, by first assessing the efficacy of trametinib preventing ERK activation using western blotting, and then to examine the effects of trametinib on triglyceride content in *Drosophila* in response to high sugar feeding and during ageing. This was achieved in two ways: biochemical measurement of TAG content using a colorimetric TAG assay and confocal microscopy of dissected fat bodies to quantify lipid droplet size.

To test whether the changes in TAG storage with trametinib treatment contribute to the lifespan extension induced by trametinib, genetic manipulations to both block lipolysis and induce lipid synthesis were used to test the hypothesis that changes in TAG storage

3.3 RESULTS

3.3.1 EFFICACY OF TRAMETINIB ON ERK INHIBITION IN RESPONSE TO DIET AND AGEING

To investigate the effects of trametinib on lipid metabolism in response to high sugar diet and during ageing, it was essential to verify that trametinib effectively inhibits MEK under these treatment conditions. Previously, it was shown that trametinib decreases ERK phosphorylation in genetic and diet-induced mouse models of obesity, (Bost *et al.*, 2005; Banks *et al.*, 2015). And while trametinib treatment of 10µM in *Drosophila* S2 cells showed a decrease in phosphorylation of ERK, these findings were not yet replicated in *Drosophila in vivo*, (Slack *et al.*, 2015). The results of trametinib's efficacy to inhibit ERK on both normal (1x) and high (8x) sugar diet are shown in Figure 3.4.



Figure 3.4: Quantification of phosphorylated-ERK (p-ERK) in response to high sugar diet and trametinib treatment.

Western blot analysis of p-ERK protein levels in flies fed with normal (1x) or high (8x) diets, in both presence (+) and absence (-) of Trametinib. (A): Representative image of western blots made using protein extracts taken from wild-type 10-day old adult female *Drosophila*. Blots were probed with antibodies against total ERK protein (t-ERK), phosphorylated-ERK (p-ERK) and Tubulin. Tubulin was used as a loading control. (B): Quantification of band density, with lowest value, median and highest value lines shown, normalised to tubulin for both t-ERK and p-ERK for both normal(1x) and high(8x) sugar fed flies; n=3, two-way ANOVA with *post-hoc* Welch's t-test, p<0.05 *, p<0.01 **.

As expected, on normal (1x) sugar diet, trametinib significantly decreased phosphorylation of ERK. High (8x) sugar diet significantly increased phosphorylation of ERK in the absence of trametinib. And on both normal (1x) and high (8x) sugar diet,

trametinib reduced the levels of phosphorylated ERK. The reduction of ERK phosphorylation observed on high (8x) sugar diet in response to trametinib treatment was a full rescue to the levels observed on normal (1x) sugar diet without trametinib, (Figure 3.2).



Figure 3.5: Quantification of phosphorylated-ERK (p-ERK) protein in response to Trametinib during ageing.

Western blot analysis of p-ERK protein levels in flies, in both presence (+) and absence (-) of Trametinib during ageing (D10-D30). (A): Representative image of western blots made using protein extracts taken from wild-type 10,20 and 30 -day old adult female *Drosophila*. Blots were probed with antibodies against total ERK protein (t-ERK), phosphorylated-ERK (p-ERK) and Tubulin. Tubulin was used as a loading control. (B): Quantification of band density, with lowest value, median and highest value lines shown, normalised to tubulin for both t-ERK and p-ERK for each time point; n=3, two-way ANOVA with *post-hoc* Welch's t-test, p<0.01 **, p<0.001***.

The effects of trametinib on ERK phosphorylation were then examined during normal ageing. In the absence of trametinib, phosphorylation of ERK showed an age-dependent increase from day 10 to day 30. Trametinib significantly decreased ERK phosphorylation at all ages and the age-dependent increase in ERK phosphorylation was no longer observed (Figure 3.5).

3.3.2 EFFECT OF TRAMETINIB ON STORAGE MOLECULES IN RESPONSE TO HIGH SUGAR FEEDING

In the previous section, it was established that trametinib effectively inhibits MEK under both high sugar diet and during normal ageing. Additionally, the preliminary data suggested that trametinib inhibits TAG storage under both high sugar diet and normal ageing, TAG being the primary macromolecule used for lipid storage in the fat body of the fly. Also, flies store energy as carbohydrates in the form of glycogen. Glycogen stores fluctuate considerably based on motor activity, feeding and environmental conditions, (Chatterjee and Perrimon, 2021). To check whether trametinib treatment also impacts glycogen storage, TAG and glycogen biochemical assays were performed on whole fly extracts after high sugar feeding. The results of trametinib's effect on triglyceride and glycogen storage are shown in Figure 3.6.



Figure 3.6: Quantification of triglyceride and glycogen in flies fed high sugar diet in response to trametinib.

Biochemical assay quantification of triglycerides and glycogen of flies fed a normal (1x) and high (8x) sugar diet in absence (-) and presence (+) of trametinib. (A): Triglyceride content measured per protein content of day 10 wild-type female flies on normal (1x) and high (8x) sugar diets in absence (-) and presence (+) of trametinib. (B): Quantification of glycogen content measured per protein content of day 10 wild-type female flies on normal (1x) and high (8x) sugar diets in absence (-) and presence (+) of trametinib. (B): Quantification of glycogen content measured per protein content of day 10 wild-type female flies on normal (1x) and high (8x) sugar diets in absence (-) and presence (+) of trametinib. The middle bar shows the mean value; n=5 pooled flies of 10 replicates, two-way ANOVA with *post-hoc* Tukey multiple comparison test, p<0.0001 ****.

Trametinib did not have a significant effect on TAG content in flies fed a normal (1x) sugar diet. TAG content significantly increased in flies fed a high (8x) sugar diet in absence of trametinib. Trametinib completely blocked the increase in TAG content in flies fed high (8x) sugar diet, (Figure 3.6A). Trametinib did not have a significant effect on glycogen content in flies fed normal (1x) diet. High (8x) sugar diet significantly increased glycogen content but unlike for TAG, trametinib did not affect this increase in glycogen content on high (8x) sugar diet, (Figure 3.6B).

As the effects of trametinib on metabolic storage molecules in response to high sugar feeding, were observed solely on TAGs and not glycogen, it was central to investigate further by looking at the fat body, the main storage organ of TAGs in *Drosophila*, (Heier and Kuhnlein, 2018). First, it was important to determine whether high sugar diet and trametinib treatment affect the feeding behaviour of flies as reduced feeding in response to trametinib could be a factor in the ability of trametinib to lower TAG levels. Therefore,

a feeding assay was carried out to quantify the proboscis extension in flies fed high sugar diet in response to trametinib, (Figure 3.7).



Figure 3.7: Proboscis extension quantification in response to high sugar diet and trametinib treatment.

Proportion feeding based on proboscis extension in wild-type female day 10 flies fed with normal (1x) or high (8x) diets, in both presence (+) and absence (-) of Trametinib; n=15, two-way ANOVA with *post-hoc* Tukey multiple comparison test, $p<0.05^*$, $p<0.001^{***}$.

Trametinib did not have a significant effect on the proportion of flies feeding on a normal (1x) sugar diet. Feeding was significantly decreased in flies fed a high (8x) sugar in the absence of trametinib but trametinib did not have a significant effect on feeding in flies fed a high (8x) sugar diet, (Figure 3.7).

3.3.3 EFFECT OF TRAMETINIB ON LIPID DROPLET SIZE IN RESPONSE TO HIGH SUGAR FEEDING AND DURING AGEING.

In the previous section, it was found that trametinib primarily affected TAG storage and not storage of glycogen in response to high sugar diet. In flies, TAG is stored in lipid droplets within the fat body. The size of the lipid droplets is determined by the amount of TAG they store, therefore the size of the lipid droplets within the fat bodies of flies fed high sugar diet in response to trametinib were quantified. The results of trametinib's effect on lipid droplet size in response to high sugar feeding and during ageing are shown in Figure 3.8 and Figure 3.9, respectively.



Figure 3.8: Quantification of lipid droplet size in response to high sugar diet and trametinib treatment.

Confocal microscopy images of fat body lipid droplets of flies fed with normal (1x) or high (8x) diets, in both presence (+) and absence (-) of trametinib. (A): Representative merged images of Nile red, and DAPI signals of fly fat body made by dissecting the fat body of wild-type 10-day old adult female *Drosophila*. Fat bodies were incubated with Nile red (Neutral lipids) and DAPI (Nucleus). Example lipid droplets are circled in red. (B): Quantification of lipid droplet size, with lowest value, median and highest value lines shown, for both normal (1x) and high (8x) sugar fed flies; n=165, two-way ANOVA with *post-hoc* Tukey multiple comparison test, p<0.001 ***.

On normal (1x) sugar diet, trametinib significantly decreased the average lipid droplet size. High (8x) sugar diet significantly increased lipid droplet size in the absence of trametinib. However, trametinib significantly reduced lipid droplet size on high sugar (8x) diet to comparable sizes to those observed on normal (1x) sugar diet, (Figure 3.8).

Changes to lipid droplet size were also examined during normal ageing. Lipid droplet size showed an age-dependent increase in the absence of trametinib from day 10 to day 30. In the presence of trametinib, there was also a significant increase in lipid droplet size, showing an age-dependent increase from day 10 to day 30. Additionally, trametinib significantly reduced the lipid droplet size at each time point from day 10 to day 30, (Figure 3.9).



Figure 3.9: Quantification of lipid droplet size in response to trametinib treatment during ageing.

Confocal microscopy images of fat body lipid droplets of flies in both presence (Tram+) and absence (Tram-) of Trametinib during ageing (D10-D30). (A): Representative merged images of Nile red, and DAPI signals of fly fat body made by dissecting the fat body of wild-type 10-day old adult female *Drosophila*. Fat bodies were incubated with Nile red (Neutral lipids) and DAPI (Nucleus). Example lipid droplets are circled in red. (B): Quantification of lipid droplet size, with lowest value, median and highest value lines shown; n=165, two-way ANOVA with post-hoc Tukey multiple comparison test, p<0.01 **, p<0.001 ***, p<0.001 ***.

3.3.4 EFFECT OF ENHANCED LYPOLYSIS AND REDUCED LIPID SYNTHESIS ON LIFESPAN EXTENDING PROPERTIES OF TRAMETINIB

Based on the previous results, trametinib was shown to decrease both lipid droplet size and TAG content in response to high sugar feeding yet had no effect on glycogen storage. The next step was to investigate the potential mechanism behind trametinib's ability to lower TAG levels and whether these effects on TAG storage contribute to the trametinibdependent lifespan extension. It was hypothesized that trametinib may extend lifespan by either enhancing lipolysis and hence decreasing lipid content and lipid droplet size in response to high sugar feeding and during ageing. On the other hand, trametinib could lower lipid storage by reducing lipid synthesis. To test each of these mechanisms, genetic manipulations were employed to either block lipolysis or enhance lipid synthesis. The justification behind this was that if trametinib extends lifespan through enhanced lipolysis then preventing lipolysis in *Drosophila* should prevent trametinib-dependent lifespan extension. On the other hand, if trametinib extends lifespan by decreasing lipid synthesis, then overexpression of a key rate-limiting enzyme within the lipid synthesis pathway should also prevent lifespan extension by trametinib.

To block lipolysis, a null mutation in *bmm* was used to prevent the hydrolysis of TAG into diglycerides and fatty acids with the LDs. To induce lipid synthesis in *Drosophila*,

overexpression of acetyl-CoA carboxylase was used. Acetyl-CoA carboxylase (ACC) catalyses the carboxylation of acetyl-CoA to produce malonyl-CoA, (Barber *et al.,* 2005). The sequential condensation of several malonyl-CoA molecules with acetyl-CoA allows for build-up of long chain fatty acids, (Heier and Kuhnlein, 2018). ACC overexpression was induced under the control of UAS using the Gene-Switch system and was activated using mifepristone (RU486).

3.3.4.1 DEPENDENCE OF TRAMETINIB LIFESPAN EXTENSION ON ENHANCED LIPOLYIS.

An initial assessment into investigating the mechanism of enhanced lipolysis by trametinib, was to look at the expression of brummer (bmm) in response to trametinib treatment. However, beforehand it was crucial to find a suitable housekeeping gene as a control for normalisation of expression of genes of interest in response to trametinib treatment. The expression of the housekeeping gene should be stable across all experimental conditions and show little difference in expression within experimental conditions. To determine a suitable housekeeping gene for gene expression analysis, the Normfinder statistical analysis software was used to rank a panel of four potential housekeeping genes: *b-cop*, *rpl-23*, *b-tub*, *rpl-9*, according to stability in reference to the experimental samples, (Kohsler et al., 2020). The lowest stability value from Normfinder represents the most stable housekeeping gene. From this analysis, expression of *b-cop* produced the lowest stability value (Table 3.1) and therefore represented the most stable housekeeping gene. Expression of *b-cop* was used in subsequent experiments to normalise the expression of genes of interest in response to trametinib treatment. B-cop is a gene that encodes a protein crucial for the formation and function of the coat protein complex involved in vesicular trafficking within cells, particularly in the Golgi apparatus.

Gene name	Stability value	
В-сор	1.57	
Rpl-23	1.86	
B-tub	2.45	
Rpl-9	2.83	

Table 3.1: Normfinder data of control housekeeping genes.

Table containing Normfinder data analysis to identify a control housekeeping gene. The stability value is calculated based on estimated intra- and intergroup variation giving a practical measure of the systematic error that is introduced when using a reference gene of choice, (Andersen *et al.*, 2004).

Differences in expression of *bmm* were then analysed by qRT-PCR in flies fed with high sugar diet and during normal ageing, both in the absence and presence of trametinib. The relative expression of *bmm* was comparable on both normal (1x) and high (8x) sugar diet. Furthermore, trametinib did not have a significant effect on *bmm* expression on either normal (1x) or high (8x) sugar diets, (Figure 3.10A). Similarly, during normal ageing there were no significant differences in the relative expression of *bmm* from day 10 to day 30 either in the absence or presence of trametinib, (Figure 3.10B).



Figure 3.10: *Brummer* relative gene expression analysed by qRT-PCR and normalised to relative expression of *b-cop* mRNA.

(A): Relative gene expression of *bmm* of flies fed normal (1x) and high (8x) sugar diets in absence (-) and presence (+) of trametinib, central bar indicating the mean. (B): Relative gene expression of *bmm* of flies during ageing from day 10 to day 30 in absence (-) and presence (+) of trametinib, central bar indicating the mean. (n=3 replicates of 15 pooled flies for each treatment condition, two-way ANOVA with post-hoc Tukey multiple comparison test).

To test whether Bmm activity was required for the ability of trametinib to reduce TAG levels in flies fed high (8x) sugar diet, *bmm* loss-of-function mutants were assayed for TAG storage on both normal (1x) and high (8x) sugar diet in the absence and presence of trametinib. The results for these TAG assays can be found in Figure 3.11.



Figure 3.11: Quantification of triglyceride in wild-type and *brummer* mutant flies fed high sugar diet in response to trametinib.

Quantification of triglycerides in wild-type (*wDah*) and *bmm* mutant (Δbmm) flies fed a normal (1x) and high (8x) sugar diets in absence (-) and presence (+) of trametinib. (**A**): Triglyceride content measured per protein content of day 10 wild-type female flies on normal (1x) and high (8x) sugar diets in absence (-) and presence (+) of trametinib. (**B**): Quantification of triglyceride content measured per protein content of day 10 *bmm* mutant female flies on normal (1x) and high (8x) sugar diets in absence (-) and presence (+) of trametinib. (**B**): Quantification of triglyceride content measured per protein content of day 10 *bmm* mutant female flies on normal (1x) and high (8x) sugar diets in absence (-) and presence (+) of trametinib. The middle bar shows the mean value; n=5 pooled flies of 10 replicates, two-way ANOVA with *post-hoc* Tukey multiple comparison test, p<0.05 *, p<0.001 ***, p<0.001 ****.

As shown earlier, trametinib did not have a significant effect on TAG content in wild-type (*wDah*) flies fed a normal (1x) sugar diet. TAG content significantly increased in wild-type (*wDah*) flies fed a high (8x) sugar diet in absence of trametinib. While in the presence of trametinib this increase in TAG content on high (8x) sugar diet was completely blocked (Figure 3.11A).

Bmm mutant flies (Δbmm) flies fed a normal (1x) sugar diet had elevated TAG levels compared to wild-type flies and TAG levels increased further after feeding *bmm* mutant flies (Δbmm) with a high (8x) sugar diet. Interestingly, trametinib treatment reduced TAG levels in *bmm* mutant flies (Δbmm) on normal (1x) sugar diet and completely blocked the increase in TAG content in *bmm* mutant flies (Δbmm) flies fed a high (8x) sugar diet, (Figure 3.11B) suggesting that trametinib reduced TAG levels independently of Bmm-dependent lipolysis.

Next, to determine whether the lifespan extension induced by trametinib depends on enhanced lipolysis, the survival of *bmm* mutant flies in response to trametinib was investigated, (Figure 3.12).



Figure 3.12: Comparing lifespan of wild-type, wDah, and Bmm lipase mutant, Δ Bmm, flies fed normal (1x) and high (8x) sugar diets in the presence (+) or absence (-) or Trametinib.

Survival analysis of **(A)** wild type, *wDah*, and **(B)** Brummer lipase mutant, ΔBmm , flies. Results are plotted as proportion of survivors as a function of time (in days). Median survival times of flies are shown in the table **(C)**. Table showing median lifespan, log rank test comparing absence(-) of trametinib to presence(+) of trametinib for both genotypes and diets, p<0.001 *** (Log-Rank test). Black arrow (Day 10) shows the points at which flies were taken for TAG analysis. Red dotted line showing the median lifespan. In wild-type (*wDah*) flies, as seen before, trametinib extended lifespan on both normal (1x) and high (8x) sugar diets, (normal (1x) sugar diet: +7% median lifespan, p = 1x10⁻⁵; high (8x) sugar diet: +19% median lifespan, p = 1x10⁻⁹), (Figure 3.10).

As previously reported, mutation of *bmm* reduced lifespan compared to wild-type flies on both normal (1x) and high (8x) sugar diet. But trametinib was still able to increase lifespan in Δbmm mutants on both normal (1x) and high (8x) sugar diets, (normal (1x sugar diet: +20% median lifespan, p = 1x10⁻⁸; high (8x) sugar diet: +23% median lifespan, p =1x10⁻⁸; high (8x) sugar diet: +23% median lifespan, p =1x10⁻⁸; high (8x) sugar diet: +23% median lifespan, p =1x10⁻⁸; high (8x) sugar diet: +23% median lifespan, p =1x10⁻⁸; high (8x) sugar diet: +23% median lifespan, p =1x10⁻⁸; high (8x) sugar diet: +23% median lifespan, p =1x10⁻⁸; high (8x) sugar diet: +23% median lifespan, p =1x10⁻⁸; high (8x) sugar diet: +23% median lifespan, p =1x10⁻⁸; high (8x) sugar diet: +23% median lifespan, p =1x10⁻⁸; high (8x) sugar diet: +23% median lifespan, p =1x10⁻⁸; high (8x) sugar diet: +23% median lifespan, p =1x10⁻⁸; high (8x) sugar diet: +23% median lifespan, p =1x10⁻⁸; high (8x) sugar diet: +23% median lifespan, p =1x10⁻⁸; high (8x) sugar diet: +23% median lifespan, p =1x10⁻⁸; high (8x) sugar diet: +23% median lifespan, p =1x10⁻⁸; high (8x) sugar diet: +23% median lifespan, p =1x10⁻⁸; high (8x) sugar diet: +23% median lifespan, p =1x10⁻⁸; high (8x) sugar diet: +23% median lifespan; p =1x10⁻⁸; high (8x) sugar diet: +23% median lifespan; high (8x) sugar diet; high

As trametinib seemed to reduce TAG levels and extend lifespan independently of lipolysis, an alternative hypothesis is that trametinib may exert its effects through reducing lipid synthesis. First, differences in the expression of acetyl-CoA carboxylase (*ACC*) was examined using qRT-PCR in flies fed with a high sugar diet and during ageing both in the absence and presence of trametinib, (Figure 3.13).



Figure 3.13: Acetyl-CoA carboxylase (ACC) relative gene expression analysed by qRT-PCR and normalised to relative expression of *b*-cop mRNA.

(A): Relative gene expression of ACC of flies fed normal (1x) and high (8x) sugar diets in absence (-) and presence (+) of trametinib, central bar indicating the mean. (B): Relative gene expression of ACC of flies during ageing from day 10 to day 30 in absence (-) and presence (+) of trametinib, central bar indicating the mean.(n=3 replicates of 15 pooled flies for each treatment condition, two-way ANOVA with post-hoc Tukey multiple comparison test).

The relative expression of *ACC* was similar on both normal (1x) and high (8x) sugar diet. Furthermore, trametinib did not have a significant effect on *ACC* expression on either normal (1x) or high (8x) sugar diet, (Figure 3.13A). Similarly, during normal ageing there were no significant differences in the relative expression of *ACC* from day 10 to day 30 either in either the absence or presence of trametinib, (Figure 3.13B).

To test whether ACC overexpression could enhance lipid synthesis and prevent trametinib-dependent lifespan extension, two ACC overexpressing fly strains were used, BL-63224 and BL-63225, which both contain the same transgene under UAS control, but the transgenes are located at different regions of the genome. Both UAS-ACC transgenic lines were crossed to Act-GS to drive their expression ubiquitously and expression induced by feeding the flies with RU486. Overexpression of ACC protein was first verified by western blotting using an ACC-specific antibody, (Figure 3.14).



Figure 3.14: Quantification of acetyl-CoA carboxylase (ACC) protein in gene switch alone and ACC overexpressing lines.

Western blot analysis of ACC protein levels in gene switch alone (actGS>) and ACC overexpressing lines, (BL-6324, BL-63225) in absence (-) and presence (+) of RU486. (A): Representative image of western blots made using protein extracts taken from wild-type and ACC overexpressing (BL-63224, BL-63225) 10-day old adult female *Drosophila*. Blots were probed with antibodies against ACC protein and Tubulin. Tubulin was used as a loading control. (B): Quantification of band density, with lowest value, median and highest value lines shown, normalised to tubulin; n=3, two-way ANOVA with *post-hoc* Welch's t-test, ns>0.05, p<0.01 **. Density of bands were determined using ImageJ Fiji software, comparing density of ACC bands to tubulin bands.

The western blot analysis revealed a faint but detectable band for the control line (actGS>) in the absence (-) and presence (+) of RU486. The detectable band was present at the expected molecular weight, indicating the presence of ACC.

RU486 treatment did not significantly change ACC protein expression in gene switch alone (actGS>) flies while RU486 treatment significantly increased ACC protein expression in both ACC overexpressing lines crossed to ActGS, by around 3-4 fold, (Figure 3.14).

Next, the ability of the two ACC overexpressing lines to induce TAG synthesis was examined by measuring TAG levels in flies after RU486 induction of their expression, (Figure 3.15).



Figure 3.15: Quantification of triglyceride in gene switch alone and *acc* overexpressing flies fed high sugar diet in response to trametinib and RU486.

Quantification of triglycerides of gene switch alone (*actGS>*) and *acc* overexpressing flies, *BL*-63224, *BL*-63225, fed a normal (1x) and high (8x) sugar diets in absence (-) and presence (+) of trametinib and in absence (clear) and presence (shaded) of RU486. (**A**): Triglyceride content measured per protein content of day 10 gene switch alone female flies on normal (1x) and high (8x) sugar diets in absence (-) and presence (+) of trametinib and in absence (clear) and presence (shaded) of RU486. (**B**): Quantification of triglyceride content measured per protein content of day 10 *acc* overexpressing female flies (*BL*-63224) on normal (1x) and high (8x) sugar diets in absence (-) and presence (clear) and presence (clear) and presence (-) and presence (+) of trametinib and in absence (clear) and presence (-) and presence (+) of trametinib and in absence (clear) and presence (-) and presence (shaded) of RU486. (**C**): Quantification of triglyceride content measured per protein content of day 10 *acc* overexpressing female flies (*BL*-63225) on normal (1x) and high (8x) sugar diets in absence (-) and presence (+) of trametinib and in absence (clear) and presence (-) and presence (+) of trametinib and in absence (shaded) of RU486. (**C**): Quantification of triglyceride content measured per protein content of day 10 *acc* overexpressing female flies (*BL*-63225) on normal (1x) and high (8x) sugar diets in absence (-) and presence (+) of trametinib and in absence (clear) and presence (shaded) of RU486. The middle bar shows the mean value; n=5 pooled flies of 10 replicates, one-way ANOVA with *post-hoc* Tukey multiple comparison test, p<0.05 *,p<0.01 ** p<0.001 ****, p<0.0001 ****.

In gene switch alone (*actGC*>) flies, the presence of RU486 did not significantly affect TAG content, nor the ability of trametinib to reduce TAG levels, on both normal (1x) and high (8x) sugar diet.

For both ACC overexpression lines, on normal (1x) and high (8x) sugar diets induction of transgene expression by RU486 resulted in significant increases in TAG content demonstrating that ACC overexpression resulted in enhanced lipid synthesis and storage. Interestingly, trametinib was able to at least partially reduce TAG levels in flies overexpressing ACC on both normal (1x) and high (8x) sugar diets suggesting that trametinib reduces TAG levels even under conditions of enhanced ACC-dependent lipid synthesis, (Figure 3.13).

To determine whether the lifespan extension induced by trametinib depends on reduced lipid synthesis, the survival of ACC overexpressing flies in response to trametinib was investigated, (Figure 3.14).



G

Median Lifespan	actGS>				
RU486	-	-	+	+	
Trametinib	-	+	-	+	
Normal diet	67.5	71.9***	67.5	71.9***	
High sugar diet	30	32.4***	30.1	35.3***	
Median Lifespan	BL-63224				
RU486	-	-	+	+	
Trametinib	-	+	-	+	
Normal diet	67.5	71.9***	67.5	71.9***	
High sugar diet	34.8	34.8	32.4	36.8***	
Median Lifespan	BL-63225				
RU486	-	-	+	+	
Trametinib	-	+	-	+	
Normal diet	67.5	67.5	67.5	69.9***	
High sugar diet	34.8	36.8***	32.4	39.4***	

Figure 3.16: Comparing survival of gene switch alone, and Acetyl-CoA overexpressing flies fed normal (1x) and high (8x) sugar diets in the presence (+) or absence (-) of trametinib.

Survival analysis of (A-B) gene switch alone, actGS>, flies, left panel fed a normal diet (A), right panel fed a high sugar diet (B). Survival analysis of (C-D) acetyl-CoA carboxylase overexpressing flies (*BL*-63224), left panel (C) fed a normal diet, right panel (D) fed a high sugar diet. Survival analysis of (E-F) acetyl-CoA carboxylase overexpressing flies (*BL*-63225), left panel I fed a normal diet, right panel (F) fed a high sugar diet. Results are plotted as proportion of survivors as a function of time (in days). Median survival times of flies are shown in the table (G). Table showing median lifespan, log rank test comparing absence(-) of trametinib to presence(+) of trametinib for both genotypes and diets, p<0.001 ***(Log-Rank test). Black arrow (Day 10) shows the points at which flies were taken for TAG analysis. Red dotted line showing the median lifespan.

As shown previously, for gene switch alone (actGS>) flies on both normal (1x) and high (8x) sugar diets, trametinib significantly extended lifespan and this was not affected by the presence RU486 (normal (1x) sugar: +7% median lifespan, $p = 1x10^{-5}$; high (8x) sugar: +16% median lifespan, $p = 1x10^{-7}$) (Figure 3.14A and B).

For *BL-63224* flies, in absence of RU486-induced transgene expression, trametinib significantly extended lifespan on normal (1x) but not high (8x) sugar diet. But upon transgene expression by RU486, trametinib significantly extended lifespan on both normal (1x) and high (8x) sugar diets (normal (1x) sugar: +7% median lifespan, p = 1×10^{-5} , high (8x) sugar: +6% median lifespan, p = 1×10^{-5}), (Figure 3.14C and D).

For *BL-63225* flies, in the absence of RU486-induced transgene expression, trametinib extended lifespan on high (8x) but not normal (1x) sugar diet. But upon induction of transgene expression by RU486, trametinib significantly extended lifespan on both normal (1x) and high (8x) sugar diets (normal (1x) sugar: +4% median lifespan, p = 1×10^{-3} , high (8x) sugar: +13% median lifespan, p = 1×10^{-5}), (Figure 3.14E and F).

Taken together, this suggests that trametinib is still able to extend lifespan in the presence of elevated lipid synthesis induced by ACC overexpression.

3.3.4.3 DEPENDENCE OF TRAMETINIB LIFESPAN EXTENSION ON REDUCED LIPID SYNTHESIS PART II

Although ACC overexpression was sufficient to induce lipid synthesis resulting in elevated TAG levels, as a rate-limiting enzyme in lipogenesis, it may have been possible that the levels of ACC overexpression were not sufficient to fully block the effects of trametinib on lipid synthesis. Therefore, the effects of SREBP overexpression were examined as SREBP is a master regulator of lipid synthesis, which functions upstream of ACC to directly activate the expression of more than 30 genes dedicated to lipogenesis, (Horton *et al.,* 2002). A previously published SREBP overexpression transgenic strain that has been shown to induce lipid droplet synthesis in glial cells when overexpressed in *Drosophila* neurons, (Liu *et al.,* 2015) was used to investigate whether SREBP overexpression could prevent the effects of trametinib on reducing TAG levels both on normal (1x) and high (8x) sugar diets, (Figure 3.15).



Figure 3.17: Quantification of triglyceride in gene switch alone and *srebp* overexpressing flies fed high sugar diet in response to trametinib and RU486.

Quantification of triglycerides in gene switch alone(*actGS*>) and *srebp* overexpressing flies, *3839RB*, fed a normal (1x) and high (8x) sugar diets in absence (-) and presence (+) of trametinib and in absence (clear) and presence (shaded) of RU486. **(A)**: Triglyceride content measured per protein content of day 10 gene switch alone female flies on normal (1x) and high (8x) sugar diets in absence (-) and presence (+) of trametinib and in absence (clear) and presence (+) of trametinib and in absence (clear) and presence (shaded) of RU486. **(B)**: Quantification of triglyceride content measured per protein content of day 10 *srebp* overexpressing female flies (*38396RB*) on normal (1x) and high (8x) sugar diets in absence (-) and presence (+) of trametinib and in absence (clear) and presence (shaded) of RU486. The middle bar shows the mean value; n=5 pooled flies of 10 replicates, one-way ANOVA with *posthoc* Tukey multiple comparison test, p<0.05 *,p<0.01 ** p<0.001 ****, p<0.0001 ****.

In gene switch alone (actGS>) flies, the presence of RU486 did not significantly affect TAG content, nor the ability of trametinib to reduce TAG levels, on both normal (1x) and high (8x) sugar diets.

On both normal (1x) and high (8x) sugar diets, induction of SREBP transgene expression by RU486 resulted in significant increases in TAG content demonstrating that SREBP overexpression resulted in enhanced lipid synthesis and storage. Trametinib was still able to reduce TAG levels in flies overexpressing SREBP on both normal (1x) and high (8x) sugar diets suggesting that trametinib reduces TAG levels even under conditions of enhanced SREBP-dependent lipid synthesis, (Figure 3.15).

To again examine whether the lifespan extension induced by trametinib depends on reduced lipid synthesis, the survival of SREBP overexpressing flies in response to trametinib was investigated, (Figure 3.16).


Ε

Median Lifespan	actGS>			
RU486	-	-	+	+
Trametinib	-	+	-	+
Normal diet	72.0	81.6***	72.0	81.6***
High sugar diet	39.5	42.0***	37.0	42.0***
Median Lifespan	38396RB			
RU486	-	-	+	+
Trametinib	-	+	-	+
Normal diet	62.9	74.5***	53.5	64.9***
High sugar diet	25.4	37.0***	20.9	29.9***

Figure 3.18: Comparing survival of wild-type, wDah, and SREBP overexpressing, 38396RB, flies fed normal (1x) and high (8x) sugar diets in the presence (+) or absence (-) of trametinib.

Survival analysis of (A-B) wild type, *wDah*, flies, left panel fed a normal diet (A), right panel fed a high sugar diet (B). Survival analysis of (C-D) SREBP overexpressing flies (*38396RB*), left panel (C) fed a normal diet, right panel (D) fed a high sugar diet. Results are plotted as proportion of survivors as a function of time (in days). Median survival times of flies are shown in the table I. Table showing median lifespan, log rank test comparing absence(-) of trametinib to presence(+) of trametinib for both genotypes and diets, p<0.001 ***(Log-Rank test). Black arrow (Day 10) shows the points at which flies were taken for TAG analysis. Red dotted line showing the median lifespan.

As shown previously, for wild-type (*wDah*) flies on both normal (1x) and high (8x) sugar diets, trametinib significantly extended lifespan and this was not affected by the presence RU486 (normal (1x) sugar: +13% median lifespan, $p = 1x10^{-20}$; high (8x) sugar: +6% (absence of RU486)/ +14% (presence of RU486) median lifespan, $p = 1x10^{-8}$) (Figure 3.16A and B).

For *38396RB* flies, in absence of RU486-induced transgene expression, trametinib significantly extended lifespan on normal (1x) and high (8x) sugar diets (normal (1x) sugar: +18% median lifespan, p = 1×10^{-15} ; high (8x) sugar: +46%, p = 1×10^{-23} . Upon transgene expression by RU486, trametinib significantly extended lifespan on both normal (1x) and high (8x) sugar diets (normal (1x) sugar: +21% median lifespan, p = 1×10^{-12} , high (8x) sugar: +43% median lifespan, p = 1×10^{-19}), (Figure 3.16C and D).

Taken together, this suggests that trametinib is still able to extend lifespan in the presence of elevated lipid synthesis induced by SREBP overexpression.

3.4 DISCUSSION

The main aim of this chapter was to determine the metabolic changes that occur in adult *Drosophila* in response to trametinib on both normal and high sugar diets as well as during normal ageing. This was investigated by first establishing the efficacy of trametinib to inhibit RAS/MAPK signalling in response to diet-induced obesity and during ageing. Second, the effects of trametinib on metabolic storage molecules were investigated including how trametinib influences changes in metabolic stores in response to diet-induced obesity and during ageing. Lastly, following the observed metabolic changes due to trametinib treatment, the mechanism by which trametinib elicits its metabolic changes was examined along with the effects of these metabolic changes on trametinib-dependent lifespan extension.

3.4.1 PHENOTYPIC CHARACTERISATION OF FLIES TREATED WITH TRAMETINIB

In this study, it was shown that trametinib inhibited the phosphorylation of ERK on both normal and high sugar diets as well as during ageing, which was used as a readout for the efficacy of trametinib to inhibit MEK. The effects of trametinib on inhibiting ERK phosphorylation seemed conserved between Drosophila and mice as similar effects were observed Banks et al., (2015). where MEK specific inhibitor by а (GSK1120212/Trametinib) was used in genetically obese mice (ob/ob), and was shown to reduce phosphorylation of ERK, (Banks et al., 2015). These results were further verified by Ozaki et al., (2018) using a MEK specific inhibitor similar in its mechanism to trametinib, PD184352, in genetically diabetic mice (db/db), (Ozaki et al., 2018). Additionally, more recently, Urena and colleagues have shown that trametinib treatment decreased phosphorylation of ERK in both fly sexes although to a lesser extent in male flies, (Urena et al., 2024). The extent to which trametinib decreased phosphorylation of ERK on a normal diet in this study and study of Urena et al., (2024) are comparable with a decrease in phosphorylation of ERK by around 50-60% in female flies. Here, it was additionally shown that trametinib is efficacious in inhibiting the RAS/MAPK pathway in response to diet-induced obesity as well as during ageing.

The Inhibition of ERK phosphorylation by trametinib was also shown to impact on the lipid stores of *Drosophila*, which was previously investigated, however mainly in mice and mammalian cells, (Bost *et al.*, 2005; Andersson *et al.*, 2006; Ozaki *et al.*, 2018). Trametinib treatment decreased the TAG content and lipid droplet size of flies fed either a normal or high sugar diet as well as during ageing. Previous studies have shown that inhibition of the RAS/MAPK pathway can influence lipid metabolism and lipid droplet formation. Bost *et al.*, (2005) created an ERK1 homozygous knockout mice and have shown that these mice have impaired adipogenesis, with reduced markers of adipocyte

differentiation such as PPAR-y, adiponectin and leptin, (Bost et al., 2005). Additionally, the total amount of TAG in ERK1 mutant mice cells was reduced by 80% compared to wild-type cells which demonstrates that ERK1 was required for adjpocyte differentiation and plays a critical role in adipogenesis, (Bost et al., 2005). Furthermore, Andersson and colleagues have shown that ERK2 in mammalian cells regulates cytosolic lipid droplet formation, (Andersson et al., 2006). Transfection of cells with ERK2 increased the formation of LDs in intact cells by around 3-fold but also increased the TAG content of cells (40%), whereas the knockdown of ERK2 by siRNA led to a reduction in the amount of lipid droplets and TAG content of cells, (Andersson et al., 2006). Trametinib did not have a significant effect on TAG content on normal sugar diet but reduced TAG content by around 60% on high sugar diet feeding. This reduction in TAG content was associated with a reduction in the size of lipid droplets within the fat body. Interestingly, lipid droplet size was also reduced by around 40% on normal sugar and an even greater reduction in TAG of around 60% was observed on high sugar diet feeding. The differences in magnitude between TAG content and lipid droplet size of flies fed high sugar diet in response to trametinib could be attributed to a specific effect of trametinib on lipid droplets of the fat body, whereas the biochemical assay to measure TAG content used whole fly extracts. Nevertheless, these results demonstrate the trametinib prevented TAG and lipid droplet size increase in response to diet-induced obesity and ageing.

A reduction in metabolic TAG stores could arise through differences in feeding behaviour. However, while flies fed with a high sugar diet showed a lower proportion of feeding, this was not further affected by trametinib treatment. The effects of high sugar diet on feeding behaviour have been previously reported but with mixed results. May and colleagues have shown that sucrose diet of 20% showed an increase in feeding behaviour by Fly-to-Liquid-Food-Interaction Counter (FLIC), (May *et al.*, 2019). However, a more recent study by van Dam *et al.*, (2020), showed that a 20% sucrose diet feeding resulted in a decrease in feeding behaviour, in flies of a comparable age to May *et al.*, (2019), measured by proboscis extension assay, (van Dam *et al.*, 2020). Here, a high sucrose diet of 40% was used and showed a decrease in feeding behaviour of around 20%, measured by proboscis extension assay which was comparable to study carried out by van Dam *et al.*, (2020). The difference in feeding behaviour observed by May *et al.*, (2019) could be due to use of liquid food compared to solid food, and a difference in methodology.

Although high sugar diet feeding decreased feeding behaviour in flies in this study, trametinib did not further affect feeding, suggesting that the observed differences in TAG storage of high sugar diet fed flies treated with trametinib could not be explained by a difference in feeding behaviour. Furthermore, trametinib did not affect glycogen storage under high sugar feeding suggesting that the effects of trametinib are specific to lipid metabolism.

This set out the precedent to explore the potential mechanism by which trametinib reduces TAG storage. It was hypothesized that trametinib may exert its effects on lipid storage by either enhancing lipolysis or inhibiting lipid synthesis. To explore these potential mechanisms, genetic manipulation of these two processes was undertaken and their effects on trametinib-dependent lipid storage and lifespan were examined.

3.4.2 POTENTIAL LIPID LOWERING MECHANISMS OF TRAMETINIB

Lipid droplets are present in all developmental stages of *Drosophila* and represent the major TAG storage form in *Drosophila*, (Parisi *et al.*, 2011). Lipolysis is the enzymatic hydrolysis of TAG into glycerol and fatty acids, a reaction which provides a major energy source in situations of energy depletion or increased energy demand, and therefore represents a crucial determinant of energy homeostasis, (Zechner, 2015). A key enzyme involved in lipolysis in *Drosophila* is Brummer (Bmm), an ortholog of mammalian ATGL, (Gronke *et al.*, 2005). Previously, it has been shown that loss of Bmm activity in flies resulted in increased lipid storage, specifically increased lipid droplet size due to *bmm* mutation, as well as impaired TAG mobilization, (Gronke *et al.*, 2005). These effects of *bmm* mutation made it a good candidate to test whether trametinib exerts its effects on lipid storage and lifespan by enhancing lipolysis.

In this study, it was shown that trametinib decreased the TAG content of *bmm* mutant flies on both normal and high sugar diets. Gronke and colleagues have shown that bmm mutant flies have increased TAG storage, as well as shortened lifespan on control diet, which was replicated in this study, (Gronke et al., 2005). Additionally, trametinib increased the median lifespan of *bmm* mutant flies to a similar extent as in wild-type flies suggesting that trametinib is still able to reduce lipid storage and extend lifespan even when lipolysis is impaired in *bmm* mutants. The effects of trametinib in reducing TAG content were greater in *bmm* mutant flies on normal sugar diet compared to control, however the extent to which TAG content was reduced on high sugar diet in *bmm* mutant flies was reduced compared to control, from 60% to 40% reduction. This suggests that the ability of trametinib to lower TAG may depend in part on lipolysis at least on high sugar diet, however independently of *bmm*-mediated lipolysis. An alternative lipolytic system exists in Drosophila which depends on Akh signalling and controls the expression of one or more, currently, uncharacterized TAG lipases, which complement Bmm function, (Gronke et al., 2007), thereby suggesting there may be systematic redundancy, (Heier and Kuhnlein, 2018).

In terms of lipogenesis, the main enzyme involved in *de novo* lipid synthesis is acetyl-CoA carboxylase (ACC) which catalyses the formation of malonyl-CoA from acetyl-CoA, (Barber *et al.*, 2005). *Drosophila* possess one *ACC* gene, which is ubiquitously expressed with highest levels in the fat body cells, (Parvy *et al.*, 2012). Additionally, ACC seems to contribute to lipid synthesis mainly in the fat body and the midgut, (Parvy *et al.*, 2012).

In this study, ubiquitous overexpression of *ACC* in adult flies resulted in both elevated TAG storage and reduced lifespan on a normal diet suggesting a pathological role for ACC overexpression under control conditions. Furthermore, these effects of *ACC* overexpression were enhanced on high sugar diet. Previous studies have shown that muscle-specific knockdown of *ACC* prevents lifespan extension by dietary restriction, (Katewa *et al.*, 2012) whereas unrestricted lipogenesis via *ACC* overexpression was shown to be pathogenic in a *Drosophila* model of cardiac dysfunction, (Lim *et al.*, 2011). Unexpectedly, here it was shown that trametinib still extended lifespan in *ACC* overexpressing flies, used as a proxy for enhanced lipid synthesis, suggesting that trametinib still extends lifespan under conditions of elevated *ACC*-dependent lipid synthesis. Furthermore, trametinib was still able to lower TAG storage in flies overexpressing *ACC*, suggesting that enhanced lipogenesis is insufficient to block the effects of trametinib on lowering lipid storage. However, it is possible that the levels of *ACC* overexpression were not sufficient to block the effects on trametinib on lipid storage and lifespan, (Wang *et al.*, 2022).

This led to an alternative approach to modulate lipid synthesis through overexpression of a key upstream regulator of lipid synthesis, the transcriptional regulator of de novo lipogenesis, SREBP. SREBP has an evolutionarily conserved function in controlling lipogenic gene expression, (Walker et al., 2011). Previously, it was shown that srebp null mutants do not contain any fatty acids and die as second-instar larvae unless supplemented with dietary fatty acids, suggesting that srebp is crucial for development, (Kunte et al., 2006). Additionally, ectopic expression or RNAi mediated knockdown of srebp has been shown to reciprocally affect lipid stores in the midgut and body TAG levels, (Son et al., 2014) suggesting that SREBP overexpression may produce similar effects to ACC overexpression on TAG storage. As trametinib extended lifespan and lowered TAG levels in ACC overexpressing flies, it was expected that trametinib would produce similar effects in srebp overexpressing flies. This was indeed observed in the results presented here. However, the extent to which trametinib lowered TAG content in srebp overexpressing flies was greater than in ACC overexpressing flies. It has been previously shown that Drosophila SREBP null mutants are fatty acid auxotrophic and die as second-instar larvae unless supplemented with dietary fatty acids, showing that SREBP is crucial for fly development, (Kunte et al., 2006). Furthermore, Drosophila mutants lacking the components of the SREBP-processing machinery (SCAP or S2P) showed a lean fly phenotype suggesting that SREBP function is required for the organismal buildup of TAG stores, (Matthews et al., 2010). In this study, srebp overexpression was shown to increase the TAG content of flies fed normal and high sugar diets, a phenotype which was rescued in presence of trametinib. SREBP, being the main regulator of lipogenesis, suggests that trametinib lipid lowering properties did not depend on inhibiting lipid synthesis. Additionally, it was shown that lipid synthesis was dispensable for trametinib effects on lifespan extension.

Taken together, the data presented in this chapter shows that trametinib impacts on lipid metabolism to lower TAG levels in response to high sugar feeding, but its effects are not as simple as enhancing lipolysis or inhibiting lipid synthesis. A potential explanation may be that trametinib acts in an independent manner to lipid metabolism and perhaps instead regulates the transport and utilisation of nutrients, or instead affects the composition of lipids leading to a 'healthier' fly lipidome which promotes lifespan extension.

Consequently, next, to investigate whether trametinib affects the global composition of lipids in flies in response to normal and high sugar feeding, liquid-chromatography coupled tandem mass spectrometry (LC-MS/MS) was used to examine changes in the fly lipidome in response to trametinib treatment.

Chapter 4

4.1 INTRODUCTION

4.1.1 THE USE OF MASS SPECTROMETRY TO STUDY THE LIPIDOME

Although there is an extensive understanding of lipids and their structure, a full definition of the function of the eukaryotic lipidome in response to high sugar diet as well as trametinib remains vague. Most recently, advances in the analysis of lipid characteristics in the context of obesity and diabetes has led to a better understanding of their role in cellular physiology and pathology. This emphasizes the importance for the need for an integrated study of cellular lipids utilising the lipidomics approach, (Hu *et al.*, 2018).

Lipidomics is a branch of metabolomics based on the large-scale study of lipids. The aim of such analyses are to address the identification and quantification of lipid species in each sample, as well as their local and global distribution, together with their relevant signalling role and the network of lipids with which they interact in a biological system, (Postle, 2012). This information can then contribute to the integration of all the branches of metabolomics to better elucidate cellular metabolism in events of pathology (Fonteh *et al.*, 2006; Suvitaival *et al.*, 2018). Recently, advances in chromatography and mass spectrometry (MS) technologies have enabled scientists to comprehensively profile lipids in biological samples, (Jurowski *et al.*, 2017). This has made MS an exceptionally valuable qualitative and quantitative technique, with increasing relevance within metabolomics research in recent years. A typical MS-based lipidomics experiment consists of sample collection, lipid extraction, lipid separation, MS data acquisition, post-acquisition data processing and clarification of physiological relevance, (Hu *et al.*, 2018).

4.1.1.1 LIPID EXTRACTION METHODS

Extraction of lipids has been accomplished with a variety of methods available for sample preparation for MS-based lipidomics. Liquid liquid extraction or Folch method (see Figure 5.1) has been the predominant extraction method, owing to its applicability to a wide range of biological samples, the simplicity of extraction and the ease with which contaminants can be separated (Folch *et al.*, 1957; Blight and Dyer, 1959). Alternative lipid extraction methods using methyl tert.butyl ether (MTBE) have been developed by Matyash *et al.*, 2008, which uses MTBE, methanol and water. The MTBE lipid extraction method offers practical advantages compared to the Folch method, as the lipid containing organic phase forms the upper-most layer during phase separation due to the low density

of MTBE, thus simplifying the sample collection and minimising contamination, (Matyash *et al.*, 2008). Furthermore, the MTBE lipid extraction method improves the recovery of most lipid classes, (Qu *et al.*, 2014). A more recent lipid extraction method was developed by Alshehry and colleagues, which involved the use of 1-butanol and methanol in 1:1 ratio. This leads to a single phase partitioning, unlike the MTBE and Folch methods, which rely on two phase partitioning. This approach also compares favourably to commonly used current methodologies, which rely on extraction in a chloroform:methanol solution, often incompatible with downstream analyses methods, (Alshehry *et al.*, 2015). Alshehry and colleagues offered a simple single layer extraction method using solvents compatible with downstream processing methods while maintaining a strong correlation of all lipid measures compared to the established chloroform:methanol extraction method, (Alshehry *et al.*, 2015). Lipid extraction methods are summarised in Figure 4.1.



Figure 4.1 A schematic outlining the differences between the lipid extraction methods in the analytical pipeline.

In the Folch method, the lipid containing organic phase (O) lies beneath a non-extractible residue (NR) and must be collected by pasting a micropipette through the aqueous (A) and NR. The Matyash MTBE method simplifies the extraction method by having the lipid collected in the upper phase. The Alshehry method extraction collects all lipids within a single phase partition with the NR being pelleted at the bottom of the tube.

4.1.1.2 MASS SPECTROMETRY METHODS FOR LIPID ANALYSES

As the lipid extraction methodologies have been improving, so to have the mass spectrometric approaches to their analysis. While lipid samples can be analysed by direction infusion "shotgun" MS, (Wang et al., 2016), it is more common to see a coupled chromatographic separation method, termed liquid chromatography mass spectrometry, or LC-MS. The use of chromatography enables an additional dimension of separation, allowing isomeric species with identical m/z values to be distinguished on the basis of their retention on a column and is used for the analysis of mixed biological samples, (Fabritius et al., 2021). In this study, lipid samples were separated by reverse-phase (RP) high performance liquid chromatography (HPLC). RP-HPLC (and the higher pressure variant, ultra-high performance liquid chromatography, UPLC) is a commonly used method for the separation and analysis of phospholipids and neutral lipids, allowing the efficient separation of species prior to MS analysis, (Harvey et al., 2023, Bielawski et al., 2021). RP-HPLC usually consists of a polar mobile phase and a non-polar stationary phase, separating components based upon the relative adsorption of the non-polar functional groups of the analytes to the hydrophobic surface of the stationary phase, (Moldoveanu et al., 2013; Zuvela et al., 2019). Aqueous initial conditions are used for the analyte elution on a RP column, with a gradient established over time to gradually progress to a higher percentage organic solution. This allows lipid separation according to the individual hydrophobicities of their fatty acyl chains, with their elution order being associated with the carbon chain length and the number of double bonds. Alternatively, lipids can also be separated according to their head groups in normal-phase chromatography allowing lipids with like polar head groups to elute at the same time, (Bang et al., 2012). Normal phase columns have a polar stationary phase, in contrast to the non-polar nature of reverse phase columns. As the analyte molecules are separated in the chromatography column, they flow into the MS where the analyte is analysed.

All mass spectrometry typically comprises three general principles: sample ionisation, ion separation and ion detection. One of the common forms of ionisation is electrospray ionisation (ESI), which is a relatively soft-ionisation technique (more likely to allow species to remain intact) in which the samples are dissolved in a volatile solvent and pumped through a fine charged capillary held at a high voltage, resulting in a spray of analyte-containing solution in tiny volatile droplets. In lipidomics LC-MS, this spray is the output from the chromatographic separation, usually comprising a mixture of aqueous with volatile polar organic solvent (most typically methanol and acetonitrile) with a non-polar organic solvent such as hexane or isopropanol. The constant stream of sample solution is directed through the capillary tube, generating a mist of highly charged droplets, with the same polarity as the capillary voltage through which the solution passes, (Wilm, 2011). The transfer of ions from this mist of droplets into the gas phase (ionisation) then

involves the solvent evaporation, known as desolvation, followed by ion ejection from the droplets as they shrink. The expelled ions are then attracted to the opposite charge of the sampling skimmer cone and are accelerated into the mass analyser, (Ho *et al.,* 2023).

Ion separation is carried out by mass analysers. Like ion sources, there are many different types applicable for different applications and budgets. One of the most common is the quadrupole mass filter (Q), a low mass resolution analyser typically used in instruments as a scanning filter, allowing accurate detection of analyte based on a particular m/z for MS2 analysis, (Li et al., 2021). For quantitative applications of mass spectrometry, triple quadrupoles (QQQ) are commonly used, allowing a targeted approach to analyte detection. Quadrupole analysers are also often coupled with "time of flight" (ToF) or ion trap instruments, often functioning to isolate a given set of m/z ranges prior to the second stage analyser, (Saleem et al., 2023). Ion trap mass analysers act as ion storage devices, in which volatile ions of a variable range of m/z values can be confined under stable electrode potential conditions, allowing capture of specific ions, (Patil et al., 2017). In the ion trap mass analyser, a specific voltage is applied which affects the trajectory of trapped ions, allowing the ions to leave the trapping field in order of the defined m/z, (Raffaelli and Saba, 2023). ToF mass analysers are high-resolution mass spectrometers based on a flight tube that is used to separate ions according to their mass and allow detection of relative abundance of the analyte in a mixed sample. In the flight tube, the origin of a beam of ions is located a fixed distance from the detector, the ions are accelerated towards the detector by an electric field of equivalent kinetic energy, (Volny, 2020). The velocity of the travelling ions is dependent on their mass, allowing separation of analyte according to the m/z ratio. For example, ions of lower mass but the same charge would travel down the flight tube faster, whereas ions of higher mass but the same charge would travel slower thus would be detected later, (Boesl, 2017). The ToF technology has been improved using reflectrons, otherwise known as ion mirrors, that reverse the trajectory of ions for the purpose of minimising the spread of kinetic energy of ions with the same m/zratio, which improves the resolution of ToF mass spectrometers, (Scherer et al., 2006).

As separated ions reach the detector, located at the end of the flight tube, consisting of a charged plate, an electric current is generated which can be computationally monitored and converted to a mass spectrum. The mass spectrum contains information of the m/z values of the ions and their relative abundances, (Tamura *et al.*, 2021).

Lipid ions can form by protonation or deprotonation, or by forming non-covalent adducts with positively (such as Na+, K+, Li+ or NH4+) or negatively charged ions (such as formates, or acetates) thereby picking up a charge. Most lipid ions are singly-charged with some having the capacity to accept two charges, depending on the chemical properties and the propensity of an individual lipid to gain or lose charge in each environment, (Gonzalez-Riano *et al.,* 2021). Adduct formation can be altered and

controlled dependant on the additives in the solvents or LC mobile phase to enhance adduct ion formation, (Kostiainen et al., 2009). Lipids such as fatty acids (FA), phosphatidic acid (PA), phosphatidylserine (PS), phosphatidylglycerol (PG), phosphatidylinositol (PI) and cardiolipins (CL), generally carry a net positive charge at physiological pH, which makes them easier to identify in the positive ion mode as [Lipid+H]⁺ adducts. However, lipids such as phosphatidylethanolamine (PE), ceramides and eicosanoids carry a net negative charge at alkaline pH, which aids in their detection in the negative ion mode as [Lipid-H]⁻ species, although they can also be detected in the positive ion mode as [Lipid+H]⁺ adducts, (Buyukpamukcu et al., 2007). Lipid species that contain nitrogen atoms, such as phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), sphingomyelin (SM), ceramides (Cer) and cholesterol (Chol) are mostly detected as protonated [M+H]⁺ species. Lipids that do not contain nitrogen such as PI,PG,CL, TAG and DAG, can be detected as [M+NH₄]⁺ adducts when ammonium acetate or formate are used in the mobile phase during the LC step, (Rush and van Breemen, 2017; Warren, 2018). Lastly, lipids that are commonly detected in positive ion mode can also be detected in the negative ion mode at a lower intensity, as [M+COO]⁻ adducts, (Gonzalez-Riano et al., 2021).

4.1.1.3 METHODS OF DATA ACQUISITION IN LIPIDOMICS

The acquisition of data in lipidomics using mass spectrometers can be targeted or untargeted. Targeted lipidomics involves the analysis of a defined group of chemically characterised analytes and can be used to solve particular biological questions, (Roberts et al., 2012). On the other hand, untargeted lipidomics is used as a comprehensive analysis of all measurable analytes in a sample mixture, aiming to identify and quantify all lipids present in a sample, and is often used to obtain a global lipid profile of the system of interest, (Hu and Zhang, 2018). The two main approaches utilised in lipidomics are data dependent acquisition (DDA) and data independent acquisition (DIA), (Rudt et al., 2023). The DDA method involves selecting a fixed number of precursor ions of interest within a specified m/z range that are then analysed by MS/MS. Most commonly, the mass spectrometer selects the most intense precursor ions of interest in the first analyser, which are then fragmented to be analysed. The DDA method offers a precise coverage of identified lipids in an unknown sample, as well as high-resolution MS/MS spectra for precursor ions, allowing more accurate quantification, (Schwudke et al., 2007). However, DDA offers relatively poor detection of low abundance precursor ions, favouring more intense species, therefore providing relatively limited coverage. On the other hand, the DIA method (specifically that used by the Select Series Cyclic instrument that features in this study) of untargeted MS/MS analyses all ions within a selected m/z range in a given sample. The main focus is on a narrow m/z range of precursor ions in each cycle and as the cycles commence, the mass range is moved across the entire m/z range in order to fragment all detected precursor ions, allowing for MS/MS data to be collected. The DIA method offers high resolution MS/MS spectra for all high and low abundance precursor ions, (Alcoriza-Balaguer *et al.*, 2019).

4.1.1.4 LIPIDOMICS METHODS USED IN THIS STUDY

For the current study, electrospray ionisation (ESI) was utilised, since it is one of the few approaches able to overcome the issue of labile macromolecules such as lipids fragmenting during the ionisation step. The soft ionisation of ESI offers the advantage of conserving the molecular ion structure, (Hu and Zhang, 2018). Minimal structural information is obtained from the MS spectra about the elemental composition from the peak corresponding to the molecular ion of each analyte, but subsequent fragmentation of these species in the MS2 mode is employed to generate informative fragments. Each peak observed in the ESI analysis could correspond to a specific lipid in a sample, but a lipid cannot be identified by its mass alone, with multiple isomeric structures possessing the same mass. These species can however be discriminated based upon their unique and diagnostic fragmentation patterns. Depending on the resolution and mass accuracy, *isobaric* species (same nominal mass, but different accurate mass) can be discriminated, as they will have slightly different masses owing to different elemental compositions, (Chen *et al.*, 2022).

Isomeric lipid species are those that have identical m/z ratio but may differ in chemical and physical properties and cannot be resolved by MS1 alone, but can be resolved by MS2 and MS3 if the precursors are selectively isolated. The complexity in the detection of lipid species reflects the high diversity of the natural lipidome with high dynamic ranges of lipid concentrations and a range of chemical properties, (Zullig *et al.*, 2021).

Mass spectrometry for lipidomics can be carried out on a variety of mass spectrometers. In this study, lipid samples were analysed using the Select Series Cyclic ion mobility (IMS) mass spectrometer (Waters Ltd., Manchester, UK) with an instrument geometry of a quadrupole mass filter (Q) coupled to a trap cell, a circular path of ion mobility (cIM), a transfer region and an extended time-of-flight (TOF) mass analyser (Figure 4.2).



Figure 4.2 Representative schematic of the Select Series Cyclic ion mobility system showing the Q-cIM-ToF machine geometry, (adapted from Giles *et al.,* 2019).

A DIA based method was used to define the global lipid profile of flies fed either a normal or high sugar diet in both the presence and absence of trametinib. Despite the current knowledge about the effects of high sugar feeding on lipid toxicity in the *Drosophila* model, there is a knowledge gap corresponding to the effects of trametinib on the lipidome, especially in response to high sugar feeding, which emphasizes the need for further research and investigation in this area.

4.1.2 EFFECTS OF HIGH SUGAR DIET FEEDING ON THE LIPIDOME

It was shown earlier, using a colorimetric TAG assay, that trametinib was able to prevent TAG accumulation in response to high sugar diet and ageing. However, a few studies have suggested that the colorimetric TAG assay approach does not provide an accurate assessment of stored fat in insects, (Al-Anzi and Zinn, 2010, Williams *et al.*, 2011), although others have shown that both TLC and colorimetric assays provide similar results, (Hildebrandt *et al.*, 2011). Therefore, it was imperative that a more sensitive method such as mass spectrometry was used in this study to investigate the effects of trametinib on the fly lipidome. It is also worth noting that colorimetric assays that use lipoprotein lipase release glycerol from mono- and diacylglycerides in addition to TAGs, making it crucial that any conclusion regarding particular forms of glycerolipids are

validated by other methodologies, (Van Veldhoven *et al.*, 1997). This opened up the opportunity to use the emerging field of lipidomics using mass spectrometry, to simultaneously analyse hundreds of lipids in trametinib treated flies in response to diet-induced obesity in this study.

Mass spectrometry-based lipidomics have been successfully employed in studies using Drosophila. Initial steps into using mass spectrometry for lipidomics based research in insects was employed by Hammad and colleagues, who have used liquid chromatography coupled tandem mass spectrometry (LC-MS/MS) to profile 74 lipids from whole adult Drosophila, enabling quantification of absolute abundance of 28 phosphatidylethanolamine (PE) and phosphatidylcholine (PC) species, (Hammad et al., 2011). Additionally, several triglyceride (TAG) species were found in the extracts. Since then, the more recent development of high mass resolution shotgun lipidomics was used to identify and quantify lipid species in individual organs of Drosophila L3 larvae including gut, brain, fat body, salivary glands, and wing discs, in response to a range of diets, (Carvalho et al., 2012). The analyses by Carvalho and colleagues demonstrated that the Drosophila lipidome is predominantly dominated by PE species rather than PC species, as compared to the more PC-centric mammalian lipidome, (Carvalho et al., 2012). Additionally, it was revealed that there are tissue specific lipid composition differences. For example, ether phospholipids were only present in the brain while hexosyl ceramides with hydroxylated fatty acid moieties were only present in the gut. Comparable results were present in lipid extracts of mammalian tissues suggesting a tissue specific conservation, (Salem et al., 2001). Also, Drosophila brain phospholipids and neutral lipids contained longer and more unsaturated fatty acids than those in other tissues, showing clear tissue specific differences in lipid composition, (Carvalho et al., 2012).

4.2 AIMS AND OBJECTIVES

In this chapter, the main aim was to characterize the lipid profile of flies fed normal and high sugar diets in both the absence and presence of trametinib. This would determine the effects of trametinib on the global lipid profile.

The main hypothesis was that trametinib may exert its phenotypic effects and lifespan extension at least in part through preventing deleterious alterations in lipid composition. Additionally, a secondary hypothesis was that trametinib prevents certain sugar-induced deleterious effects of high sugar feeding, which could be leading to the observed differences in lifespan and lipid phenotype of trametinib treated flies.

To achieve this, lipids were extracted from 10-day old adult female *Drosophila* and liquid chromatography coupled lipid mass spectrometry was then performed to analyse the abundance of identified lipid species.

4.3.1 NORMALISATION OF THE LIPID MASS SPECTROMETRY DATA

The first step for analysing the lipid mass spectrometry data from *Drosophila* extracts was normalization. Lipid intensities can be scaled by the minimum and maximum values; however, this method is sensitive to outliers (Amagata, 2023), . A better approach to scaling lipidomics data involves scaling by the standard deviation or by the root of standard deviation (Pareto scaling), (Eriksson, 1999). The data is then centered which is based on subtracting the mean or median intensity from all values. Lastly, log transformation is usually applied to produce a scaling-like effect making features more comparable. Following this method the lipidomics data was normalized for both positive and negative ion mode as shown in Figure 4.3 and Figure 4.4, respectively.

For both positive and negative ion mode data, Pareto scaling and normalization resulted in a normal distribution curve of the normalized intensity for all features, which allowed for reliable subsequent analyses. Pareto scaling was used as a common data preprocessing method in lipidomic. Pareto scaling centers each variable (lipid) by subtracting the mean of that variable across all samples (mean-centering). After meancentering, each variable is scaled by dividing it by the square root of its standard deviation. The reason for using Pareto scaling is to balance the signal variation in the lipidomics dataset which often contains lipids of varying abundances. This scaling method reduces the dominance of highly variable lipids while preserving meaningful variation, preventing certain lipids from dominating the analysis. Additionally, Pareto scaling minimizes noise amplification by not overemphasizing small differences which is particularly important for lipidomics where abundance of trace lipid measurements can be significant.





The intensity values of each identified feature were divided by the square root of standard deviation shown as before scaling (Intensity) and after scaling (Normalised Intensity).





The intensity values of each identified feature were divided by the square root of standard deviation shown as before scaling (Intensity) and after scaling (Normalised Intensity).

4.3.2 ANALYSIS OF DIFFERENTIALLY ABUNDANT LIPIDS

The changes in abundance between the treatment conditions were then analysed using Metaboanalyst 3.0 using raw abundances for each lipid species identified in the Progenesis QI software. Progenesis QI is a powerful software that allows for rapid, objective, and reliable discovery and identification of compounds of interest using multigroup experimental designs with a capability to handle large sample sets, (Waters, UK).

Initial analysis of the differentially abundant lipids was carried out using principal component analysis (PCA) of transformed lipidomics data. Principal component analysis (PCA) of the transformed lipidomics abundance data in both positive and negative ion modes is shown in Figure 4.5.



Figure 4.5: Principal component analysis (PCA) for flies fed a high sugar diet in response to trametinib in positive and negative ion mode.

Scatter plot of Principal component analysis (PCA) of transformed count data from Metaboanalyst of flies fed normal (1x) and high (8x) sugar diets in absence (T-) and presence (T+) of trametinib in both positive (A) and negative (B) ion modes. Samples are clustered according to their diet (PC1) and trametinib treatment (PC2) (n=4 independent biological replicates).

In the positive ion mode, the PCA plot revealed that 86% of total variance amongst the 4 treatment groups was represented by two principal components. Samples were separated along the first principal component showing 49% of the variation between samples was explained by the sugar diet that was fed to the flies. Samples were also separated along the second principal component, with 37% of the variation between samples caused by the trametinib treatment.

In the negative ion mode, the PCA plot revealed that 69% of total variation amongst the 4 treatment groups was represented by two principal components. Sample were

separated along the first principal component showing 45% of the variation between samples was explained by the sugar diet that was fed to flies. Samples were also separated along the second principal component, with 24% of the variation between samples caused by the trametinib treatment.

In the positive ion mode, the high sugar fed trametinib treated flies did not group with normal sugar fed control flies, but instead grouped separately from the other treatments suggesting that trametinib did not revert the lipidome to its control state, instead eliciting a separate metabolic response. The effect of trametinib on separation in the negative ion mode was less clear compared to positive ion mode, yet there was still clear sugar-dependent separation along the PC1 in negative ion mode.

To further dissect which lipid classes were differentially abundant in flies fed a high sugar diet in response to trametinib, the normalized abundance of identified lipid classes in positive and negative ion modes were investigated shown in Figure 4.6.

In the positive ion mode, on normal (1x) sugar diet, trametinib significantly increased the abundance of PC lipid species with no significant change in PE, TAG, DAG or Cer lipid species abundances.

High sugar diet significantly increased the abundance of PE, DAG and Cer lipid species. Treatment with trametinib on high sugar diet did not have any significant effect on the abundance of lipid species identified in the positive ion mode, although there was a trend for reduced TAG and DAG species.

In the negative ion mode, there were no significant changes in abundance of any lipid classes in response to either high sugar diet or trametinib treatment. Although there was a trend for PA, PS, PG PI, PE and SM lipid classes to increase in response to high sugar diet which were further increased in the presence of trametinib, these effects were not statistically significant.



Figure 4.6: Lipid class composition of identified lipids in positive and negative ion modes of flies fed high sugar diet in response to trametinib.

Lipid samples were analysed by LC-MS/MS in ESI positive (A) and negative (B) ion modes in flies fed normal (1x) and high (8x) sugar diets in absence (T-) and presence (T+) of trametinib. In positive ion mode, phosphatidylcholine (PC), phosphatidylethanolamine (PE), triglyceride (TAG), diglyceride (DAG) and ceramide (Cer) were identified. In negative ion mode, phosphatidic acid (PA), phosphatidylserine (PS), phosphatidylglycerol (PG), phosphatidylinositol (PI), PE and sphingomyelin (SM) were identified. The relative normalised abundance was calculated as a percentage (%) of total lipid class abundance. Data are presented as a mean of all replicates, (n=4, p<0.01 **. Two-way ANOVA with Tukey's multiple comparison test).

To further analyse the differences in lipid abundances in high sugar diet fed flies in response to trametinib, enrichment analysis was performed on lipids differentially abundant in the positive (89 lipids) and negative (51 lipids) ion modes based on chemical structures similarity, using Metaboanalyst, the false discovery rate was controlled using Benjamini-Hochberg procedure. The results of enrichment analysis are displayed in Figures 4.7 and Figure 4.8 for positive and negative ion modes, respectively.





В	Enrichment FDR	Lipid Class
	9.32E-21	Diacylglycerol
	7.02E-17	Glycerophosphocholines
	7.02E-17	Glycerophosphoethanolamines
	1.70E-08	Triacylglycerol

Figure 4.7: Enrichment analysis of differentially abundant lipids in positive ion mode.

(A) Lipid enrichment analysis of the lipids found to be differentially abundant in the positive ion mode, showing the enrichment ratio based on the degree to which the lipid in the lipid class falls into a specific category (generalised linear model). (B) Each enriched lipid class shows the enrichment false discovery rate (FDR) correction (Significance of the enrichment score adjusted for false positive) with a cut off of 0.05, and lipid class showing the lipid class for which the lipid likely falls into based on chemical structure. This list is not exhaustive and shows the overrepresented categories for specific classes.

Α

Metabolite Sets Enrichment Overview



R	Enrichment FDR	Lipid Class
U	6.57E-59	Glycerophosphoethanolamines
	3.57E-28	Glycerophosphoserines
	2.97E-18	Glycerophosphoinositols
	2.98E-17	Glycerophosphoglycerols
	4.83E-10	Glycerophosphates

Figure 4.8: Enrichment analysis of differentially abundant lipids in negative ion mode.

(A) Lipid enrichment analysis of the lipids found to be differentially abundant in the negative ion mode, showing the enrichment ratio based on the degree to which the lipid in the lipid class falls into a specific category (generalised linear model). (B) Each enriched lipid class shows the enrichment false discovery rate (FDR) correction (Significance of the enrichment score adjusted for false positive) with a cut off of 0.05, and lipid class showing the lipid class for which the lipid likely falls into based on chemical structure. This list is not exhaustive and shows the over-represented categories for specific classes.

Enrichment analysis of the differentially abundant lipids in the positive ion mode included enriched lipid classes such as diacylglycerol, glycerolphosphocholines, glycerophosphoethanolamines and triacylglycerol. It was encouraging to observe enrichment in triacylglycerol lipid class as this was consistent with earlier findings demonstrating quantitative differences in TAGs using a biochemical assay.

Enrichment analysis of the differentially abundant lipids in the negative ion mode included enriched lipid classes such as glycerophosphoethanolamines, glycerolphoserines, glycerolphoinositols, glycerolphoglyceroles, and glycerolphosphates. Interestingly, this demonstrated that a range of additional lipid classes other than TAGs were also differentially abundant in response to trametinib treatment on a high sugar diet.

To explore this idea further and identify sets of lipid species which were differentially abundant in response to high sugar diet feeding and trametinib treatment heatmaps of differentially abundant lipids in both positive and negative ion modes were generated, as shown in Figure 4.9 and 4.10, respectively.



Figure 4.9: Heatmap of differentially abundant lipid species in flies fed high sugar diet in response to trametinib in the positive ion mode.

The heat map produced using the positive ion mode data of flies fed high sugar diet in response to trametinib. Heat map of differential lipids found in the positive ion mode. Data underwent variance scaling utilised for heat map plot. The blue colour represents the trend of increase, orange represents a decrease trend, (n=4 independent biological replicates). Red star show a significant decrease in the abundance, (p<0.05). Green star show a significant increase in the abundance, (p<0.05).n=4, Two-way ANOVA with Tukey's multiple comparison test.



Figure 4.10: Heatmap of differentially abundant lipid species in flies fed high sugar diet in response to trametinib in the negative ion mode.

The heat map produced using the negative ion mode data of flies fed high sugar diet in response to trametinib. Heat map of differential lipids found in the negative ion mode. Data underwent variance scaling utilised for heat map plot. The blue colour represents the trend of increase, orange represents a decrease trend, (n=4 independent biological replicates). Red star show a significant decrease in the abundance, (p<0.05). Green star show a significant increase in the abundance, (p<0.05). n=4, Two-way ANOVA with Tukey's multiple comparison test.

In the positive ion mode, trametinib significantly decreased the abundance in a range of TAG species ranging from TG(45-54), on both normal and high sugar diets. Surprisingly, high sugar diet itself did not significantly increase the abundance of any TAG species in contrast to earlier data in which high sugar diet increased TAG levels and lipid droplet size.

In the negative ion mode, trametinib significantly increased the abundance of a range of phospholipid species such as PA, PS, PG, PI, PE and SM species on both normal and high sugar diets. On normal sugar diet, trametinib also decreased the abundance of PG(34:1) PE(34:1) species. High sugar diet itself increased the abundance of phospholipid species such as PA, PS, PG, PI, PE and SM species while in the presence

4.4 DISCUSSION

The aim of this chapter was to characterize the effects of trametinib on global lipid composition in flies fed with a high sugar diet using advanced mass spectrometry equipment and methodologies. To this end, the work reported in this chapter aimed to gain insights into changes in lipid species that may explain the phenotypic effects trametinib on high sugar diet.

The key findings of this chapter were that trametinib decreased the abundance of a range of TAG species on flies fed with both normal and high sugar diets while increasing the abundance of a range of phospholipid species.

The analysis of normalized abundance of lipid species in positive and negative ion modes has shown that high sugar diet significantly increased the abundance of DAGs and ceramides. This is consistent with previous findings by Tuthill et al., 2020 who also observed increased DAGs and a trend to increased ceramides on high sugar diet. However, although the normalized abundance of TAGs in response to high sugar diet showed an increased trend, this was not statistically significant. Tuthill et al., 2020 observed that the abundance of even-chained TAGs increased whilst odd-chained TAGs decreased in response to high sugar diet so combining even- and odd-chained TAGs for analysis perhaps explains why an overall change in abundance was not apparent. This shift from odd-chained to even-chained TAGs on high sugar diet is thought to reflect a reduction in lipogenesis by the enteric microbiota within the gut which are known to predominantly make odd-chain fatty acids, (Sultana et al., 2011). It may therefore be interesting to investigate whether trametinib changes the commensal gut microbiome of Drosophila particularly in flies fed a high sugar diet that could lead to changes in the lipidome. Further analysis of individual lipid species in the positive ion mode showed that high sugar diet significantly decreased the normalized abundance of odd-chained TAGs including TG(51:1), TG(49:0) and TG(49:1) consistent with Tuthilll et al., 2020, but an increase in abundance of even-chained TAGs was not observed.

Earlier findings in the thesis showed that high sugar diet increased TAG levels in fly homogenates using a biochemical assay and increased lipid droplet size within the fat body. However, the mass spectrometric analysis presented here did not detect a difference in the overall abundance of TAG species with high sugar diet. The colorimetric TAG assay used in this study to measure TAGs in *Drosohpila* extracts uses lipoprotein lipase-mediated hydrolysis of TAG to generate glycerol and free fatty acids which is then enzymatically reacted to generate a color change reaction, however in addition to TAG, mono- and diacylglycerides are also hydrolyzed, therefore it is possible that the results of

the colorimetric assay presented earlier reflect combined differences in these lipid species. Interestingly, an increase in the overall abundance of DAGs was observed in the lipidomics data in response to high sugar diet and both TAGs and DAGs showed a decreased trend in response to trametinib treatment, although this was not statistically significant. Moreover, the ability to detect differences in the abundance of individual TAG and DAG species across treatment conditions demonstrates that the lipidomics approach adopted here provides a more thorough understanding of the effects of high sugar and trametinib on lipid composition in *Drosophila*.

The analysis of lipids in the negative ion mode showed that high sugar diet and trametinib affect the abundance of a range of phospholipid species. For example, high sugar diet increased PS(34:4), PS(36:2), PS(36:4) and PS(38:2) while trametinib increased PS(36:4), PS(38:4), PG(32:2), PI(32:1), PI(34:2), PE(O-36:1), PE(36:2) and SM(35:1). PS phospholipids has been previously implicated in the maintenance of appropriate muscle mass in flies. Kim and colleagues have shown that phosphatidylserine synthase (Pss), involved in base-exchange reaction in which the polar head group of PE is replaced by L-serine to form PS, is crucial for muscle health in *Drosophila* whereby muscle-specific knockout of *pss* enhanced apoptosis and autophagy rate leading to sarcopenic phenotype in *Drosophila*, (Kim *et al.*, 2024). This suggests that perhaps trametinib prevents the deleterious effects of high sugar diet by maintaining muscle homeostasis in *Drosophila* or by adapting the lipidome to support muscle maintenance. The observed increase in PE species in response to trametinib treatment could be to support the increased synthesis of PS species.

Both PG and PI are involved in a range of biological processes acting as membrane lipids, but their role ageing or obesity in *Drosophila* has not been studied extensively.

One of the well-researched kinases involved in phosphorylation of PIs, is phosphatidylinositol 3-kinase, PI-3K, genetic inhibition of which have been shown to increase lifespan across species. For example, in *C.elegans*, mutation of *age-1*, encoding PI-3K in worms, extends lifespan, (Klass, 1983; Morris *et al.*, 1996). Similarly, down-regulation of PI-3K signaling has been shown to extend lifespan in *Drosophila* in *Drosophila* in a range of studies, (Partridge *et al.*, 2011). The effects of trametinib on the abundance of PI and PG lipid species may therefore affect PI-3K activity and the biological processes it regulates leading to lifespan extension.

On the other hand, it may be that trametinib shifts lipid metabolism in *Drosophila* fed a high sugar diet from TAG synthesis to phospholipid synthesis thereby decreasing the LD size and overall TAG content. In *Drosophila*, PA is metabolized into either DAG or PI and PG species, acting as an important precursor of TAG and phospholipid synthesis. Tian and colleagues have shown that salivary gland specific knockout of *cdsA* in larvae,

encoding CDP diglyceride synthetase involved in synthesis of cytidine diphosphate diacylglycerol, which is a precursor of PI and PG, caused ectopic LD accumulation in the salivary glands, (Tian *et al.*, 2011). This suggests that perhaps trametinib decreases LD size and TAG content of flies fed a high sugar diet by directing the dietary fatty acids to phospholipid synthesis instead of TAG synthesis.

Whole fly lipid extracts were analysed here and so the spatial effects of trametinib and high sugar diet on the *Drosophila* lipidome were not investigated which is one of the limiting factors of this study. Previous tissue-specific analyses have shown that the relative double bond content of fatty acids is different across different tissues in a lipid-specific manner, (Tuthill *et al.*, 2020). For example, the double bond content of neutral lipids is lower in the fat body and hemolymph compared to the heart while the double bond content in DAGs in the heart is considerably lower compared to the fat body and hemolymph (Tuthill *et al.*, 2020). A more tissue-specific analysis of the effects of trametinib on lipid content may therefore reveal more subtle changes to the lipidome.

For example, previous work has suggested that lipid species such as DAGs are lipotoxins associated with myocardial lipotoxicity and obesity, (Sletten *et al.*, 2018). *Drosophila* fed a high sugar diet showed increased saturation of the fatty acid substituents in both DAGs and TAGs, (Tuthill *et al.*, 2020) which is strongly correlated with lipotoxicity in cultured human cells, (Piccolis *et al.*, 2019). The increase in overall abundance in DAGs shown here in response to high sugar diet may therefore warrant a further, more targeted investigation into the effects of trametinib on DAGs specifically within the heart as perhaps trametinib prevents the deleterious effects of high sugar diet by preventing myocardial lipotoxicity in *Drosophila*.

The use of whole fly extracts for this lipidomics analysis may also have introduced high variability within the treatment conditions which perhaps could explain the lack of significant differences between the overall abundances of lipid classes. Although LC-MS/MS allows for accurate lipid measurements and identification of low-abundance species, being a highly specific and sensitive technique, variability introduced at sample preparation and lipid extraction can affect the reproducibility of the results, (Piehowski *et al.*, 2013). Also, the LC/MS/MS experiments generate complex datasets that can be challenging to analyse, especially when it comes to the *Drosophila* lipidome which is not well defined, leading to certain biologically relevant lipid species left unidentified. Consequently, misidentification can arise due to isomeric and isobaric species, (Zullig *et al.*, 2021). Also, different adduct forms can lead to the generation of a number of different ions from the sample analyte leading to misidentification of the lipid species and the use of a non-targeted method of acquisition, such as the one used in this study, (Hu *et al.*, 2020). Furthermore, identification of lipid species required a lipid database of known lipid

species, yet the current lipid databases do not encompass all possible lipid species, leading to a failure to accurately identify them, as well leading to false-positive identifications, (Hu *et al.*, 2020). Manual curation of lipid identification is possible but is time-consuming and may still result in misidentification even for experienced researchers requiring further validation. Future analysis should therefore employ the use of internal standards to aid in the accurate identification of the analyte, (Drotleff and Lammerhofer, 2019).

In conclusion, an in-depth analysis of the lipidome of trametinib-treated flies fed a high sugar diet was performed for the first time using LC-MS/MS methodology, employing the high-sensitivity Select Series Cyclic ion mobility (IMS) system. Although overall abundances among lipid classes did not show dramatic changes in response to trametinib treatment, investigations into changes within individual lipid species revealed interesting differences across treatment groups that may contribute to the pro-longevity effects of trametinib. This analytical data therefore provides an important foundation for further studies into the contribution of the *Drosophila* lipidome to longevity.

Consequently, next, to investigate whether trametinib influences the transcriptome in flies in response to normal and high sugar feeding, RNA-sequencing was used to examine the transcriptome changes in flies in response to trametinib treatment.

Chapter 5

5.1 INTRODUCTION

5.1.1 DIET INDUCED OBESITY PHENOTYPE IN DROSOPHILA

In *Drosophila*, high sugar diet (HSD) induces an obese phenotype with increased TAG storage, (Musselman *et al.*, 2011; Woodcock *et al.*, 2015). Previous studies have shown that the hormones involved in energy metabolism are conserved between vertebrates and *Drosophila*. For example, the functional homolog of insulin is encoded by *insulin-like peptides*, the functional homolog of leptin is encoded by *unpaired2*, and the functional homolog of glucagon is encoded by *adipokinetic hormone*, all of which respond to dietary interventions in a manner such that obesity-associated morbidities can develop, (Hariri and Thibault, 2010). Therefore, *Drosophila* is a great organism to model diet-induced obesity and its associated morbidities.

Typically, a HSD refers to a diet containing an elevated amount of sucrose, glucose or fructose, often with total sugar content of around 20-30% (w/v), (Galikova and Klepsatel *et al.*, 2018). Based on nutritional content, the sugar content of a HSD is proportionally higher than levels of proteins and fats, (Raubenheimer *et al.*, 2015). A range of studies have investigated the metabolic consequences of HSD in flies by measuring their sugar and fat content. It has been shown that HSD leads to increased levels of glucose, trehalose, glycogen and TAGs, (Skorupa *et al.*, 2008; Musselman *et al.*, 2011), whilst decreasing protein levels, (Skorupa *et al.*, 2008). In addition to changes in storage molecule content, HSD leads to increased food intake, impaired carbohydrate homeostasis and insulin resistance, (Skorupa *et al.*, 2008; Colinet *et al.*, 2013).

HSD has been shown to delay larval development, reduce fecundity and increase ageindependent mortality, however the overall effect of HSD on ageing is not clear, (Reis, 2016; Lushchak *et al.*, 2014). Several studies report a lifespan-shortening effect of HSD, (Musselman *et al.*, 2011; Na *et al.*, 2013; Lushchak *et al.*, 2014). However, Galenza and colleagues have shown that despite causing obesity and hyperglycaemia, HSD led to a median lifespan extension of 31%, (Galenza *et al.*, 2016). The study suggests that flies reared on HSD showed increased early mortality, yet overall lifespan was increased in most flies, (Galenza *et al.*, 2016). On the other hand, a large range of studies have shown that HSD results in detrimental changes to health and fitness, including reductions in flight and climbing ability as well as survivorship, (Na *et al.*, 2013; Villanueva *et al.*, 2019). Even exposure to HSD at a young age for a short term, leads to long-term decline in lifespan due to nutritional programming by the dFOXO transcription factor, (Dobson *et al.*, 2017).

Even though it was shown that HSD causes deleterious metabolic effects such as insulin resistance and fat accumulation, , van Dam et al., (2020) were able to uncouple these metabolic changes from the lifespan shortening effects of HSD. Shortened lifespan associated with HSD was reversed by water supplementation while metabolic changes were unaffected suggesting that HSD limits survival by causing dehydration independently of metabolic changes, (van Dam et al., 2020). Furthermore, HSD has been shown to induce tissue-specific metabolic changes. Na and colleagues have shown that HSD induced dysregulation of the hexosamine synthesis pathway in the heart leading to increased hexosamine production and heart defects, characterised by irregular beating patterns and reduced contractility, (Na et al., 2020). Also, HSD has been associated with morphological and functional changes of the Malpighian tubules, characterised by cytoskeletal deformities, increased oxidative stress and apoptosis, (Rani et al., 2020). Van Dam and colleagues showed that HSD increased the deposition of uric acid stones in the Malpighian tubules and hindgut, thereby reducing tubule secretion rates, (van Dam et al., 2020). HSD induces neurodegenerative modifications of the eye, such as increased disorganisation of ommatidia, enhanced apoptosis and reduced autophagy partially caused by increased oxidative stress by reactive oxygen species leading to increased light sensitivity and visual disturbances, (Catalani et al., 2021). Lastly, HSD decreases reproductive capacity and leads to genetic programming, (Ost et al., 2014; Brookheart et al., 2017). Buescher and colleagues showed that exposure of the maternal F0 generation to HSD led to male larvae of F1 and F2 generations with increased whole-body glucose and trehalose levels, while F2 females showed increased trehalose and decreased TAG levels, suggesting that HSD induces transgenerational gene changes in carbohydrate homeostasis and nutrient storage, (Buescher et al., 2013).

Even though a large amount of work has been carried out to characterise the phenotypes of diet-induced obesity, more recently, several studies have investigated the transcriptome changes of flies in response to HSD to decipher the underlying molecular mechanisms of these phenotypic changes.

5.1.2 TRANSCRIPTOMIC CHANGES OF DIET-INDUCED OBESITY

HSD was shown to reduce the development of flies especially at larval stages. To find the underlying mechanism, Musselman and colleagues, have analysed whole-body gene expression changes by microarray in male *Canton*-S larvae. To identify the transcriptional changes between control diet and HSD (34% sugar), flies were exposed to the different diets for a short-term of 12 hours or long-term from egg to L3 stage, (Musselman *et al.,* 2011). It was discovered that both short- and long-term exposure to HSD increased transcript levels of genes involved in glucose transport, fatty acid and trehalose synthesis

and TAG storage, (Musselman *et al.*, 2011). Additionally, short term exposure to HSD increased the transcription of genes involved in glycogenolysis and reduced the expression of genes involved in glycolysis, whereas long-term exposure to HSD elevated the expression of genes that regulate gluconeogenesis and β -oxidation. The findings indicated that changes in gene expression were of target dFOXO genes suggesting a link between HSD and the development of an insulin resistant phenotype in L3 larvae, (Musselman *et al.*, 2011).

Loreto and colleagues reared *Oregon-R* flies on 30% sucrose starting from eggs and used RNA-sequencing to investigate whole-body gene expression changes in 7-day old adults. HSD resulted in up-regulation of genes involved in ribosomal biogenesis and down-regulation in genes involved in energy metabolism including glycolysis, TCA cycle and ATP synthesis, and genes involved in development, (Loreto *et al.,* 2021). Additionally, actin genes were down-regulated leading to disruption of muscle development and mitochondrial dysfunction, which is phenotypically evident in developmental delay and muscle atrophy, (Loreto *et al.,* 2021).

A more tissue-specific RNA-sequencing analysis was carried out by Hemphill and colleagues using isolated heads from 7-day old *Oregon R-C* female flies fed with 20% HSD, which showed increased expression of genes involved in carbohydrate and peptide metabolism, predominantly those genes involved in carbohydrate processing, (Hemphill *et al.*, 2018). An increase in the transcription of heat shock proteins, antimicrobial defence genes and genes involved in apoptotic processes were also shown in animals fed with HSD, indicating that these flies experienced stress, (Hemphill *et al.*, 2018). And in accordance with increased TAG storage, *akh* expression was down-regulated by high sugar feeding, (Hemphill *et al.*, 2018).

Lastly, RNA-sequencing analysis of outbred females (*Drosophila* synthetic population resource) fed with HSD has shown an increase in expression of genes encoding proteins of the insulin/insulin-like signalling pathway, the mTOR pathway and the sirtuin pathway, including *dilp5, rheb* and *sirt2*. However, these expression changes were not limited to key genes but rather HSD affected a wide range of pathways and cofactors, (Ng'oma *et al.,* 2020).

5.1.3 EFFECTS OF DIET-INDUCED OBESITY ON RAS/MAPK PATHWAY

The effects of diet-induced obesity and RAS/MAPK inhibition on the transcriptome of flies is not well characterized However, some work has been done looking at the effects of diet-induced obesity on the RAS/MAPK pathway and associated effectors of the pathway.

Previous research has shown that HSD leads to early life transcriptomic changes in Drosophila that affect lifespan. Dobson and colleagues have shown that high sugar feeding in Drosophila inactivated dFOXO driving changes in expression of a number of epigenetic modifiers including acf. encoding chromatin assembly factor subunit, d12. encoding a subunit of the ATAC histone acetyltransferase complex, egg, encoding a histone methyltransferase, hdac1, encoding a histone deacetylase, and hmt4-20, encoding a histone methyltransferase. Expression of all of these genes was reduced in both wild-type and dFOXO mutant flies fed with HSD, which likely have a substantial consequence on the epigenome and therefore the transcriptome, (Dobson et al., 2017). Also, previous work has pointed at the role of ETS transcription factors in diabetes, (Seeter et al., 2009; Chen et al., 2015). Seeter and colleagues have shown that blood derived progenitor cells from diabetic patients and vasculogenic progenitor cells treated with high levels of glucose ex vivo have increased transcriptional activation of ETS transcription factors, (Seeter et al., 2009). Additionally, Chen and colleagues have used rodent models to show that high glucose treatment induced up-regulation of Ets-1 expression, the mammalian homologue of Drosophila pnt, as well as hyperacetylation of H3 and H4 histones in the promoter region of Ets-1 further enhancing its activation, (Chen et al., 2015). It was also shown that excess activation of Ets-1 led to a defect in pancreatic beta cells whereas knockdown of Ets-1 prevented hyperglycemia-induced dysfunction of beta cells, (Chen et al., 2015). Lastly, Dobson and colleagues have recently shown that AOP, an ETS transcriptional repressor, binds the same genomic loci and affects a similar transcriptional programme in vivo as dFOXO, (Dobson et al., 2019). It was shown that Pnt, an ETS transcriptional activator, promotes catabolism of energy stores, and that overexpression of pnt restored TAG levels in flies fed HSD to those observed on normal sugar diet suggesting a role for Pnt in metabolism, (Dobson et al., 2019). Additionally, Dobson and colleagues have shown that the transcriptional effects of dFOXO and Aop oppose the gene-regulatory role of Pnt suggesting the presence of an Aop-Pnt-dFOXO feedback loop which may respond to metabolic cues to modify ageing a range of organs and tissues, (Dobson et al., 2019). The current literature points to a direct link between high sugar-induced diabetes, regulation of metabolism and the role of ETS transcription factors. Therefore, it was critical to investigate whether trametinib, an inhibitor of the RAS/MAPK pathway, would play a role in alleviating the deleterious transcriptional effects of high sugar feeding through regulation of metabolic genes. This could potentially identify suitable drug targets and biomarkers for sugar-induced obesity to prevent deleterious changes.

One way of assessing the transcriptional effects of trametinib in an in-depth manner is to use RNA-sequencing. The transcriptome consists of all the gene transcripts in an organism and the quantity of the transcripts present at a given time, (Wang *et al.*, 2009b). RNA-sequencing technology takes extracted RNA from an organism and converts it to complementary DNA (cDNA), which is subsequently sequenced using high throughput processes from both ends in a process called paired-end sequencing, creating forward and reverse reads of around 150bp, (Wang *et al.*, 2009b). Paired-end sequencing offers advantages over single-end sequencing, as it offers a more accurate mapping to a reference genome, (Risca & Greenleaf, 2015). The reads are then aligned and mapped to a reference genome, Berkeley *Drosophila* Genome Project Release 6 Genome, *Drosophila* dm6 in the case of this study, from which counts of each read are produced allowing for the analysis of differentially expressed genes between treatment groups. In this project, the use of RNA-sequencing was key as there are no previous studies investigating the effects of trametinib in response to high sugar feeding, thereby offering an unbiased method to analyse the transcriptome, (Marguerat and Bahler, 2010).

5.2 AIMS AND OBJECTIVES

In this chapter, the main aim was to assess the effects of trametinib and HSD on gene expression of wild type *Drosophila*. This would determine if the genes found to be differentially expressed between conditions could be used to identify the potential lifespan extending and lipid lowering mechanisms of trametinib in response to high sugar feeding by investigating the sugar-responsive ERK-dependent genes. Additionally, the analyses would be used to investigate the conservation in the transcriptional response to trametinib between normal and HSDs.

The hypothesis was that any differences in gene expression between trametinib-treated flies on normal and HSDs would be the same, indicative of a common mechanism at play. Additionally, a secondary hypothesis was that trametinib would prevent certain deleterious sugar-induced gene expression changes which could cause the observed differences in lifespan and lipid storage phenotypes of trametinib-treated flies.

To achieve this, RNA was extracted from 10-day old adult female *Drosophila* that were fed on either normal and HSDs in both the absence and presence of trametinib. RNA-sequencing was then performed to analyse any differences in gene expression in three biological replicates. Quantitative reverse transcription polymerase chain reaction (qRT-PCR) was used to validate gene expression changes identified by RNA-sequencing analysis.

This set of experiments helps to fulfil the overall aim of the project by investigating the transcriptional changes associated with trametinib treatment on normal and HSD.

5.3 RESULTS

5.3.1 RNA-SEQUENCING OF DROSOPHILA FED HSD IN RESPONSE TO TRAMETINIB

RNA-sequencing of flies fed with HSD was carried out to investigate the changes in gene expression that occur in response to trametinib treatment and classify changes in gene expression that are sugar-responsive and ERK-dependent.

To investigate this, adult female flies were fed either a normal or HSD in both the absence and presence of trametinib. A similar HSD treatment was carried out before by Dobson *et al.*, 2017 therefore allowing for a direct comparison between the data of this study and the one published by Dobson *et al.*, 2017.

5.3.2 QUALITY CONTROL OF THE RNA-SEQUENCING DATA

The first step for analysing the RNA-sequencing data was quality control of the raw sequencing reads which is a crucial part of RNA-sequencing analysis as it allows for any errors introduced during sequencing to be identified and dealt with avoiding misinterpretation and bias that could arise due to misalignment or loss of raw reads.

FASTQC was therefore used to provide a simple overview of the quality of the raw data and was carried out for all replicates across all the conditions, (Figure 5.1).


В	Condition	% Dups	%GC content	M Seqs
	1xT- Forward	38.3	49	22.9
	1xT- Reverse	28.4	49	22.9
	1xT+ Forward	37.7	49	26.4
	1xT+ Reverse	29.5	49	26.4
	8xT- Forward	39	48	24.5
	8xT- Reverse	30.1	48	24.5
	8xT+ Forward	37.9	48	24.5
	8xT+ Reverse	28.8	48	24.5

Figure 5.1: Raw sequence quality control for a range of conditions used in RNA sequencing.

(A) Mean quality sequence scores, (B) Table of overall sequence statistics for the raw sequencing data (Forward and Reverse) for flies fed a normal (1x) and high (8x) sugar diets, in presence (+) and absence (-) of Trametinib. Mean quality scores show Phred scores across the sequence length, where Phred scores in the green area represent a 'passable' sequence quality and yellow/red areas indicating different degrees of sequence quality 'failure'. Overall sequence data statistics shows the average duplication levels, average GC content and average total amount of sequences in millions (M Seqs) for each condition. Quality control data was generated using FastQC and compiled using MultiQC, (Andrews, 2010; Ewels et al., 2016).

The mean quality scores showed that all sequences were the above the quality threshold Phred score of 28. The Phred score measured the quality of the identification of the nucleotide bases at each position. All the sequences maintained a consistently high Phred score between 34 and 36 across the sequence length indicating that the raw RNAsequencing reads were of good, acceptable quality along their entire length.

Overall, sample data showed on average 34% duplication of sequences, which is

expected for RNA-sequencing data due to the presence of highly abundant transcripts. GC content was also consistent across the sequences with an average of 48-49% GC indicating a normal distribution of GC content across all the sequences. This indicates that there was no apparent contamination or unwanted bias in the library. Lastly, the M Seqs content showed on average 24-25 million sequences present for each replicate, which is a representatively sized data set that seemingly was not affected by RNA degradation, allowing for more accurate downstream analyses.

Altogether, quality control shows seemingly no contamination, bias, or degradation in the library and overall high-quality reads. This allows for continuation of the downstream analysis without concerns for accuracy or representability.

5.3.3 MAPPING AND ALIGNMENT OF THE RNA-SEQUENCINGUECING DATA

After the quality control of the sequencing data, the sequences were mapped and aligned to the *Drosophila* reference genome, the *Drosophila melanogaster* Release 6 (dm6) produced by the Berkeley *Drosophila* Genome Project in 2014, (dos Santos *et al.*, 2014) using the HISAT2 tool, (Kim *et al.*, 2015).

The quality of mapping was assessed by analysing gene body coverage to show the gene regions to which the reads were aligned and read distribution which shows the distribution of reads across the genome. This was carried out for all samples across all treatments, (Figure 5.2).



Figure 5.2: Quality assessment of the mapping of paired-end reads for flies fed HSD in response to trametinib.

Assessment of the mapping for flies fed normal (1x) and high (8x) sugar diets in absence (T-) and presence (T+) of trametinib showing **(A)** the total number of reads successfully assigned to genes in each replicate for each treatment condition, **(B)** the overall average alignment statistics showing % of uniquely mapped genes and % of mapped reads aligned to coding DNA sequences (CDS) of exons, **(C)** the coverage of the reads across the gene body. **(D)** Percentage of reads aligned to gene exons in a genome.

All replicates for each treatment condition showed a consistent number of assigned read counts between 18-24 million total assigned reads. HISAT2 scores for all samples showed that 96% of all reads were uniquely mapped, with the remaining 4% unmapped. Of the uniquely mapped reads, between 75-78% of reads were mapped to CDS of exons with the remaining being unmapped, multimapped, non-feature or ambiguous.

Gene body coverage assessed the location of the mapped reads across the genes and for all replicates in all treatment conditions showed a consistent and even coverage of reads across the length of the gene body with no bias. Most of the reads were evenly mapped between the 2nd, 3rd and X chromosomes with some reads mapped to 4th chromosome. No transcripts were mapped to the Y chromosomes, as the RNA was isolated from female flies.

Altogether, this analysis indicates mapping was successful with a high level of uniquely mapped genes to CDS exons across the whole genome length.

5.3.4 ANALYSIS OF DIFFERENTIAL GENE EXPRESSION

Changes in gene expression between the treatment conditions were then analysed using read counts for each gene normalized using the DESeq2 tool. DESeq2 has strong statistical power due to its high sensitivity, precision, and ability to connect any variability within the measurements to give a finer estimate of significance between the different treatments, (Love *et al.*, 2014). Initial analysis was carried out using principal component analysis (PCA) of transformed RNA-sequencing count data, (Figure 5.3).



Principal Component Scores

Figure 5.3: Principal Component Analysis (PCA) for flies fed a HSD in response to trametinib.

Scatter plot of Principal Component Analysis (PCA) of transformed count data from DESeq2 of flies fed normal (1x) and high (8x) sugar diets in absence (Control) and presence (Treated) of trametinib. Samples are clustered according to their diet (PC1) and trametinib treatment (PC2) (n=3 independent biological replicates).

PCA revealed that 51% of the total variance amongst the 4 treatment groups was represented by two principal components. Samples were separated along the first principal component showing 29% of the variation between samples was explained by the sugar diet that was fed to flies. Samples were separated along the second principal component, with 22% of the variation between samples caused by the trametinib treatment.

Surprisingly, high sugar fed trametinib-treated flies did not group with normal sugar fed control flies but instead grouped separately from the other treatments suggesting that trametinib did not revert the transcriptome to its control state but instead elicits a separate transcriptional program.

Genes that were significantly differentially expressed in response to trametinib on normal and HSDs as well as differentially expressed genes in response to HSD were identified alongside their fold change in expression, (Figure 5.4)



Figure 5.4: Visualisation of differentially expressed genes for flies fed HSD in response to trametinib.

Volcano plots comparing (A) trametinib treated flies on normal sugar diet, (B) normal sugar diet fed flies to HSD fed flies, (C) trametinib treated flies on HSD. Plots show the -log10(Adjusted p value) for each gene plotted against log2 fold change. Significantly upregulated genes (logFC>1) are highlighted in blue and downregulated genes (logFC<1) in orange. Genes that show no significant differences in expression are in grey.

Many genes were significantly (q<0.05) differentially expressed in response to high sugar feeding (1308 genes) as well as trametinib treatment on high sugar feeding (3135 genes). Yet trametinib treatment only showed a fraction of significantly differentially expressed genes on normal sugar diet, (25 genes). Out of the differentially expressed genes in response to high sugar feeding, 617 were down-regulated and 691 were up-regulated. Trametinib treatment on high sugar feeding showed a down-regulation of 1696 genes and up-regulation of 1439 genes. On normal sugar diet, trametinib treatment showed a down-regulation of 5 genes and up-regulation of 20 genes, (Figure 5.4).

To validate these gene expression changes, the sugar responsive genes identified in the dataset compiled here were compared to a publicly available dataset from Dobson *et al.,* 2017 which used a comparable dietary protocol. Sugar responsive genes were classified

here as genes that showed significant differential expression in response to HSD, by comparing the transcripts of flies fed normal vs HSDs in absence of trametinib. The overlap in DEGs between the two data sets were compared and visualized using a Venn diagram (Figure 5.5).



Figure 5.5: Comparison of the sugar-responsive gene expression changes to publicly available data.

Proportional Venn diagram comparing the sugar-responsive differentially expressed gene lists identified in a public dataset (Dobson *et al.*, 2017) and sugar-responsive differentially expressed gene list identified in this study. A significant overlap was identified between the two data sets, ($p<1x10^{-47}$, hypergeometric analysis). Numbers indicate the number of genes in each category.

The number of genes that showed significant differential expression in response to HSD was much smaller than Dobson *et al.*, 2017. However, a significant proportion of those sugar responsive genes(65.3%, p<1x10⁻⁴⁷), showed a significant overlap to the sugar responsive genes identified by Dobson *et al.*, 2017. Also, investigating the directionality of changes in those shared sugar responsive genes showed 99% (1120 out of 1127) similarity in the direction by which HSD changed their expression. The similarity of these two data sets therefore validates the gene expression changes observed in this study.

To further verify the gene expression changes identified by RNA-sequencing analysis, the expression of a particular gene of interest, *akh*, was selected for expression analysis by qRT-PCR. The expression of *akh* is of particular interest as Akh is known to regulate nutrient storage and energy homeostasis in *Drosophila* as well as being ERK-dependent on causing hyperglycaemia in response to HSD feeding (Galikova *et al.*, 2015; Li *et al.*,

2023). The fold change expression values of *akh* from this study and Dobson *et al.*, 2017 are displayed in Table 5.1.

Gene name	High sugar effect log2(FC)	High sugar trametinib effect log2(FC)
akh (This study)	0.8	-1.0
akh (Dobson et al., 2017)	1.1	

Table 5.1: Comparison of differential expression *Akh* in response to HSD and presence of trametinib in this study and Dobson *et al.*, 2017.

Results of RNA-sequencing analysis comparing log fold change (FC) in expression of *Akh* gene, in flies fed HSD (High sugar effect FC), and in presence of trametinib on HSD (High sugar trametinib effect FC). Fold changes shown were significant based on adjusted p-value (adjusted using Benjamini-Hochberg procedure to reduce false positives). The significant threshold used was p < 0.05, with up-regulation shown as FC >0 and down-regulation shown as FC < 0.

The table shows that the directionality of expression of *akh* is the same as well as the amplitude in both this study and Dobson *et al.*, 2017. Also, the expression of *akh* is downregulated in presence of trametinib on HSD. Therefore, it would be intriguing to investigate whether *akh* is a sugar responsive ERK-dependent gene.

Additionally, for further verification, qRT-PCR analysis of *akh* expression using *b-cop* as a reference gene was performed, shown in Figure 5.6.



Figure 5.6: Analysis of the expression of *akh* by qRT-PCR.

mRNA expression was analysed by qRT-PCR and normalised relative expression of *b-cop* mRNA in 10-day old adult female *Drosophila* of the indicated treatment conditions. All flies were fed a normal (1x) or high (8x) sugar diet in absence (T-) or presence (T+) of trametinib. Standard curves were created using 10-day old adult female *Drosophila*. Data represents relative *akh* mRNA levels for the indicated treatment conditions. (n=4 replicates of 15 pooled flies for each treatment condition, p < 0.05 *, Two-way ANOVA with Tukey's multiple comparison test).

On normal (1x) sugar diet, there was no significant effect of trametinib treatment on *akh* expression. High (8x) sugar diet significantly increased the relative expression of *akh* but in the presence of trametinib, the relative expression of *akh* was restored to a comparable level as observed on normal (1x) sugar diet, (Figure 5.6). Therefore, *akh* expression measured using qRT-PCR showed similar changes in response to HSD and trametinib as observed in the RNA-sequencing analysis.

Next, a set of genes were identified that respond to HSD but the expression of which is rescued by trametinib on HSD. These were classified as those DEGs that change in expression in flies fed with HSD in the absence of trametinib (sugar responsive) but their expression changes in the opposite direction in the presence of trametinib on HSD. These genes are of particular interest as they potentially provide a molecular explanation for the phenotypic effects of trametinib in flies fed HSD. The DEGs were compared and visualized using a Venn diagram, (Figure 5.7).



Figure 5.7: Comparison of sugar-responsive genes and trametinib-responsive genes after high sugar feeding.

Proportional Venn diagram comparing (A) the genes that are transcriptionally upregulated in response to HSD or downregulated in response to trametinib on HSD. The overlap is composed of those genes that require ERK activity for upregulation on HSD. (B) Comparison of the genes that are transcriptionally downregulated in response to HSD or upregulated in response to trametinib on HSD. The overlap represents those genes that require ERK activity for downregulated the number of genes in each category. (25.9% (p <4.16E-215); 12.2% (p<4.70E-73), hypergeometric analysis).

The comparison of upregulated sugar responsive genes to genes downregulated by trametinib on HSD, showed a significant 25.9% overlap. The comparison of downregulated sugar responsive genes to genes upregulated by trametinib on HSD showed a significant 12.2% overlap. The genes identified in the two overlaps were combined to create a set of 488 genes of interest that may explain the molecular mechanisms behind trametinib's phenotypic effects in flies fed HSD.

These genes were investigated further using gene ontology (GO) analysis to assess their biological function (Table 5.2).

Fold Enrichment	Enrichment FDR	Functional category
6.1	5.0E-03	Neuropeptide hormone activity
3.3	2.0E-02	Regulation of behavior
3.3	4.0E-02	Neuropeptide signaling pathway
2.5	2.0E-04	Regulation of transport
1.6	6.0E-04	Regulation of biological quality

Table 5.2: Gene ontology (GO) analysis of the genes of interest.

Gene ontology (GO) analysis of the genes found to be the subset of genes of interest. Each enriched GO term shows the fold enrichment based on the degree to which the genes in the gene list fall into a specific category (hypergeometric analysis), and enrichment false discovery rate (FDR) correction (Significance of the enrichment score adjusted for false positive) with a cut-off at 0.05, and functional category showing the biological process for which the genes likely fall into. This list is not exhaustive and shows the over-represented categories for specific processes.

Enriched GO terms for the genes of interest included those involved in neuropeptide hormone activity, regulation of behaviour, regulation of transport and regulation of biological quality. Surprisingly, the GO analysis did not reveal any enrichment in biological pathways involved in lipid metabolism, suggesting that trametinib may not prevent lipid accumulation in response to HSD by ERK-dependent transcriptional regulation. Instead, pathway enrichment analysis suggested that trametinib may elicit its effects on lowering TAG levels and extending lifespan by regulating the transport of nutrients and/or regulating feeding behaviour and satiety.

To explore this idea, genes involved in the pathways highlighted in Table 4.2 were further investigated using K-means clustering to analyse the interaction of those genes. Each cluster identified is explored in the figures below in a descending number of genes.



Figure 5.8: Interaction network of genes of interest, cluster 1.

(A): Cluster map showing interaction between genes of interest found based on pathways in Table 4.1. Genes are clustered based on similarity in their biological function, the strength of associated function is defined by the thickness of the edges that connect the nodes based on text mining, experimental data, databases, co-expression, genome location, co-occurrence. (B): Table of genes in cluster 1 containing logFC in flies in response to HSD (High sugar effect FC) and in presence of trametinib on HSD (High sugar trametinib effect FC).

The Interaction network for cluster 1 contained mostly genes coding for neuropeptides and neuropeptide processing including the genes encoding short neuropeptide F (*snpf*), pigment dispersing factor (*pdf*), adipokinetic hormone (*akh*), diuretic hormone 31 (*dh31*), myoinhibiting peptide (*mip*), which all encode neuropeptides. Genes such as Clock (*clk*), FMRFaR, amontillado (*amon*) and sex peptide receptor (*spr*) encode proteins involved in regulation of the circadian rhythm and neuropeptide processing, (Figure 5.8).



Figure 5.9: Interaction network of genes of interest, cluster 2.

(A): Cluster map showing interaction between genes of interest found based on pathways in Table 4.1. Genes are clustered based on similarity in their biological function, the strength of associated function is defined by the thickness of the edges that connect the nodes based on text mining, experimental data, databases, co-expression, genome location, co-occurrence. (B): Table of genes in cluster 1 containing logFC in flies in response to HSD (High sugar effect FC) and in presence of trametinib on HSD (High sugar trametinib effect FC).

The interaction network for cluster 2 mostly included genes coding for proteins involved in the oxidative stress response, such as nitric oxide synthase (*nos*) and vitamin D_3 upregulated protein 1 (*vdup1*). Also, genes such as thin (*tn*), myosin heavy chain (*mhc*), upheld (*up*), and wings up A (*wupa*), encode proteins involved in muscle maintenance, (Figure 5.9).



Α

Figure 5.10: Interaction network of genes of interest, cluster 3.

(A): Cluster map showing interaction between genes of interest found based on pathways in Table 4.1. Genes are clustered based on similarity in their biological function, the strength of associated function is defined by the thickness of the edges that connect the nodes based on text mining, experimental data, databases, co-expression, genome location, co-occurrence. (B): Table of genes in cluster 1 containing logFC in flies in response to HSD (High sugar effect FC) and in presence of trametinib on HSD (High sugar trametinib effect FC).

The Interaction network for cluster 3 included genes involved in lysosome fusion, straitjacket (*stj*); fluid homeostasis, lon transport peptide (*itp*); Sleep regulation, hypokinetic (*hk*) and resistant to dieldrin (*rdl*) as well as genes involved in excitotoxicity such as Grik, glutamate-gated chloride channel subunit alpha (*gluclapha*), and glutamate receptor IID (*glurIID*), (Figure 5.10).



Figure 5.11: Interaction network of genes of interest, cluster 4.

(A): Cluster map showing interaction between genes of interest found based on pathways in Table 4.1. Genes are clustered based on similarity in their biological function, the strength of associated function is defined by the thickness of the edges that connect the nodes based on text mining, experimental data, databases, co-expression, genome location, co-occurrence. (B): Table of genes in cluster 1 containing logFC in flies in response to HSD (High sugar effect FC) and in presence of trametinib on HSD (High sugar trametinib effect FC).

The interaction network for cluster 4 included genes involved in oxidative phosphorylation, such as the genes encoding *nd-23*, *nd-24* and *nd-b14.5b* as well as genes encoding proteins of complex I of the electron transport chain (ETC) and *uqcr-6.4*, *uqcr-14* encoding proteins of complex III of the ETC, (Figure 5.11).

These results showed that some of the sugar-responsive ERK-dependent gene expression changes fall into the categories associated with negative regulation of feeding behaviour, muscle fibre assembly, action potential, and regulation of synaptic activity among others. Genes such as *amon, akh,* and *snpf* are involved in the production of neuropeptides that have been shown regulate metabolic storage in *Drosophila* carbohydrate and lipid metabolism, (Rhea *et al.,* 2010, Malita *et al.,* 2022, Li *et al.,* 2023). *Amon* encodes a proteolytic enzyme which cleaves propeptides to produce bioactive neuropeptides such as *Akh* and *sNPF.* Genes such as *dh31, mip, and spr* are involved in regulation of reproductive behaviour, satiety and defecation, (Min *et al.,* 2016, Kotronarou *et al.,* 2023).

5.4 DISCUSSION

In the previous chapters, it was determined that trametinib has an inhibitory effect on ERK which is a key transcriptional effector of RAS/MAPK signalling both in response to HSD and during ageing. It was additionally discovered that the ability of trametinib to extend lifespan and lower lipid storage did not depend on reduced lipogenesis nor enhanced lipolysis. Therefore, the main aim of this chapter was to further investigate the mechanisms by which trametinib mediates its effects in response to HSD by examining the transcriptional response to trametinib treatment.

5.4.1 RNA-SEQUENCING OF HIGH SUGAR FED *DROSOPHILA* IN RESPONSE TO TRAMETINIB TREATMENT

5.4.1.1 QUALITY CONTROL OF THE RNA-SEQUENCING DATA, MAPPING, AND ALIGNMENT

Quality of raw sequencing data was initially assessed using FastQC and MultiQC quality control tools, to minimize the number of errors produced during the analyses. The errors are typically caused by limitations in the sequencing techniques used, however as an Illumina sequencing platform (Illumina 1.9) was used to generate the sequence reads for this project, errors due to quality control were less of a concern. The Illumina sequencing platform uses an ensemble-based sequencing by synthesis technique, which produces an error rate of <1% with most of the errors being single nucleotide mismatches, (Kukurba and Montgomery, 2015). It was, therefore, expected that all reads showed good overall quality across different replicates of all the treatment conditions with all samples being equally high quality. A way of checking PCR amplification bis is to look at the duplication rate. FastQC can be used to track the first 200,000 short reads in each file to determine duplication levels. By default, FastQC will raise a warning if there is more than 20% duplication in total and a failure if duplication reaches over 50%, indicating a seriously biased sequencing library, (Li et al., 2014). The duplication rate of samples in this study spanned between 20-30% which was deemed acceptable for downstream analysis. Downstream analyses showed successful mapping of reads to the reference genome, as nearly all reads mapped to a unique region of the reference genome, most being in the exonic regions, confirming good quality RNA-sequencing data with no genomic DNA contamination. The uniformity in gene body coverage suggests that there was no 5' or 3' end bias, supporting the assumption that there was no RNA degradation occurring during sequencing, (Wang et al., 2016a). Most genes were mapped to chromosome 2,3, and X, which was expected of female Drosophila samples, where there would be no Y chromosome present. Out of all the chromosomes (2,3,4 and X), 2 and 3 carry the most genes from the genome, as the 4th chromosome, known as the dot chromosome due to its small size, only carries around 3.5% of the genome. Therefore, a large percentage of genes mapped to the 4th chromosome would be unexpected, (Sun *et al.,* 2000).

Conclusively, this indicated that both sample and library preparations were carried out appropriately and the resulting gene expression analysis would draw accurate conclusions.

5.4.1.2 VERIFICATION OF THE RNA-SEQUENCING RESULTS AND ANALYSIS

To ensure that the RNA-sequencing data was analysed appropriately various verification steps were taken, including comparison to a publicly available, previously published data set and by qRT-PCR. Firstly, the differentially expressed genes that were identified as sugar-responsive genes from this study were compared to those identified as differentially expressed genes in response to HSD in an independently derived equivalent study in which flies of the same genetic background were fed with a similar diet, (Dobson et al., 2017). The RNA seq data generated in Dobson et al., 2017 study was derived from 4 biological replicates for each treatment group, (one sample from HSD fed flies (8xS) and its correspondent from the normal sugar diet (1xS) fed flies were removed from the analyses following preliminary assessment with PCA), and an Illumina Nexseq 500 platform which identified a total of 6,435 genes that were differentially expressed in response to high sugar (8xS) feeding, (Dobson et al., 2017). The comparison showed a significant overlap in the genes differentially expressed between the two sets of sugarresponsive differentially expressed genes. Detection of lowly differentially expressed transcripts is key when studying the effects of HSD and trametinib treatment, as activity of enzymes involved in sugar metabolism are mostly regulated post-translationally, (Yang et al., 2023) and therefore changes in activity based on transcript changes are often hard to interpret and require further validation.

As well as the significant overlap of DEGs between Dobson *et al.*, (2017) and this study's dataset, it was also shown that the directions in which expression of those genes changed in the two datasets were relatively consistent, with ~99% of the shared DEGs showing expression changes in the same direction. Also, in both studies despite the differences in the total number of DEGs in response to high sugar, a similar number of genes were up- and down-regulated in each data set. It should be noted that the flies in the Dobson *et al.*, (2017) study were 21-day old adults as compared to 10-day old adults in this study, (Dobson *et al.*, 2017). The longer-term HSD may induce certain transcriptional effects which would be under-represented or undetected after 10 days of exposure to HSD, as used in this study, which may be reflected in the smaller number of sugar-responsive DEGs compared to the gene set from Dobson *et al.*, (2017). Some of the differences in

the Dobson *et al.*, (2017) and this study's datasets, might be due to differences in aligners and read quantification methods used. Dobson *et al.*, (2017) have used Tophat2 for the alignment as opposed to HISAT2, used in this study. Based on simulation-based benchmarking of RNA-sequencing aligners, it was shown that Tophat2 was shown to be one of the slowest aligners based on runtime performance yet both HISAT2 and Tophat2 showed consistently high percentage of aligned reads, (Baruzzo *et al.*, 2016). Additionally, a different read quantification was used, Htseq was used in Dobson *et al.*, (2017) study, whereas featurecount was used in this study. The different counting methods used are consistent as previously shown in a comparative study of Liao *et al.*, (2014). Both studies used DESeq2 for downstream data analyses, allowing for reliable comparison of the two datasets. Additionally, future re-analysis of these datasets may show increased correlation between the two datasets as computational data analysis methods develop.

In combination with RNA-sequencing analysis, qRT-PCR analysis was used to independently verify one of the gene expression changes observed. The directionality and magnitude in gene expression changes of Akh were comparable between RNAsequencing and gRT-PCR analyses. The topic of RNA-sequencing validation is widely debated with many studies suggesting that in most cases validation of RNA-sequencing data with gRT-PCR is redundant. The focus of validation of genome-scale expression studies likely stemmed from work with microarrays. Even though microarrays allow for gene expression studies on a scale not seen before with overall high-level performance, a couple of studies raised concerns about reproducibility and bias, (Balazsi and Oltvai, 2007; Zhang et al., 2009). For instance, Zhang et al., (2009), carried out a comparative microarray study of two different gene chip arrays. Although these studies were identical in probe sequences, nearly 70% of the probes in the two arrays showed slightly different experimental hybridization values from the same samples suggesting array specific differences, (Zhang et al., 2009). Additionally, Balazsi and Oltvai, (2007) have studied the relative probe position on the microarrays in a series of cDNA microarray experiments and showed that the relative probe position can impose an effect on the measured gene expression value, (Balazsi and Oltvai, 2007). Due to this, researchers feel compelled to validate microarray results with qRT-PCR. However, RNA-sequencing does not suffer from the same issues as microarrays, which was addressed in a comparative analysis by Everaert et al., (2017). Everaert and colleagues carried out analysis of five RNAsequencing datasets compared to qRT-PCR results for >18000 protein-coding genes, even though the RNA-sequencing samples were from human origin, there was nothing to indicate that the results would be different for Drosophila samples. The comparative analyses demonstrated that about 85% of all genes showed consistent expression between RNA-sequencing and qRT-PCR data, (Everaert et al., 2017). It was revealed

that a small but specific gene set showed inconsistent expression measurements. These were genes that were typically smaller, had fewer exons and were lowly expressed compared to genes with consistent expression measurements, (Everaert *et al.*, 2017). It is suggested that for investigation of small, lowly expressed genes, qRT-PCR is used for RNA-sequencing analysis validation. There are a variety of other comparative studies that showed a good correlation between results obtained with RNA-sequencing and qRT-PCR, (Shi and He, 2014; Wu *et al.*, 2013; Griffith *et al.*, 2010; Asmann *et al.*, 2009). If all experimental steps and data analyses are carried out according to the state-of-the-art, results of RNA-sequencing data are likely to be reliable if enough biological replicates are used, and the added validation from qRT-PCR is likely to be low.

5.4.2 ANALYSIS OF DIFFERENTIAL GENE EXPRESSION

To identify differentially expressed genes the DESeq2 tool was used as compared to other forms of RNA-sequencing analysis tools, DESeq2 showed a marginal performance advantage with a higher detection accuracy (Rapaport *et al.*, 2013). Also, as there was little variation between replicates of the same treatment condition on PCA plot, there was little concern over batch effect affecting the resulting differentially expressed genes. Subsequent filtering showed there were a large number of significant differentially expressed genes found to change in response to HSD, even though the sugar-responsive gene set identified in this study was marginally smaller than the gene set identified by Dobson *et al.*, (2017), around 65.3% of sugar-responsive genes from this study were shared with the gene-set of Dobson *et al.*, (2017). Additionally, qRT-PCR analysis was used to analyse the expression of *akh*, and the the directionality and relative amplitude of changes in on *akh* expression were comparable to those identified within the RNA-sequencing data.

In this study, flies were fed HSD in response to trametinib therefore it was crucial to determine those genes that respond to HSD but are dependent on ERK activity due to trametinib's inhibitory effect on ERK phosphorylation. This was achieved by comparing the overlap of genes that were differentially expressed in response to HSD (sugar responsive) with those genes that changed in expression in the opposite direction on HSD in the presence of trametinib. And genes that significantly changed in expression in flies fed HSD in presence of trametinib. This identified 488 genes that were classified as sugar responsive and ERK-dependent. Gene ontology and cluster analysis of these genes identified several interesting groups of genes based on their molecular function.

5.4.3 INVESTIGATION OF POTENTIAL MOLECULAR ANALYSIS - CLUSTER 1

Previously in this study, the effects of HSD and trametinib were investigated. It was shown the HSD led to diet-induced obesity in *Drosophila*, which was comparable to previous work, (Musselman et al., 2011; van Dam et al., 2020; Baenas and Wagner, 2022). Also, it was shown that the presence of trametinib in flies fed a HSD prevented diet-induced obesity, yet it was shown through genetic manipulation that the ability of trametinib to lower lipid storage was not dependent on lipolysis nor lipogenesis. RNA-sequencing data exploration generated a sugar-responsive ERK-dependent gene list on which cluster analysis was performed to analyse gene interactions as potential molecular mechanisms that could explain the effects of trametinib in flies fed HSD.

One potential mechanism by which trametinib elicits its phenotypic effects is through the regulation of neuropeptides and neuropeptide processing enzymes, such as *akh*, *clk*, *amon*, *mip*, *dh31*and *snpf* all which were shown to play a role in regulating feeding behaviour, sugar homeostasis and gut motility. The exploration of genes of interest from cluster one is therefore discussed below.

5.4.3.1 AKH

Akh is a metabolic neuropeptide, mediating mobilisation of energy substrates from the fat body in a range of insects. Some of the early studies have shown that starved wildtype flies displayed prolonged hyperactivity prior to death, which was a novel behaviour attributed to Akh, (Lee and Park, 2004). Lee and Park have shown that ectopic expression of akh lowered levels of TAGs which was a consequence of increased hydrolysis of TAGs, supported by increased levels of glycerol in the haemolymph, (Lee and Park, 2004). It was therefore suggested that Akh is responsible for regulation of starvation-induced foraging behaviour. More recently, it has been shown that akh is responsible for dietary sugar induced activation of mTOR, a known regulator of lifespan, and the selective secretion of *dilp3*, (Kim and Neufeld, 2015). Sugar levels are sensed directly by Akh-producing cells in the corpora cardiaca and Kim and Neufeld, (2015) showed that Akh signalling is required for the sugar-dependent release of Dilp3 in the larval fat body, (Kim and Neufeld, 2015). A characterisation study by Galikova et al., (2015) of *akh* has shown that Akh signalling is dispensable for ontogenesis, locomotion, oogenesis and homeostasis of lipid and carbohydrate storage until the end of metamorphosis. However, during adulthood, Akh regulates fat body content and haemolymph sugar levels and nutritional response, (Galikova et al., 2015). Galikova and colleagues have shown that akh mutants survived longer in response to starvation stress, and contained higher amounts of TAGs, compared to wild-type controls, (Galikova et al., 2015). However, akh mutants were shown to be hypoglycaemic, having reduced circulating sugar levels, while increased TAG levels due to subsequent use of dietary sugars for lipogenesis, (Galikova *et al.*, 2015). Song and colleagues have shown that enhanced *akh* signalling is essential for high-sugar diet-induced hyperglycaemia in *Drosophila*, (Song *et al.*, 2017). It was shown that *akh* overexpression in the fat body of flies led to higher glycaemic level suggesting that ectopic *akh* expression in the fat body results in hyperglycaemia in an autocrine manner, (Song *et al.*, 2017). Additionally, more recently Li and colleagues have shown that HSD-induced hyperglycaemia is Akh dependent via ERK translocation into peroxisomes to increase carbohydrate production in the fat body, (Li *et al.*, 2023). Also, it was shown that *erk* knockdown or trametinib feeding to suppress ERK activity alleviated HSD-induced hyperglycaemia, (Li *et al.*, 2023). Lastly, a study by Malita and colleagues has shown that *akh* knockdown resulted in reduced intake of sugar and increased yeast intake in starved flies, suggesting that Akh is not only responsible for high-sugar diet induced hyperglycaemia, but also regulates the food preference in starved flies, (Malita *et al.*, 2022).

5.4.3.2 CLK

Circadian clocks are inherent oscillators found in almost all organisms, in Drosophila the major loop involving circadian rhythm is a transcriptional activator Clk, (Hardin, 2005). Xu and colleagues have shown that disruption of *clk* expression in the fly fat body, led to higher food consumption compared to control flies, (Xu et al., 2008). However surprisingly, disruption of *clk* was shown to be more sensitive to starvation, likely due to defects in glycogen storage, alternatively, due to lipid stores being affected differently in flies lacking fat body *clk* expression, under starvation, (Xu et al., 2008). More recently, it was shown that *clk* mutation in the fat body of flies did not affect the TAG content of flies, yet inhibition of *clk* specifically in cells expressing the neuropeptide, *pigment dispersing* factor (pdf), increased TAG content of female and male flies, (DiAngelo et al., 2011). Even though specific knockdown of *clk* in PDF neurones decreased food consumption, that does not account for the increased lipid phenotype when the circadian function of PDF neurons is altered, (DiAngelo et al., 2011). Most recently, a clk mediated expression of gart, a trifunctional enzyme involved in de novo purine synthesis pathway, has been shown to regulate feeding and energy homoeostasis in Drosophila, (He et al., 2023). He and colleagues have shown that CLK regulated Gart expression was crucial for maintenance of feeding rhythm and food intake, but also regulation of energy homeostasis, (He et al., 2023). It was shown that flies with Gart knockdown in the whole body ate significantly less, than those with upregulated Gart which ate more, (He et al., 2023). To understand gart function on feeding in different tissues, He and colleagues knocked down *gart* in glial cells, the fat body, the gut/salivary glands and the salivary glands. It was shown that the feeding was maintained in glia-specific knockdown of gart

however feeding was diminished in fat body- and gut-specific knockdowns of *gart* but not in the salivary glands, (He *et al.*, 2023). Additionally, flies with downregulated *gart* in the whole body ate significantly less, while flies with upregulated *gart* ate more which was also shown to be in genetic manipulation of gut-specific *gart* but not in the glia, the fat body nor salivary gland, (He *et al.*, 2023). Also, since food intake and energy storage are intrinsically linked, it was shown that tissue specific knockdown of *Gart* significantly decreased TAG content, however the tissue specific knockdown of *Gart* decreased lifespan of flies, (He *et al.*, 2023).

5.4.3.3 PDF

Pdf is yet another circadian clock gene that was identified as a negative regulator of the master circadian regulator Clk, and expression of *pdf*, the main circadian neuropeptide, (Mezan *et al.*, 2016). Mezan and colleagues have shown that Clk suppressed *pdf* transcription whereas Pdf was required for maintenance of normal oscillation pattern of Clk transcription in a feedback loop, (Mezan *et al.*, 2016).

5.4.3.4 AMON

Amontillado is a homolog of mammalian PC2 protein, involved in proteolytic processing of neuropeptides. Rhea and colleagues have shown that Amon is required for processing of Akh, a regulatory hormone produced by corpora cardiaca (cc) endocrine cells, (Rhea et al., 2010). It was shown that amon knockdown flies have significantly reduced haemolymph sugar levels and phenotypically copy flies where the Akh-producing cells were ablated, (Rhea et al., 2010). Additionally, it was shown that amon knockdown in Akh-producing cells resulted in hypoglycaemia, which was rescued by expression of the akh gene, (Rhea et al., 2010). It was suggested that the Akh propepride is a proteolytic target of Amon which in turn is responsible for glucose regulation in Drosophila larvae, therefore it is important to interpret this study with caution as Akh plays a different role during and post development as shown before, (Rhea et al., 2010; Galikova et al., 2015). Reiher and colleagues performed a peptidomic LC/MS analysis of fly midguts containing enteroendocrine cells, involved in neuropeptide production, to identify the presence and abundance of key neuropeptides, (Reiher et al., 2011). Analysis of fly midguts in amon mutant flies showed impaired peptide detectability and decreased localization of neuropeptides in *amon*-driver GFP reporter, suggesting that *amon* plays a crucial role in peptide processing, (Reiher et al., 2011). Lastly, more recently, it was shown that amon is involved in processing of Pdf, (Lee et al., 2023).

5.4.3.5 MYOINHIBITORY PEPTIDE

Myoinhibitory peptides are neuropeptides which have been shown to be tightly linked to body weight, (Min *et al.*, 2016). It was shown that silencing of Mip neurons significantly increased body weight through increased feeding and increased TAG content, (Min *et al.*, 2016). Silencing of MIP neurons and *mip* gene expression led to obesity in flies mainly by increasing their feeding, suggesting that Mip is involved in regulation of feeding behaviour and satiety in flies because activation of MIP neurons made flies moderately avoidant of food odours, producing a characteristic anorexic symptom, (Min *et al.*, 2016).

5.4.3.6 SMALL NEUROPEPTIDE-F

Small neuropeptide-F (sNPF) is a regulatory peptide produced by endocrine cells in the midgut and found in neurones in the hypocerebral ganglion protruding to the anterior midgut, (Veenstra et al., 2008). sNPF shows structural similarity to vertebrate NPY, but is much smaller, consisting of 11 amino acids. Hong and colleagues have shown that sNPF regulates food intake in Drosophila, (Hong et al., 2012). It was shown that snpf knockdown showed a trend in reducing relative food consumption whereas overexpression of *snpf* showed an increasing trend in relative food consumption, (Hong et al., 2012). Hong and colleagues demonstrated that food intake is regulated through an evolutionary conserved pathway involving sNPF and dFOXO, in Drosophila and mammals, (Hong et al., 2012). The roles of sNPF in feeding are different, it was shown that sNPF signalling has been found to stimulate food intake and growth during larval development and might signal in circuits that regulate the rate of feeding and release of insulin-like peptides, (Lee et al., 2008; Hong et al., 2012). Root and colleagues have shown that sNPF receptor is expressed in the olfactory sensory neurones and is required for food-search behaviour in response to starvation, (Root et al., 2011). The increase in snpf in response to starvation, increased sensitivity to food-related odours and so there was a higher probability of flies reaching a food odour target. However, neither snpf nor its receptor were found in the olfactory system in the larva, as opposed to adults, (Carlsson et al., 2013). Oh and colleagues more recently have shown that sNPF is a functional neurotransmitter, (Oh et al., 2019). It was determined through knockdown of snpf and snpfr, that sNPF alters the activity of insulin producing cells (IPC) and/or the cc. The activity of IPCs was significantly stimulated by application of *snpf* whereas cc activity was significantly inhibited by snpf suggesting a regulatory role of sNPF in glucose homeostasis, (Oh et al., 2019).

5.4.3.7 FMRFAR

FMRFamide is a neuropeptide which had been shown to simultaneously promote energy expenditure and suppress food intake through its cognate receptor FMRFaR, both contributing to fat loss in *Drosophila*, (Song *et al.*, 2023). Song and colleagues have demonstrated the effects of FMRFamide in response to protein-rich diet, which were attributed to dietary cysteine intake, (Song *et al.*, 2023). It was shown that dietary cysteine promoted the production of FMRFa, which acts on its cognate receptor FMRFaR. The knockout of *fmrfa* and knockdown of *fmrfar* blocked the fat loss effects of dietary cysteine, but also decreased the feeding suppression mediated by dietary cysteine, (Song *et al.*, 2023). It might be speculative to think that trametinib treatment may affect the expression of *akh* leading to preferential feeding of yeast instead of sugar in flies fed HSD, thereby increasing FMRFaR signalling leading to fat loss and suppression of feeding.

5.4.3.8 DIETARY HORMONE 31

An alternative explanation to the trametinib induced phenotype is through the regulation of excretion, specifically sugar excretion. Dietary hormone 31 (*dh31*), has been studied in the isolated guts of *Drosophila* larvae where DH31 increased the contraction frequency in the anterior midgut, (Lajeunesse *et al.*, 2010). Dh31 was required for peristaltic movement in the junction region between the anterior portion and acidic region of the larval midgut of *Drosophila*, (Lajeunesse *et al.*, 2010). Additionally, it was suggested that Dh31 stimulates fluid secretion across the Malpighian tubules, (Vanderveken and O'Donnell, 2014). In a more context-dependent manner, Dh31 has been shown to trigger intestinal contractions and foster bowel emptying gut movements, especially in the infected status, to eliminate harmful bacteria, (Benguettat *et al.*, 2018). However, it would be interesting to investigate whether expression of *dh31* is affected by HSD and trametinib treatment to excrete dietary sugar, leading to trametinib-associated phenotypes.

The exploration of genes in cluster one suggests that trametinib may elicit its phenotypic effects by regulation of neuropeptide production and processing, which has been previously associated with feeding behaviour and regulation of storage molecules. Alternatively, and perhaps additionally, trametinib may influence the gut mobility of flies fed HSD, leading to excess sugar being excreted rather than stored.

5.4.4 INVESTIGATION OF POTENTIAL MOLECULAR ANALYSIS – CLUSTER 2

The exploration of the genes identified in the second cluster suggested that a potential mechanism by which trametinib elicits its phenotypic effects is through the regulation of oxidative stress, glycolytic flux and muscle homeostasis through changes in the expression of genes such as nitric oxide synthase (*nos*), Vitamin-D₃-Upregulated-Protein 1 (*vdup1*) involved in oxidative stress; *thin* (*tn*) involved in glycolytic flux and *Myosin heavy chain* (*mhc*), *upheld* (*up*) involved in muscle homeostasis and the antimicrobial response, respectively. The exploration of genes of interest from cluster two are described below.

5.4.4.1 NITRIC OXIDE SYNTHASE

Nitric oxide synthase (*nos*) is an enzyme involved in the production of nitric oxide (NO), which is suggested to cause DNA damage, protein modification and cell toxicity mainly via the reactive species, peroxynitrite, (Hunot *et al.*, 1996; Liberatore *et al.*, 1999). In *Drosophila* models of Parkinson's disease, flies show loss of dopaminergic neurons,. NO has been previously implicated in Parkinson's disease (Steinert *et al.*, 2010; West and Tseng, 2011), and in a specific context of a *Drosophila* model of Parkinson's disease, *nos* has been shown to increase dFOXO-mediated dopaminergic neuron loss, whereas administration of the Nos inhibitor, L-NAME, and reduction of Nos ameliorated the deleterious effects of dFOXO mediated Parkinson's disease, (Kanao *et al.*, 2012). A study by Kim and colleagues has tested the role of cofactor tetrahydrobiopterin (BH4), a known co-factor of Nos, and showed that BH4 induces satiety in *Drosophila* by inhibiting NPF neurons, (Kim *et al.*, 2017). However, it was shown that NPF neuron specific knockdown of *nos* did not affect feeding behaviour, suggesting that Nos elicits the phenotypic effects, observed in this study, through an alternative mechanism, (Kim *et al.*, 2017).

NO at a high concentration is an effector to kill pathogens during the insect immune response, and functions as a messenger at a low concentration to regulate antimicrobial peptide (AMP) production insects, (Chen *et al.*, 2022). Chen and colleagues have showed that bacterial infection in *Drosophila* increased the transcription of *nos* which subsequently increased NO concentration in larval haemolymph whereas knockdown of *nos* reduced NO-induced expression of AMPs, (Chen *et al.*, 2022). The role of the innate immune system in *Drosophila* has been previously shown to shift anabolic lipid metabolism from TAG storage to phospholipid synthesis to support immune function, (Martinez *et al.*, 2020). Martinez and colleagues have shown that expression of constitutively active *toll*, master regulator of AMPs, in the larval fat body, reduced whole-animal TAG levels, (Martinez *et al.*, 2020). Additionally, it was shown that *toll* expression negatively regulated TAG synthesis, by decreasing the expression of genes such as *lipin*, involved in metabolising phosphatidic acid into DAG, and *midway*, involved in

metabolising DAG into TAG, (Martinez *et al.,* 2020). On the other hand, expression of *toll* led to increased expression of genes involved in phospholipid synthesis of phosphatidylethanolamine and phosphatidylcholine, (Martinez *et al.,* 2020). Altogether, this may suggest that trametinib may increase the expression of AMPs via *nos* leading to decreased TAG synthesis and increased phospholipid production.

5.4.4.2 VDUP1

Vdup1, otherwise known as TXNIP, is a negative regulator of thioredoxon-1 (TRX) that has been shown to play a crucial role in the response to oxidative stress, (Oberacker *et al.*, 2018). Oberacker and colleagues have shown that knockdown of TXNIP increased median lifespan in *Drosophila*, whereas overexpression of TXNIP decreased survival, (Oberacker *et al.*, 2018). An indicator of DNA double strand breaks and oxidative DNA damage is phosphorylation of histone H2a, which was shown to increase when TXNIP was overexpressed, in S2 cells, where treatment of S2 cells with ROS-scavenger N-acetyl cysteine blocked the H2a phosphorylation indicating that TXNIP-mediated DNA damage was ROS dependent, (Oberacker *et al.*, 2018).

5.4.4.3 THIN

Thin (tn), is a known factor that promotes muscles development and a candidate for coordinating metabolism with cell growth, (Lazzari and Meroni, 2016). Bawa and colleagues have shown that Tn binds glycolytic enzymes such as Pgi, Ald, Pgk, Pglym, and PyK, (Bawa *et al.,* 2020). Additionally, it was shown that *tn* mutation in larvae reduced the abundance of glycolysis intermediates such as lactic acid, pyruvic acid, glycerol-3-phosphate and 2-hydroxy-glutarate, (Bawa *et al.,* 2020). The effects of *tn* mutation were shown to reduce the glycolytic flux and subsequent reduction of cellular biomass and cell growth; however, the effects were tested in larvae, therefore it would be interesting to measure whether *tn* mutation would have a similar effect on glycolytic flux in *Drosophila* adults, (Bawa *et al.,* 2020).

5.4.4.4 MYOSIN HEAVY CHAIN

The *Myosin heavy chain (mhc)* gene encodes a muscle-specific motor that mediates contractions in *Drosophila*. Early work by Montana and Littleton, (2004), showed that a single point mutation in the ATP binding/hydrolysis domain of *mhc* led to degeneration of the flight muscles, (Montana and Littleton, 2004). *Mhc* mutation induced hypercontractile myopathy in *Drosophila* third instar larvae, (Montana and Littleton, 2004). Perkins and colleagues carried out an RNAi-mediated knockdown screen in *Drosophila*, targeting fly

cytoskeleton and associated proteins and showed that knockdown of *mhc* led to severe impairment of climbing ability in flies, (Perkins *et al.*, 2014). Furthermore, it was shown that Mhc is required for the maintenance of correct sarcomere and thin filament length, (Perkins *et al.*, 2014). Mutagenesis analysis by Dahl-Halvarsson and colleagues, generated an *mhc* mutation, which was predicted to disrupt the heptad repeats of the alpha-helical coiled coil C-terminal rod domain of myosin leading to disruption of the sarcomere structure and myofibril damage resembling those observed in Laing distal myopathy, (Dahl-Halvarsson *et al.*, 2018). Also it was shown that expression of the mutated protein in the indirect flight and jump muscles reduced movement and decreased lifespan, (Dahl-Halvarsson *et al.*, 2018). However, the overexpression of *tn* was able to improve the muscle function in *mhc* mutant animals as jump ability was restored in flies overexpressing muscle-specific *tn*, showing a relationship between *tn* and *mhc* in muscle maintenance, (Dahl-Halvarsson *et al.*, 2018).

5.4.4.5 WUPA

Most mutations affecting the indirect flight muscles were isolated during mutagenesis screens for flightless behaviour, summarized by Oas et al., (2014). Most of the flightless mutants are known to show muscle phenotypes categorized as 'hypercontraction' mutants. Hypercontraction of muscle fibres is often associated with muscle defects like thinning and tearing, following uncontrolled acto-myosin interactions in normally assembled sarcomeric structure, (Nongthomba et al., 2003). The wings-upA (wupa) gene encodes the single troponin I in Drosophila genome. A range of wupa mutations have been generated such as wupa^{hdp-2} and wupa^{hdp-3}. Wupa^{hdp-2} is a recessive mutation involving a single amino-acid change A116V in the region of Wupa that interacts with troponin C. This mutation is recessive in nature and homozygous flies have been shown to have muscle hypercontraction, but also show muscle tearing in the context of up mutation, (Firdaus et al., 2015). The wupa^{hdp-2} mutation in larvae has been shown to cause muscle defects leading to decreased crawling and feeding compared to wild-type larvae, but the behavioural phenotypes of *wupa^{hdp-2}* mutation have not been tested in adult flies, (Naimi et al., 2001). On the other hand, wupahdp-3 mutation is a splice mutation in the intron preceding exon 6b1, which is specifically expressed within the indirect flight muscles. The wupa^{hdp-3} mutation leads to a troponin I-null condition in indirect flight muscles, The heterozygous mutants of wupa^{hdp-3} are flightless and show hypercontraction muscles in 30% of all flies, (Nongthomba et al., 2003).

5.4.4.6 UPHELD

Upheld (up), gene encodes the striated muscle protein Troponin T. In Drosophila the regulation of striated muscle contraction is mediated by calcium-dependent myosin cross-bridge cycling on actin by the thin filament troponin-tropomyosin complex, alterations of which can induce contractile dysregulation and disease, (Viswanathan et al., 2013). For instance, mutations in the troponin T tropomyosin-binding region causes cardiomyopathy in Drosophila, (Viswanathan et al., 2013). Viswanathan and colleagues utilised live imaging of Drosophila cardiac tubes to reveal that an up heart-specific knockout mutant led to cardiac dysfunction and showed remodelling of heart tissue comparable to human restrictive cardiomyopathy, (Viswanathan et al., 2013). It was suggested that mutation of up led to perturbations of critical intermolecular associations of tropomyosin-troponin leading to myopathy in Drosophila, (Viswanathan et al., 2013). More recently, Chatteriee and colleagues has showed that indirect flight muscles are an immune-responsive tissue. Flies with up and wupa knockout showed reduced survival in response to bacterial infection, (Chatterjee et al., 2016). It was shown that flies lacking intact indirect flight muscles, via knockout of genes such as up and wupa reduced the production of antimicrobial peptides, suggesting that intact indirect flight muscles are required for production of antimicrobial peptides, (Viswanathan et al., 2013).

Previously, it has been shown that HSD in *Drosophila* leads to cardiac dysfunction, (Na *et al.*, 2013; Gera *et al.*, 2024). Therefore, it is intriguing to speculate that trametinib may elicit its life extending properties by preventing the diet-induced cardiac dysfunction, coupled with increased expression of antimicrobial peptide production due to direct effects on indirect flight muscles and nitric oxide synthase, leading to lipid phenotypes as observed in trametinib-treated flies fed a HSD.

The exploration of genes in cluster two therefore suggests that trametinib may elicit its phenotypic effects by regulation of genes involved in muscle maintenance and the innate immune response.

5.4.5 INVESTIGATION OF POTENTIAL MOLECULAR ANALYSIS – CLUSTER 3

The exploration of the genes identified in the third cluster suggested that a potential mechanism by which trametinib elicits its phenotypic effects is through the regulation of feeding behaviour or excretion as well as by the regulation of sleep and glutamate processing. The *straighjacket* (*stj*), *Resistant to dieldrin* (*rdl*), *Shaker* (*sk*), *Grik*, glutamate-gated chloride channel subunit alpha (*glucalpha*), and glutamate receptor IID (*glurIID*) involved in glutamate processing; *Ion transport peptide* (*itp*) is involved in regulation of feeding behaviour and excretion; *Hyperkinetic* (*hk*) is involved in sleep.

5.4.5.1 STRAIGHTJACKET

Straitjacket (stj), encodes a subunit of the Drosophila voltage-gated calcium channel (VGCC) that is required for synaptic vesicle fusion with the plasma membrane and neurotransmitter release, (Tian et al., 2015). Tian and colleagues have shown that stj knockout resulted in autophagic defects and a great accumulation of autophagic vacuoles suggesting that VGCC is required for complete autophagy, (Tian et al., 2015). Additionally, stj knockout led to accumulation of synaptic vacuoles suggesting that autophagy defect affects the release of neurotransmitters, yet a loss of neuronal synaptobrevin, a master regulator of neurotransmitter release, led to accumulation of synaptic vesicles without the autophagy phenotype of stj knockout, indicating that the loss of neurotransmitter release is not causing the autophagy phenotype of stj mutants yet may be a secondary effect of sti knockout, (Tian et al., 2015). Alternatively, sti shows expression in the fat cells of adult flies based on Fly Cell Atlas, (Li et al., 2022). The role of autophagosome-based lipophagy has been shown as an alternative mechanism to lipid droplet breakdown in yeast, (Toulmay and Prinz, 2013). Even though the exact contact sites between lipid droplets and autophagosomes remain unclear, it is intriguing to speculate that perhaps stj could influence lipid autophagy in the fat body of Drosophila leading to the trametinib-associated phenotype of reduced TAG on HSD.

5.4.5.2 ION TRANSPORT PEPTIDE

Galikova and colleagues have identified the first thirst-promoting and anti-diuretic hormone in Drosophila encoded by Itp, (Galikova et al., 2018). It was discussed that functionally, Itp, acts analogously to the human vasopressin and renin-angiotensin systems, where the expression of *ltp* is elevated by dehydration and the peptide increases thirst while repressing excretion, thereby promoting the conservation of water resources, (Galikova et al., 2018). The over-expression of Itp resulted in increased water content whereas knockdown resulted in decreased water content, (Galikova et al., 2018), showing that Itp, plays a diuretic role. Additionally, Itp was shown to regulate food intake with *itp* knockdown increasing food intake and *itp* overexpression reducing it (Galikova et al., 2018). A more recent study by Galikova and colleagues showed that *Itp* codes for a catabolic regulator which regulates energy expenditure, feeding behaviour and transit of the meal through the digestive tract, (Galikova and Klepsatel, 2022). It was shown that overexpression and knockdown of *ltp* led to reduced starvation survival in both male and female flies, associated with a reduction in both stored fat and glycogen in these flies, (Galikova et al., 2022). The ability of Itp to regulate glycemia was shown to be Akh dependent, but the requirement of Itp for lipid and glycogen homeostasis were Akh independent, (Galikova *et al.*, 2022). Itp was shown to act upstream of Akh to regulate its secretion as well as regulating transcription of the Akh receptor gene, *akhr*, (Galikova *et al.*, 2022).

5.4.5.3 HYPERKINETIC

Hyperkinetic (*hk*) is a beta modulatory subunit of the protein *Shaker* which encodes a structural subunit of alpha subunit of the voltage-gated potassium channel. It has been shown that *Shaker* mutant flies are short sleeping, suggesting that *Shaker* plays a regulatory role in regulating daily sleep in *Drosophila*, (Bushey *et al.*, 2007). Bushey and colleagues have shown that loss-of-function mutation of *hk* leads to reduced sleep however the necessity of *sh* for normal sleep is not known yet, (Bushey *et al.*, 2007). Sleep is thought to be important for health, cognition and memory, (Durmer and Dinges, 2005), whereas HSD in *Drosophila* has been shown to reduce sleep, (Catterson *et al.*, 2010). Therefore, it may be that trametinib prevents the deleterious effects of HSD on sleep throught regulation of *sh* and *hk* transcription. Additionally, it was shown that loss-of-function mutation of *hk* decreased lifespan in *Drosophila* while increasing waking activity, (Bushey *et al.*, 2010).

5.4.5.4 GRIK

Grik encodes a glutamate receptor in *Drosophila* that is mostly analogous to the human kainite receptor family. It is ubiquitously expressed in the central nervous system with considerable evidence indicating its presence at both the pre- and postsynaptic side regulating transmission of information and excitability in a synaptic-specific manner, (Lerma, 2006). It is widely accepted that, in mammals, presynaptic kainite receptors control the release of aminobutyric acid (GABA) in the hippocampus, (Mulle *et al.*, 2000; Maingret *et al.*, 2005). Furthermore, inhibition of the kainite receptor prevented epileptic activity in rats, suggesting that the excitability of the neuron network is under fine control by the kainite receptor, (Smolders *et al.*, 2002). In *Drosophila*, Aw and colleagues have shown that increased expression of *Grik* elevated glutamate receptor levels and increased excitotoxicity leading to decreasing glial numbers and defective climbing ability, (Aw *et al.*, 2017).

5.4.5.5 RESISTANT TO DIELDRIN

Resistant to dieldrin (rdl) encodes a widely expressed ligand-gated chloride channel and ionotropic GABA receptor, which mediates the fast inhibitory effects of GABA. Kim and

colleagues have shown that *sh* promotes sleep via GABA transmission. Rdl acting downstream of Sh, playing an antagonizing role in sleep promotion, (Kim *et al.,* 2020).

5.4.5.6 GLUCALPHA AND GLURIID

Glucalpha encodes a glutamate-gated chloride channel subunit alpha which, together with *Rd*, plays an important role in mediating synaptic inhibition and regulates cellular excitability, yet no glutamate-gated chloride channels have been identified in vertebrates, (Cully *et al.*, 1996; Knipple and Soderlund, 2010). *GlurIID* encodes a subunit of the muscle glutamate receptor that responds to neurotransmitter glutamate and the neuromuscular junction, (Han *et al.*, 2015).

There is not a lot of literature looking at the effects of diet-induced obesity and ERK inhibition on *glucalpha* and *glurIID*, however it is part of the glutamate metabolism network therefore further investigation may be warranted.

The exploration of genes in cluster three suggests that trametinib may elicit its phenotypic effects through the regulation of genes involved in glutamate metabolism, regulation of thirst and feeding but also sleep.

Previously, it has been shown that HSD in *Drosophila* leads to thirst as well as the formation of Malpighian tubule deposits which shorten the lifespan of *Drosophila* which could be rescued with water supplementation, (van Dam *et al.*, 2020). The effects of HSD on glutamate metabolism is not well established in *Drosophila*, however a study by Sketriene and colleagues in rats has shown that high-fat high-sugar food is associated with "addiction-like" glutamatergic dysfunction in obesity prone rats, suggesting an aversion role of glutamate in animals, albeit requiring further investigation in *Drosophila*, (Sketriene *et al.*, 2022). Additionally, trametinib may prevent the deleterious effects of HSD on reduced sleep by regulating the expression of *sh* and *hk* genes leading to normal sleep in HSD flies.

5.4.6 INVESTIGATION OF POTENTIAL MOLECULAR ANALYSIS - CLUSTER 4

The exploration of the genes identified in the fourth cluster suggested that a potential mechanism by which trametinib elicits its phenotypic effects is through the regulation of the electron transport chain and subsequently oxidative phosphorylation. The NADH dehydrogenase subunits 23, 24, B14.5b and 49, (ND-23, ND-24, ND-B14.5B and ND-49, respectively) form the functional and structural subunits of the Complex I of the electron transport chain (ETC). The ubiquinol-cytochrome c reductase subunits -6.4 and 14 (UQCR-6.4, UQCR-14) and oxen (Ox) are part of the Complex III of the ETC. Additionally,

croquemort (crq) encodes a lipid specific scavenger receptor.

5.4.6.1 NADH DEHYDROGENASE (-23, -24, -B14.5B, -49)

The first step of the ETC is catalysed by complex I being NADH oxidation upon which electrons are transferred along a chain of seven iron-sulphur (FeS) clusters to a ubiquinone binding pocket at the base of complex I, (Sousa *et al.*, 2018). One of the subunits in the binding pocket is ND-49, forming the hydrophilic arm of complex I, (Sousa *et al.*, 2018). The hydrophilic arm consists of the dehydrogenase domain involved in the oxidation of NADH and transfer of electrons to quinone and the quinone-binding domain. One of the subunits of the dehydrogenase domain is encoded by *Nd-24*. The interface between the membrane and hydrophilic arm of Complex I is comprised of a quinone-binding domain, with ND-23 being a structural subunit, (Sousa *et al.*, 2018). Lastly, *nd-b14.5b* may encode an accessory subunit of complex I but its role is not well established.

In *Drosophila*, mutation in mitochondrial oxidative phosphorylation complex I are associated with pathologies leading to complex I being recognised as a crucial regulator of animal longevity. For example, Tong and colleagues have shown that increased activation of mitochondrial complex I reduced ROS production and oxidative damage, leading to increased lifespan in *Drosophila*, (Tong *et al.*, 2007). Much of the work characterising the adult lifespan effects of decreased complex I subunit expression in *Drosophila* involved RNAi knockdown of *cg9172* and *cg9762*, (Copeland *et al.*, 2009). Copeland and colleagues have shown that adult-specific RNAi knockdown of *cg9172* and *cg9762* increased median lifespan in female flies by 11% and 46%, respectively, while neuron-specific RNAi knockdown of *cg9172* and *cg9762* increased lifespan by 6% and 24%, respectively, (Copeland *et al.*, 2009). The reduction of complex I subunit expression, can therefore prolong lifespan in flies, and there is a clear relationship between complex I function and ageing.

On the other hand, simply overexpressing complex I to increase activity is currently technically impossible, as complex I is a holoenzyme comprised of more than 40 subunits, some of which are encoded by the mitochondrial genome, (Carroll *et al.*, 2006). However, several studies have investigated the impact of stimulating mitochondrial activity in aging flies by two independent approaches: upregulation of the *Drosophila* PGC-1 homolog or by transgenic expression of alternative single-subunit complex I enzymes.

In mammals, the PGC-1 family of transcriptional activators play a regulatory role in mitochondrial biogenesis and activity, (Lin *et al.*, 2005). Recently, Rera and colleagues showed that overexpression of the *Drosophila* PGC-1 homolog, *spargel*, was sufficient to increase mitochondrial activity based on three independent mitochondrial markers: amount of mitochondrial DNA, enzymatic activity of citrate synthase, a key enzyme in the

Krebs cycle, and abundance of HSP60, a mitochondrial matrix protein, all of which were upregulated upon ubiquitous overexpression of *spargel*, (Rera *et al.*, 2011). The ubiquitous overexpression of *spargel* also increased TAG storage and decreased lifespan, yet fat body- and gut-specific overexpression of *spargel* increased lifespan, (Rera *et al.*, 2011). In addition to increased lifespan, overexpression of *spargel* reduced ROS levels and improved gut integrity in the aging fly, (Rera *et al.*, 2011).

Additionally, several studies have investigated the function of alternative enzymes that bypass or replace the proton-translocating complexes of the mitochondrial respiratory chain. Alternative oxidates to NADH dehydrogenase are members of the Ndi family. Sanz and colleagues expressed alternative yeast NADH dehydrogenase Ndi1 in Drosophila which increased median lifespan of flies fed both normal and restrictive diets, as well increasing substrate oxidation and reducing ROS production, thereby decreasing oxidative damage, (Sanz et al., 2010). This suggested that modulation of ROS production at complex I offers a way to manipulate the lifespan of Drosophila by mitigating key sources of age-associated damage, (Sanz et al., 2010). A more recent study by Gospodaryov and colleagues expressed a different alternative NADH dehydrogenase (aNADH) in Drosophila from Ciona intestinalis which extended median lifespan by 17-71% depending on the protein: carbohydrate ratios in the diet, (Gospodaryov et al., 2020). Lifespan extension in aNADH-expressing flies was suggested to be due to increased resistance to xenobiotics such as 2,4-dichlorophenoxyacetic acid, alloxan and catechol, but also to inorganic compounds such as potassium iodate, sodium molybdate and sodium chromate, (Gospodaryov et al., 2020). The aNADH from yeast, Ndi1, has also been studied by Vilain and colleagues in a pink1-mediated Parkinson's disease model in Drosophila. Expression of aNADH rescued the deleterious mitochondrial defects of pink1 mutation by improving mitochondrial fission, but failed to improve enzymatic deficiency at the level of complex I suggesting a complex I-independent role of aNADH which improves mitochondrial morphology in this disease model, (Vilain et al., 2012). The effects of high caloric diets on mitochondrial morphology and function have been studied in a range of model organisms. For example, Alcantar-Fernandez and colleagues fed a range of HSDs to C.elegans and showed that HSD led to reduced NADH dehydrogenase activity, (Alcantar-Fernandez et al., 2019). In Drosophila, high fat diet was shown to cause a reduction in mitochondrial respiration, while increasing production of ROS, (Cormier et al., 2021). High fat diet in Drosophila was also shown to change the lipid profile of flies, reducing the proportion of most long chain saturated fatty acids, and decreasing long chain monounsaturated fatty acids, while all measured long chain polyunsaturated fatty acids increased in abundance, (Cormier et al., 2021). It was shown that high fat diet reduced the activity of complex I, explaining the metabolic inflexibility in processing complex I substrates, leading to higher free radical leak in Drosophila fed high fat diet,

(Cormier et al., 2021).

It Is currently hypothesised that lifespan extension results from both decreased and increased complex I function in part due to discrete mechanisms. The increase in complex I function could improve mitochondrial homeostasis through increase NAD+ production, which has been shown to increase mitochondrial stress response and lifespan through activation of sirtuins, (Mouchiroud *et al.*, 2013). On the other hand, the disease in complex I function could increase ROS production leading to lifespan extension through a hermetic mechanism leading to activation of mitochondrial stress response, (Hur *et al.*, 2014).

5.4.6.2 CROQUEMORT

Croquemort/CD36 (*cqr*) encodes a member of the scavenger receptor family which promotes apoptotic cell clearance and plays a role in phagosome maturation but is also implicated in lipoprotein scavenging, fatty acid transport and innate immune signalling, (Woodcock *et al.,* 2015). Woodcock and colleagues have shown that a chronic lipid-rich diet in *Drosophila* leads to activation of JAK-STAT signalling and reduced insulin signalling, hyperglycaemia and shortened lifespan, which was shown to be mediated by expression of the cytokine *upd3* in a *cqr*-dependent manner, (Woodcock *et al.,* 2015). Expression of *upd3* in flies fed with a high fat diet was dependent on *cqr* expression as *crq* knockdown diminished the expression of *upd3* in flies fed high fat diet, (Woodcock *et al.,* 2015).

5.4.6.3 UBIQUINOL-CYTOCHROME C REDUCTASE (UQCR-6.4, UQCR-14)

Ubiquinol-cytochrome C reductase is predicted to be part of the complex III of the ETC and is involved in mitochondrial electron transport from ubiquinol to cytochrome C. Complex III is the third enzyme complex in the ETC which couples the electron transfer from ubiquinol to cytochrome C with the translocation of protons across the intermitochondrial membrane, (Trumpower and Gennis, 1994). Complex III is a symmetrical dimer, with three core subunits that are highly conserved from bacteria to mammals, (Yang and Trumpower, 1986). Additionally, complex III contributes to the production of ROS in the cell. In contrast to complex I which generates superoxide exclusively in the matrix, complex III releases ROS in the inter-mitochondrial spaces and possibly the metric, (Muller *et al.*, 2004). Studies investigating complex III have shown evidence of complex III-derived ROS contributing to the cellular response to hypoxia and related cardioprotective ischemic preconditioning, (Chandel, 2010) and adipocyte differentiation, (Tormos *et al.*, 2011).

The exploration of genes in cluster four suggests that trametinib may elicit Its phenotypic effects by regulating complex I and III subunit expression, previously shown to play a role in mitochondrial homeostasis. Softic and colleagues have shown that high fructose diet in mice led to lower NADH levels and increased ROS levels suggesting that fructose promotes mitochondrial dysfunction, (Softic et al., 2019). Additionally, high fructose diet led to the most significant decrease in transcripts of ubiquinol-cytochrome C reductase subunits and NADH:ubiquinone oxidoreductase subunits, (Softic et al., 2019). The NAD/NADH ratio is an important factor for ageing and development of age-related diseases, as they are cofactors in redox reactions and coenzymes in metabolic processes. Additionally, NAD/NADH act as substrates for sirtuins, previously shown to play a role in ageing, (Poljsak and Milisav, 2016; Poljsak et al., 2023). For instance, lowering of the NAD/NADH ratio and subsequent ATP depletion has been implicated in mitochondrial dysfunction and senescence in mammalian cells, (Wiley et al., 2016). Most studies have investigated the effects of NAD+ depletion, previously described to cause mitochondrial dysfunction, accumulation of ROS and consequent decrease of sirtuin levels, promoting ageing, (Guarante, 2014). However, it may be interesting to investigate whether trametinib elicits its effects through regulation of NAD/NADH ratio leading to prevention of mitochondrial dysfunction and subsequent lifespan and healthspan benefits.

A potential mechanism by which hyperglycaemia induces mitochondrial dysfunction and causes deleterious effects is through the activation of protein kinase C (PKC) via increased *de novo* synthesis of DAG, (Koya and King, 1998). In diabetic mice, abnormal activation of PKC led to decreased glomerular production of nitric oxide and decreased production of nitric oxide in smooth muscle cells, (Ishii *et al.*, 1996; Craven *et al.*, 1994). The abnormal activation of PKC led to abnormalities in retinal and renal blood flow possibly through the depression of nitric oxide production, (Ishii *et al.*, 1996). On the other hand, treatment of diabetic mice with PKC inhibitors, significantly reduced diabetes-induced retinal abnormalities and normalized glomerular filtration rate, showing that PKC inhibition may ameliorate some diabetes-associated complications, (Brownlee, 2001).

It Is Intriguing to think that perhaps trametinib elicits Its effects through the regulation of transcripts encoding functional components of the ETC, thereby preventing the deleterious effects of HSD on the ETC, leading to reduced ROS production and subsequent healthspan effects.

5.4.7 POST TRANSLATIONAL EFFECT OF TRAMETINIB TREATMENT OF HIGH SUGAR FED DROSOPHILA

Altogether, this chapter has demonstrated that trametinib affects a range of discrete gene networks which could potentially explain the phenotypic effects of trametinib observed in this study. The four gene clusters investigated suggest that trametinib elicits a global transcriptional effect instead of acting through a discrete mechanism. However, most interestingly, the RNA-sequencing analysis did not show any DEGs encoding proteins directly involved in lipogenesis nor lipolysis. The compendium of ERK targets generated by Unal et al., (2017) has shown that as a kinase, ERK phosphorylates enzymes such as Fasn, a multifunctional protein involved in synthesis of palmitate from acetyl-CoA and malonyl-CoA, acting downstream of acetyl-CoA carboxylase, investigated earlier in this study, (Unal et al., 2017). Additionally, ERK phosphorylates sphingosine kinase 1/2 involved in the phosphorylation of sphingosine to form sphingosine-1-phosphate, which acts as a lipid mediator for both intra- and extracellular function. This suggests that perhaps trametinib elicits both transcriptional and post-translational effects on lipid metabolism which is expected since ERK acts both transcriptionally and posttranslationally, (Ram et al., 2023; Yildiz and Kaya, 2021). Therefore, it may be necessary to decipher both the transcriptional and post-translational effects of trametinib treatment to determine a more unified mechanism of action of trametinib against diet-induced obesity and other associated phenotypes.
Chapter 6

6.1 INTRODUCTION

6.1.1 MALPIGHIAN TUBULES

6.1.1.1 STRUCTURE

The purpose of excretion in organisms is to maintain physiological homeostasis through the elimination of potentially harmful substances. The renal system of Drosophila is comprised of two anatomically and functionally discrete organs, nephrocytes and Malpighian tubules. Nephrocytes are a group of specialized cells clustered near the heart that filter the fly's haemolymph, removing waste products through endocytic processes, analogous to the human glomerulus, (Weavers et al., 2009). The Malpighian tubules are an analogous organ to human nephrons, involved in urine constituent production, part of excreta, through active transport of ions, water and organic solutes from the haemolymph into the Malpighian tubule lumen, (Miller et al., 2013). The insect renal tubules were first described by Marcello Malpighi in the 17th century, (Malpighi, 1669). In Drosophila there are two pairs of tubules, with each pair feeding into a common ureter that connects to the junction of the midgut and hindgut. The tubules are not identical: the pair on the right are longer and positioned anteriorly associated with the midgut, whereas the pair on the left are shorter and positioned posteriorly associated with the hindgut, (Rodan, 2019). The tubules primarily and actively produce urine constituents, part of excreta, (potassium ions, water, urate ions, sugar and amino acids) into the main segment of the tubule, comprised of two cell types, principal cells and stellate cells, that is acted on by the lower tubule and hindgut, (Miller et al., 2013). The rapid flow of secretions allows for rapid removal of waste and toxic solutes at the cost of ions, water and solute loss that is balanced by selective hindgut reabsorption, (Cohen et al., 2020). Malpighian tubules are genetically and functionally segmented with majority of the urine constituent generation occurring in the blind-ended tubules in the main segment, (Dow et al., 1994). Subsequently further reabsorption occurs in both lower segments and hindgut, (Yerushalmi et al., 2018), (Figure 6.1).



Figure 6.1: Schematic of *Drosophila* ion and water regulatory Malpighian tubule.

Drosophila Malpighian tubules are located at the bottom of the abdomen. The right pair of tubules projects forward from the insertion point within the hindgut and lie at the anterior end of the abdomen, and the left pair of tubule project backwards from the insertion point within the hindgut and lie at the posterior part of the hindgut. The pair of Malpighian tubules (anterior and posterior), together with the hindgut regulate ionic and osmotic homeostasis of the fly. Urine constituents are generated by the transepithelial movement of ions and water across the main segments of the anterior and posterior tubules resulting in a potassium and chloride-rich fluid. The secreted fluid can contain sodium, and secretions can also occur without potassium. Excreta flows through the downstream lower segment where potassium and water, secreted from the main segment, are reabsorbed. The excreta passes through the ureter and enters the hindgut where more reabsorption of ions and water occurs to match the composition of the excreta dependant on the physiological state of the animal. *Figure adapted from Rodan, 2019.*

6.1.1.2 FUNCTION

Even though the configuration of transporters and channels in the Malpighian tubules differ from the mammalian tubules, the transporters and signalling pathways that regulate them are conserved.

One of the main functions of Malpighian tubules is fluid secretion by the main segment, (Figure 6.2). Fluid secretion involves transepithelial cation flux through the principal cells, while chloride ion flux occurs through the neighbouring stellate cells, (O'Donnell *et al.*, 1996). Fluid secretion is powered by the apical vacuolar proton ATPase (V-ATPase), a multi-subunit transporter homologous to mammalian V-ATPase, (Dow *et al.*, 1994). The V-ATPases generate a positive transepithelial potential which is thought to drive proton/cation exchange across the apical membrane, (O'Donnell *et al.*, 1996).

Drosophila contains cation-chloride cotransporters (NKCC), inducing the sodiumpotassium-2-chloride, encoded by *Ncc69*, (Leiserson *et al.*, 2011). *Drosophila* tubules have two inwardly rectifying potassium channels, encoded by *Irk1* and *Irk2* expressed in the tubules, (Wu *et al.*, 2015). The sodium/potassium-ATPase is required for transepithelial potassium flux by recycling the sodium entering the principal cells through NKCC, (Rodan *et al.*, 2012). The net transepithelial flux of potassium across the principal cell causes a major charge imbalance which is balanced by a flow of chloride. The stellate cells are primarily responsible for chloride ion transport, transported by CLC family chloride channels in *Drosophila*, encoded by *Clc-a*, (Cabrero *et al*, 2014). The mechanism of chloride transport has yet to be defined.

The *Drosophila* genome encodes a family of aquaporin (AQP) proteins which are responsible for transcellular water transport suggested to take place in both stellate and principal cells, (Kaufmann *et al.*, 2005). Calcium transport occurs predominantly in the initial segment which is larger in anterior tubules compared to posterior tubules, (Sozen *et al.*, 1997). Rich deposits of magnesium and calcium have been found in the distal tubules of *Drosophila*, found to be excreted in the initial segment of anterior tubule, (Terhzaz *et al.*, 2005). A phosphate transporter in *Drosophila* is encoded by *Mfs13* and is enriched in the Malpighian tubules, yet phosphate flux has not been measured, (Sozen *et al.*, 1997).

Other functions of Malpighian tubules play a role in innate immunity, detoxification, and circadian regulation. The tubules contain a complete innate immune response pathway which suggests that tubules might play a role in detecting and signalling or even directly defending against bacterial pathogens, (McGettigan *et al.*, 2005). Overexpression of nitric oxide synthase, an enzyme involved in the immune response, in tubules, elevates *Diptericin* levels, an antimicrobial peptide, however other antimicrobial peptide genes were also found to be significantly expressed in the tubules such as *attacin, Metchninikowin* and *Drosomycin,* (McGettigan *et al.,* 2005; Chintapalli *et al.,* 2012).

The Malpighian tubules are also capable of handling toxic molecules but also those that the insect might not have experienced previously such as insecticides. ABC transporters, such as those involved in multidrug resistance, have been documented to be highly expressed in tubules, (Wang *et al.*, 2004). One such gene, *Cyp6g1*, implicated in resistance to the insecticide DDT, has been shown to be abundantly expressed in tubules, (Daborn *et al.*, 2002). An epistasis experiment of *Cyp6g1* expression in tubule principal cells has showed inverse sensitivity to DDT, (Daborn *et al.*, 2002).

Lastly, Malpighian tubules in *Drosophila* contain all elements of the circadian clock, which operate autonomously *in vitro* in isolation from the fly, (Giebultowicz *et al.,* 2000). It is therefore likely that the tubules maintain their own time to optimize their function in anticipation of the insect's needs over a day.



Figure 6.2: Cell model of the Malpighian tubule main segment.

Transporters and channels described in the text are shown in the principal cells and stellate cells. Additionally, Nha1 and Nha2 are apically expressed chloride/proton and sodium/photon exchanges, respectively, (Chintapalli *et al.*, 2015). The apically expressed ligand-gated chloride channel, pHCL-2, has a functional role in fluid secretion, (Feingold *et al.*, 2016). NKCC is required for normal transpithelial potassium flux. The SLC4 family sodium-driven anion exchanger NDAE1 is localised basolateral in the principal cells, (Sciortino *et al.*, 2001). The lkr1/2 are potassium channels shown to have a functional role in transpithelial ion flux and fluid secretion, (Wu *et al.*, 2015). Aquaporin family of proteins (AQP1-7) are responsible for transpithelial water transport and osmotic regulation.

6.1.1.3 MALPIGHIAN TUBULES AS A MODEL FOR RENAL DISEASE

Even though there are significant differences in the origin and function of Malpighian tubules compared to mammalian nephrons, it is still possible to model a range of renal diseases in *Drosophila*. This is due to both systems being functionally analogous: both generate and process urine constituents for the excreta, facilitate the maintenance of ionic and osmotic homeostasis as well as the excretion of waste compounds. Additionally, there is sequence homology between a range of *Drosophila* renal genes and their human orthologs especially in genes coding for transport ATPases, ion channels and ion exchangers.

Kidney stones or nephrolithiasis, and stones in the urinary tract or urolithiasis, are increasing in their prevalence and incidence, and are associated with substantial morbidity, (Ziemba and Matlaga, 2017). Most common forms of stones are of calcium oxalate, (Shoag *et al.*, 2015). The treatment of oxalate stones is crude and limited in its effectiveness because of a lack of a good animal model. Currently, most treatment

involves dietary interventions based on causes of risk factors for calcium oxalate stones. For instance, low urine volume risk factor could be caused by excessive sweating and low fluid intake among others, thereby a suitable treatment to prevent stone formation is to increase fluid intake, (Borghi et al., 1996). High calcium levels in urine (hypercalciuria) could be idiopathic or caused by high vitamin D body content with an appropriate treatment being reducing sodium rich foods or thiazide diuretic therapy, (Borghi et al., 2002). Excess uric acid excretion (hyperuricosuria) is a risk factor for calcium stones caused by excessive purine ingestion as animal protein, which is treated by reducing purine ingestion and/or allopurinol therapy, (Ettinger et al., 1986). However, allopurinol has been discontinued since due to causing renal impairments, gastrointestinal issues, and acute gout flares, (Farshad et al., 2022). Additionally, the use of thiazide diuretics to treat oxalate stones are often ridden with adverse effects such as hyperglycaemia, hyperlipidaemia, metabolic alkalosis and decrease in total blood volume, requiring the use of combined therapeutics to treat side effects, (Akbari and Khorasani-Zadeh, 2023). Fortunately, Drosophila Malpighian tubules have been developed as a model for stone formation using dietary and genetic approaches, (Miller et al., 2013). In particular, oxalate stones can be modelled easily and reproducibly in flies, simply by supplementing the diet with oxalate leading to crystals of oxalate that form within days, (Miller et al., 2013). Additionally, contamination of food with ethylene glycol or melamine can produce renal stones in humans both of which can trigger the formation of stones in Drosophila tubules, (Chen et al., 2011, 2012). One of the treatments available to humans for kidney stones is the consumption of metal citrate salts which are highly soluble. Administration of citrate in Drosophila has been shown to reduce stone burden and extend lifespan, (Chen et al., 2011; Ho et al., 2013). The similarities in stone causation and treatment allows for the possibility that *Drosophila* tubules can be used in chemical screens to identify treatments against stone formation, and such screens have already identified compounds that are effective against lithiasis, (Yang et al., 2018).

More recently, *Drosophila* have been used to study another form of kidney stone, uric acid kidney stones, known as uric acid nephrolithiasis. The formation of uric acid kidney stones is strongly correlated with elevated uric acid levels, leading to low pH, thereby encouraging uric acid kidney stone formation, (KC and Leslie, 2024). Previously, it was shown that elevated uric acid levels are associated with obesity and diabetes, (Masuo *et al.*, 2003; Boyko *et al.*, 2000; Dehghan *et al.*, 2008; Johnson *et al.*, 2009). Masuo and colleagues have used linear-regression analysis to investigate the link between uric acid and obesity and found that uric acid is a significant determinant factor of changes in basal body mass index, (Masuo *et al.*, 2003). Additionally, high uric acid levels were shown to be a strong and independent risk factor for diabetes, (Dehghan *et al.*, 2008, Bhole *et al.*, 2011). Likewise, diet-induced obesity has been associated with type 2 diabetes, and has

become a major global issue commonly attributed to increased consumption of sugary drinks and foods, and many co-morbidities were associated with type 2 diabetes, such as nephropathy, (Chatterjee *et al.,* 2017). A common symptom of hyperglycaemia associated with diet-induced obesity is thirst and it was previously shown that thirst is associated as a risk factor for nephrolithiasis, (Wang *et al.,* 2022).

Due to evolutionary conservation of central signalling pathways regulating metabolism and energy homeostasis, *Drosophila* makes a powerful model system to study dietary interventions associated with metabolic disorders, (Padmanabha and Baker, 2014). Van Dam and colleagues have shown that a high sugar (20% sucrose) diet leads to significant dehydration in *Drosophila* based on decreased haemolymph volume, as well as reduced median lifespan. Interestingly, water supplementation completely rescued both the dehydration and shortened lifespan phenotypes associated with high sugar diet, (van Dam *et al.*, 2020). Additionally, van Dam and colleagues showed that high sugar diet was associated with tubule dysfunction with high uric acid levels and decreased tubule pH leading to the formation of tubule deposits, (van Dam *et al.*, 2020).

High sugar diet was suggested to provide precursors for purine biosynthesis, leading to increased levels of uric acid, and similar phenotypes of elevated uric acid levels and tubule deposits were observed with purine supplementation of the fly diet, (van Dam *et al.*, 2020). Furthermore, the use of allopurinol, a purine analogue which inhibits xanthine oxidase to block uric acid production (Bratty *et al.*, 2011) was found to inhibit tubule deposit formation and was able to partially restore reduced lifespan on high sugar diet. It was therefore suggested that high sugar diet reduces lifespan partially due to increased purine biosynthesis leading to Malpighian tubule deposit formation and dehydration, (van Dam *et al.*, 2020).

The role of the RAS/MAPK pathway in uric acid nephrolithiasis is not well studied although RAS/MAPK signalling has been previously linked to *de novo* purine synthesis, (Ali *et al.,* 2020). The ERK signalling network, when active, promotes anabolic metabolism typically through the transcription factor, c-MYC, in mammalian systems, (Kerkhoff *et al.,* 1998). The ERK-MYC axis controls a range of metabolic processes including nucleotide metabolism, (Liu *et al.,* 2008). ERK signalling also regulates *de novo* pyrimidine synthesis via phosphorylation of the rate-limiting enzymes in this metabolic pathway, (Graves *et al.,* 2000). More recently, Ali and colleagues identified that ERK directly phosphorylates a canonical purine synthesis enzyme, PFAS, involved in *de novo* purine synthesis. Ali and colleagues, have shown that physiological and oncogenic activation of ERK, using epidermal growth factor (EGF) and mutations of RAS (RAS^{G12}) respectively, enhanced *de novo* purine synthesis in mammalian cells, (Ali *et al.,* 2020). Additionally, enhanced *de novo* purine synthesis in response to activation of ERK, was shown to be inhibited by a MEK specific inhibitor U0126 and ERK specific inhibitor SCH772984, (Ali *et al.,* 2020).

The use of MEK and ERK specific inhibitors, decreased phosphorylation of ERK as well as decreased the abundance of purine synthesis intermediate metabolites which was shown to be due to the decrease in activation of a key *de novo* purine synthesis enzyme, PFAS, a direct substrate of ERK, (Ali et al., 2020). Additionally, studies in mammalian systems have shown that ERK1/2 is a key initiator of renal injury. Collier and colleagues have shown that ERK1/2 phosphorylation leads to an increase in the expression of kidney injury molecular-1 (KIM-1). KIM-1 is a glycoprotein that is expressed at low levels in healthy kidneys and is upregulated during injury, (Collier et al., 2018). ERK1/2 phosphorylation and KIM-1 mRNA expression were shown to be increased following kidney injury, which was attenuated by the MEK-specific inhibitor, trametinib, (Collier et al., 2018). It was further revealed that ERK1/2 phosphorylation during kidney injury leads to a decrease in the rate-limiting NAD⁺ enzyme, NAMPT, involved in production of NAD⁺, a decrease which was attenuated in response to ERK1/2 inhibition by trametinib which prevented renal dysfunction, (Collier and Schnellmann, 2020). Lastly, a study by Andrikopoulos and colleagues has shown that trametinib treatment ameliorated kidney fibrosis by suppressing ERK1/2 phosphorylation in mice fed with an adenine-rich diet, suggesting a link between the inhibition of ERK1/2 activation by trametinib and alleviation of the deleterious effects of kidney injury or high purine diet, (Collier et al., 2018, Andrikopoulos et al., 2019).

6.1.2 RAS/MAPK PATHWAY AND DE NOVO PURINE SYNTHESIS

The *de novo* purine synthesis pathway involves 10 enzymatic steps for the synthesis of inosine monophosphate (IMP), followed by one of two paths to produce either adenosine monophosphate (AMP) or guanosine monophosphate (GMP), (Holland et al., 2011; Souckova et al., 2020) (Figure 6.3). Glucose diet contributes to the purine biosynthesis through glucose entry into the pentose phosphate pathway which forms ribose-5phosphate, (Gooding et al., 2015). Ribose-5-phosphate ring structure can then be used to synthesize nucleotides onto from glycine, glutamine, and aspartate, (Fan et al., 2019). Additionally, glycine can be derived from glucose via de novo synthesis from serine allowing for direct conversion of glucose into nucleotides, (Lane and Fan, 2015). Alternatively, products of ATP metabolites, adenine, hypoxanthine, and ribose-1phosphate can be recycled into AMP which utilizes two enzymes: adenine phosphoribosyltransferase (APRT) and hypoxanthine-guanine phosphoribosyltransferase (HPRT), (Gessner et al., 2023). Both APRT and HPRT reaction depend on ribose-5-phosphate supply to react adenine and hypoxanthine, respectively, (Gessner et al., 2023). A crucial importance of purine salvage pathway is it's the primary route for adenine synthesis in the mammalian brain but also enzymes involved are cytosolic, allowing for enzymatic operation under mitochondrial dysfunction, (Panayiotou *et al.*, 2014). Once IMP is formed, some of the IMP is converted to allantoin to degrade the excess purines. Allantoin is the end product of the purine degradation pathway in *Drosophila*. In humans, excess purines are metabolized into uric acid and excreted from the body, whereas *Drosophila* has a functional urate oxidase (Uro) enzyme which enables further degradation of uric acid into allantoin. IMP is first converted to hypoxanthine and then into xanthine by the bifunctional enzymes, xanthine oxidase and xanthin dehydrogenase. The xanthine is further converted to uric acid in the degradation process, and uric acid is then converted to allantoin which is excreted, (Figure 6.3).



Figure 6.3: Pathway of purine synthesis in Drosophila.

Pathway intermediate abbreviations are: PRPP, 5'-phosphoribosyl-1-pyrophosphate; PRA, 5'phosphoribosyl-1-amine; GAR, 5-phosphoribosylglycinamide; FGAR, 5'-phosphoribosyl-Nformylglycinamide; FGAM, 5'-phosphoribosyl-N-formylglycinamidine; AIR, 5'-phosphoribosyl-5aminoimidazole: CAIR, 5'-phosphoribosyl-5-aminoimidazole carboxylate; SAICAR. 5'-5'-phosphoribosyl-4phosphoribosyl-4-(N-succinocarboxamide)-5-aminoimidazole; AICAR, carboxamide-5-aminoimidazole; FAICAR, 5'-phosphoribosyl-4-carboxamide-5formamidoimidazole; IMP, inosine monophosphate; AMP, adenosine monophosphate; GMP, guanosine monophosphate. Enzymes mentioned in the text are shown in gray. ERK is highlighted in red.

6.2 AIMS AND OBJECTIVES

Previously, it has been shown that high sugar diet feeding leads to reduced survival in flies, which was primarily attributed to dehydration and increased uric acid deposit formation in the Malpighian tubule of flies, (van Dam *et al.*, 2020). Additionally, a recent study in mammalian cell model has shown that one of ERK's phosphorylation substrates is a canonical and rate-limiting enzyme involved in *de novo* purine synthesis, PFAS, (Ali *et al.*, 2020).

Therefore, the main aim of this chapter was to investigate whether lifespan extension effects of trametinib, at least in part, are mediated by prevention of Malpighian tubule deposits on high sugar diet feeding.

Initial experiments were conducted to examine the morphology of the Malpighian tubules of flies fed with a high sugar diet both in the absence and present of trametinib for tubule deposits. Alongside, uric acid levels were quantified in these flies to determine any effects on purine metabolism. To investigate whether trametinib also influences the osmotic homeostasis of flies fed a high sugar diet, the water content of flies was examined by measuring wet and dry body weights. To test whether the effects of trametinib on a high sugar diet were dependent on *de novo* purine synthesis, the fly diet was supplemented with purines both in the absence and presence of trametinib. Malpighian tubule deposits and uric acid levels were then measured alongside lifespan assays to determine if trametinib extends lifespan at least in part through a reduction in purine biosynthesis.

6.3.1 EFFECT OF TRAMETINIB ON MALPIGHIAN TUBULE DEPOSIT FORMATION IN RESPONSE TO HIGH SUGAR FEEDING.

To investigate the effects of trametinib on the formation of Malpighian tubule deposits in response to high sugar feeding, the Malpighian tubules were dissected and imaged live using light microscopy after which tubule deposits were scored based on the severity of the phenotype. The results are shown in Figure 6.4.



Figure 6.4: The severity of Malpighian tubule deposits in response to high sugar diet and trametinib treatment.

(A): Representative light microscopy images of Malpighian tubules of 28-day old flies fed a normal (1x) and high (8x) sugar diets, in absence (-) and presence (+) of trametinib. (B): 28-day old wild-type flies fed with normal (1x) and high (8x) diets, in absence (-) and presence (+) of trametinib. N=56, Kruskal-Wallis with *post-hoc* Dunn's test, p<0.05 ***.

In wild-type flies on normal (1x) sugar, the severity of Malpighian tubule deposits was relatively low with ~40% of tubules showing no or very mild deposits, and trametinib did not significantly affect this. Feeding flies with a high (8x) sugar significantly increased the severity of Malpighian tubule deposits with ~80% showing severe deposit formation but not in the presence of trametinib. Trametinib treatment on high sugar (8x) diet, showed a full rescue of Malpighian tubule phenotype, (Figure 6.4).

Previous studies have shown that the Malpighian tubule deposits that form in response to high sugar diet are uric acid-based and that the uric acid content of flies fed with a high sugar diet is also increased, (van Dam *et al.*, 2020). Therefore, uric acid levels were measured in flies fed with a high sugar diet both in the absence and presence of trametinib, (Figure 6.5).



Figure 6.5: Uric acid content of whole flies in response to high sugar diet and trametinib treatment.

28 day old wild-type flies fed with normal (1x) and high (8x) diets, in absence (-) and presence (+) of trametinib. N = 13, 2-way ANOVA with *post-hoc* Tukey multiple comparison test, $p<0.001^{**}$.

In wild-type flies on normal (1x) sugar diet, trametinib did not have a significant effect on uric acid content. High (8x) sugar diet significantly increased uric acid content but not in the presence of trametinib. Trametinib-treated flies showed a full rescue in uric acid content on high (8x) sugar diet, (Figure 6.5).

High sugar diet has been shown to induce water imbalance in adult *Drosophila* causing dehydration which was proposed to be one of the main factors involved in increasing the formation of Malpighian tubule deposits, (van Dam *et al.*, 2020). Therefore, the effects of trametinib on the water content of flies fed with a high sugar diet were examined as a potential mechanism by which trametinib decreases the formation of Malpighian tubule deposits in response to high sugar diet. The water content of flies was determined by comparing wet and dry body weight measurements, (Figure 6.6).



Figure 6.6: Measurement of body mass of whole flies in response to high sugar diet and trametinib treatment.

Measurement of dry (shaded) and wet (clear) body mass of 28 day old adult female *Drosophila* fed normal (1x) and high (8x) sugar diets in absence (-) and presence (+) of trametinib. Floating bars plot shows the mean at the middle line (n=20 individual flies, p <0.001 ***, p<0.0001 ****, One-way ANOVA with *post-hoc* Tukey multiple comparison test).

On normal (1x) sugar diet, trametinib significantly increased wet weight but had no effect on dry weight. On high (8x) sugar diet, wet weight was significantly reduced in both presence and absence of trametinib indicative of reduced water content in flies fed with a high (8x) sugar diet, however dry weight was comparable to normal (1x) sugar diet. Trametinib did not significantly affect the wet weight of flies fed with a high sugar diet showing that trametinib does not prevent the decrease in water content induced by high sugar diet, (Figure 6.6).

6.3.2 EFFECT OF TRAMETINIB ON MALPIGHIAN TUBULE DEPOSIT FORMATION IN RESPONSE TO PURINE SUPPLEMENTATION

The metabolic breakdown of dietary sugars such as glucose and fructose can lead to enhanced purine production (via *de novo* purine biosynthesis) and purine breakdown forming uric acid, (Lennicke *et al.,* 2020). Additionally, Ali and colleagues have recently shown that PFAS, a canonical and rate limiting enzyme of *de novo* purine synthesis, is a direct phosphorylation substrate of ERK, (Ali *et al.,* 2020). Therefore, it was important to determine whether the effects of trametinib on Malpighian tubule deposit formation and lifespan extension, at least in part, depended on *de novo* purine synthesis. To determine if trametinib decreases tubule deposit formation through reduced purine production, the diet was supplemented with purines, adenine, and guanine, to bypass the *de novo*

biosynthesis pathway. First, the appropriate concentration of purines to use was determined by measuring lifespan after dietary supplementation with a range of purine concentrations in the food, (Figure 6.7).



Figure 6.7: Comparing the survival of flies after dietary purine supplementation at a range of purine concentrations.

Survival analysis of (A) flies fed a range of purine supplementation from 0 mM to 40 mM. Results are plotted as proportion of survivors as a function of time (in days). Median survival times of flies are shown in the table (B). Table showing median lifespan, log rank test comparing survival with increasing purine concentration to the control (0mM), p<0.001 *** (Log-Rank test). Red dotted line showing the median lifespan.

Purine supplementation at all three concentrations (10 mM, 20 mM and 40 mM) decreased median lifespan similarly, (-7% median lifespan, p<1x10⁻⁷⁻⁸). Unexpectedly, this reduction in survival was not dose dependent, (Figure 6.7). Therefore, the lowest dose of 10 mM purines was used for subsequent experiments.

First, the effects of dietary purine supplementation on tubule deposits was examined in the absence and presence of trametinib, as shown in Figure 6.8.



Figure 6.8: Formation of Malpighian tubule deposits in response to purine supplementation and trametinib treatment.

(A): Representative light microscopy images of Malpighian tubules of 28 day old flies without purine supplementation (PU-) and with purine supplementation (PU+), in absence (-) and presence (+) of trametinib. (B): 28 day old wild-type flies without purine supplementation (PU-) and with purine supplementation (PU+), in absence (-) and presence (+) of trametinib. N=50, Kruskal-Wallis with *post-hoc* Dunn's test, p<0.05 ***.

In wild-type flies on control diet without purine supplementation (PU-), trametinib did not have a significant effect on the severity of Malpighian tubule deposits. Dietary purine supplementation (PU+) significantly increased the severity of Malpighian tubule deposits even in the presence of trametinib. Trametinib did not have any effect on Malpighian tubule deposits on both control (PU-) and purine supplemented (PU+) diets, (Figure 6.8).

Earlier, it was shown that trametinib significantly reduced both the severity of Malpighian tubule deposits and the uric acid content of flies fed with a high sugar diet. As trametinib did not reduce the severity of Malpighian tubule deposits with dietary purine supplementation, the effects of trametinib on uric acid levels in these flies was also examined, (Figure 6.9).



Figure 6.9: Uric acid content of whole flies in response to dietary purine supplementation and trametinib treatment.

28 day old flies fed with control (PU-) and purine supplemented (PU+) diets, in absence (-) and presence (+) of trametinib. N=10, 2-way ANOVA with *post-hoc* Tukey multiple comparison test, $p<0.00001^{****}$.

In wild-type flies fed a control diet without purine supplementation (PU-), trametinib did not have a significant effect on uric acid content. Dietary purine supplementation (PU+) significantly increased uric acid content. Trametinib had no significant effect on uric acid content of flies on both control (PU-) and purine supplemented diet (PU+), (Figure 6.9).

Together, these data suggest that trametinib may prevent Malpighian tubule deposit formation on high sugar diet by inhibiting *de novo* purine synthesis thereby reducing uric acid production. Next, it was determined whether a reduction in *de novo* purine synthesis contributes to the ability of trametinib to increase lifespan on high sugar diet. The survival of flies after dietary purine supplementation was measured both in the absence and presence of trametinib, (Figure 6.10).



Figure 6.10: Comparing the survival of wild-type female flies fed control (PU-) and purine supplemented (PU+) diets in the absence (-) or presence (+) of trametinib.

Survival analysis of **(A)** flies fed control (PU-) and purine supplemented (PU+) diets in the absence (T-) or presence (T+) of trametinib. Results are plotted as proportion of survivors as a function of time (in days). Median survival times of flies are shown in the table **(B)**. Table showing median lifespan, log rank test comparing absence (-) of trametinib to presence (+) of trametinib for control (PU-) and purine supplemented (PU+) diets, p<0.001 *** (Log-Rank test). Red dotted line showing the median lifespan.

Purine supplementation (PU+) significantly decreased median lifespan in flies in the absence of trametinib, (-4% median lifespan, p<1x10⁻³) which was consistent with the previous data, (Figure 6.6). Unexpectedly, trametinib significantly increased lifespan both without purine supplementation (PU-: +7% median lifespan, p= $1x10^{-4}$) and after dietary purine supplementation (PU+: +4% median lifespan, p= $1x10^{-5}$), (Figure 6.10).

Therefore, although purine supplementation prevented trametinib from reducing the formation of Malpighian tubule deposits, it did not block the ability of trametinib to extend lifespan, suggesting that the effects of trametinib on purine metabolism in response to high sugar diet do not contribute to trametinib-dependent lifespan extension.

To summarise, the main aim of this chapter was to determine whether trametinib lifespan extension on high sugar diet, at least in part, was due to reduction in formation of Malpighian tubule deposits. Once established, the underlying mechanism of trametinib was investigated, determining whether trametinib elicits its effect by affecting water homeostasis or/and affecting *de novo* purine synthesis.

6.4.1 CHARACTERISATION OF MALPIGHIAN TUBULE PHENOTYPE OF FRUIT FLIES FED HIGH SUGAR DIET IN RESPONSE TO TRAMETINIB.

In this study, it was shown that trametinib decreased both the formation of Malpighian tubule deposits and elevated uric acid content of flies in response to high sugar diet. Previous studies have reported that high sugar diet leads to the formation of Malpighian tubule deposits (van Dam *et al.*, 2020, Rani *et al.*, 2020). Rani and colleagues showed that high sugar diet (~30%) increased the uric acid content of flies as well as uric acid deposits in the Malpighian tubules, (Rani *et al.*, 2020) comparable to those demonstrated in this study. Additionally, it was shown that high sugar diet impaired fluid secretion in the Malpighian tubules, (Rani *et al.*, 2020). Van Dam and colleagues showed that chronic high sugar diet (20%) induced a water imbalance in flies leading to elevated uric acid levels and the formation of Malpighian tubule deposits, (van Dam *et al.*, 2020). Again, the results presented here were comparable although the higher sugar content of the diet (40% versus 20%) led to a slightly more severe phenotype compared to van Dam *et al.* (2020).

A potential explanation to why trametinib lowered the severity of Malpighian tubule deposits and uric acid content was by modulating the water homeostasis of flies thereby preventing deposit formation. The effects of hydration status and water intake were previously associated with risks of kidney stones in humans. An association study by Wang and colleagues have shown a linear protective relationship between fluid intake and risk of nephrolithiasis (kidney stones), (Wang *et al.*, 2022). Additionally, a positive dose-response was associated with reduction of nephrolithiasis suggesting insufficient hydration as a potential marker for kidney stones, (Wang *et al.*, 2022).

A similar study on dehydration was carried out by van Dam and colleagues in *Drosophila*, which demonstrated that chronic high sugar diet induced a water imbalance in flies, leading to dehydration and Malpighian tubule deposits. Haemolymph volume was significantly decreased in these flies which could be rescued by water supplementation, (van Dam *et al.*, 2020). Here, high sugar diet also induced a decrease in water content of

around 30-40% which was comparable to the decrease in haemolymph volume observed by van Dam *et al.* (2020). However, while trametinib increased the water content of flies fed a normal sugar diet, no significant changes in water content were observed for flies fed on high sugar diet.

Interestingly, trametinib treatment therefore prevented the formation of Malpighian tubule deposits on high sugar diet despite these flies showing a similar reduced water content to untreated flies suggesting that trametinib may prevent deposit formation through a water-independent mechanism.

The study by van Dam (2020), suggests that water supplementation of flies fed high sugar diet, thereby hydration, can rescue the Malpighian tubule deposits, elevated uric acid levels and subsequently rescue the shortened lifespan associated with high sugar consumption, (van Dam *et al.*, 2020). The results presented here suggest that the effects of high sugar diet on water content and tubule deposits can be uncoupled because trametinib treatment rescues tubule deposits with no effect on water content. In this study, flies fed high sugar diet remained dehydrated even in presence of trametinib suggesting that removal of Malpighian tubule deposits was not dependent on trametinib's effect on water homeostasis, or perhaps trametinib was insufficient in affecting water homeostasis in flies fed high sugar diet. Therefore, it was investigated whether trametinib played a role in regulating *de novo* purine synthesis as the primary mechanism by which trametinib might impact uric acid production and tubule deposit formation.

Supplementation of the diet with purines can bypass the rate limiting enzymes in the *de novo* purine synthesis pathway, which leads to higher uric acid levels and Malpighian tubule deposit formation, (Lang *et al.*, 2019; van Dam *et al.*, 2020). Lang and colleagues have shown that purine supplementation within the fly diet from 10-40 mM concentrations led to a dose-dependent increase in Malpighian tubule deposit formation, (Lang *et al.*, 2019). Likewise, van Dam and colleagues have shown that purine supplementation of 10mM in the fly diet produced a comparable increase in Malpighian tubule deposit formation of 10mM in the fly diet produced a comparable increase in Malpighian tubule deposit formation, (van Dam *et al.*, 2020). In this study, purine supplementation of 10mM was also used and similar effects on Malpighian tubule deposits were observed as to those previously described, (Lang *et al.*, 2019; van Dam *et al.*, 2020).

However, trametinib failed to have a significant effect on Malpighian tubule deposits in purine supplemented flies, suggesting that the ability of trametinib to reduce Malpighian deposits were primarily dependent on inhibition of *de novo* purine synthesis rather than enhancing purine degradation

Previously, it was shown by Ali and colleagues in mammalian cells that PFAS, a key *de novo* purine synthesis enzyme, is a direct phosphorylation substrate of ERK at threonine 619, (Ali *et al.,* 2020). In *Drosophila*, purine *de novo* synthesis is a conserved pathway,

and is vital to development as gene mutations in *de novo* synthesis enzymes have been shown to cause arrest in development from pupal to adult stages, (Ji and Clark, 2006) and reduction of fertility and lifespan, (Malmanche and Clark, 2004). The enzyme PFAS is encoded by the gene *ade2* in *Drosophila* and plays a conserved role of metabolizing FGAR to FRAM in the *de novo* purine synthesis pathway, (Holland *et al.*, 2011). However, not much is known about the role of *ade2* in Malpighian tubule physiology nor the effect of high sugar diet on *ade2* expression. Considering the functional conservation of ERK and PFAS between mammals and *Drosophila*, it is expected that the ERK phosphorylation site in Ade2 may be conserved.

Ade2/PFAS may not be the only or primary target of ERK phosphorylation within the *de novo* purine synthesis pathway. A study by Unal and colleagues compiled a comprehensive ERK target phosphor-site archive based on biochemical and genetic approaches, in an online database, (Unal *et al.*, 2017). A search of *de novo* purine synthesis enzymes in the phosphor-site archive has shown that ERK potentially phosphorylates an additional *de novo* purine synthesis enzyme, PAICS, (Unal *et al.*, 2017). PAICS is a bifunctional enzyme involved in the metabolism of AIR to CAIR and further into SAICAR, (Holland *et al.*, 2011). In *Drosophila*, PAICS is encoded by one gene, *ade5*, yet its role in response to high sugar diet or trametinib treatment has not been investigated. Assuming the conservation in ERK phosphorylation sites *in Drosophila* Ade2 and Ade5, trametinib treatment may also elicit its effects on reducing Malpighian deposits by preventing ERK-dependent phosphorylation of both enzymes.

The study in this chapter has shown that trametinib prevented Malpighian tubule deposits and uric acid increase in response to high sugar diet by inhibiting *de novo* purine synthesis. As trametinib also extended lifespan on high sugar diet, it was crucial to investigate whether this trametinib-dependent lifespan extension was also mediated by inhibition of *de novo* purine synthesis. Therefore, this led to the investigation of lifespan in purine supplemented flies, showing that trametinib extended lifespan in the presence of excess dietary purines to a similar extent to that of control flies. This suggests that even though trametinib reduces Malpighian tubule deposits by inhibiting *de novo* purine synthesis, the pro-longevity effects of trametinib are not dependent on these changes. A possible explanation is that trametinib plays a role in other metabolic processes, such as reduction of lipid droplets and TAG content of flies, limiting the metabolic burden on the fly. Additionally, trametinib may affect enzymes/proteins act upstream of the *de novo* purine synthesis to extend lifespan.

6.4.2 TRANSCRPITIONAL RESPONSE OF TRAMETINIB ON MALPIGHIAN TUBULE PHENOTYPE

The RNA-sequencing exploration from this study showed that there are no sugarresponsive ERK-dependent gene changes involved in purine metabolism, suggesting that perhaps the effects of trametinib on purine metabolism are independent of transcriptional effects of ERK and are dependent on post-translational modifications mediated by ERK. Interestingly, the RNA-sequencing data identified two genes of interest involved in osmoregulation and body fluid secretions, Diuretic hormone-31 (Dh31) and Ion transport *peptide (Itp)*. It has been shown that the principal cells of Malpighian tubules express Dh31 receptors which are crucial for regulating body fluid secretion via Dh31, (Johnson et al., 2005; Hector et al., 2009), yet the molecular mechanism involved in Dh31 mediated fluid secretion is not known. Additionally, Dh31 has been shown to trigger intestinal contractions and bowel emptying gut movement, especially in an infected status, (Benguettat et al., 2018). Galikova and colleagues have shown that overexpression of Itp ubiquitously led to fly dehydration as the peptide increased thirst while repressing excretion, thereby conserving water resources, (Galikova et al., 2018). Perhaps on high sugar diet, trametinib elicits its effects on Malpighian tubule deposits at least in part by regulating the thirst of the fly leading to reduced water loss through excretion thereby conserving water.

Lastly, trametinib was shown to influence water homeostasis in flies fed a normal sugar diet yet had no effect on water content in flies fed with a high sugar diet. It may be that high sugar diet imposes strong effects on water homeostasis, which trametinib is not able to rescue. This suggests that trametinib's role in water homeostasis is secondary/dispensable for the rescue of Malpighian tubule deposits in flies fed a high sugar diet. Although flies were shown to exhibit a reduced feeding behaviour on high sugar diet, feeding rates were comparable between trametinib-treated and untreated flies. As such, these flies consume equivalent amounts of food so reduced sugar intake or enhanced ingestion of water does not explain the rescue of Malpighian tubule deposits and elevated uric acid levels by trametinib in flies fed high sugar diet, and these flies remain dehydrated. Finally, this study suggests that the ability of trametinib to rescue Malpighian tubule deposits and elevated uric acid levels in flies fed a high sugar diet can be uncoupled from the ability of trametinib to extend lifespan. Trametinib was able to extend lifespan in flies fed with a high purine diet despite the presence of tubule deposits. Therefore, trametinib extends lifespan independently of its ability to inhibit de novo purine synthesis. It would still be interesting to investigate whether modulating the activity of either Ade2 or Ade5 would be sufficient to extend lifespan on high sugar diet and whether phenotypes observed in presence of trametinib are dependent on the activity of these purine synthesis enzymes.

7.1 THE EFFECT OF TRAMETINIB ON LIPID METABOLISM IN RESPONSE TO DIET-INDUCED OBESITY AND DURING AGEING.

Overall, the main aim of this project was to further investigate the metabolic effects of pharmacological inhibition of RAS/MAPK signalling using trametinib in response to dietand age-induced obesity. Trametinib is a specific inhibitor of MEK and currently is used to treat metastatic melanomas, (Hoffner and Benchich, 2018). However, RAS/MAPK pathway inhibition has also been shown to have metabolic effects. Bost *et al.*, (2002) showed that MEK inhibition prevents adipocyte differentiation and, furthermore, mice lacking ERK showed lower body lipid content, (Bost *et al.*, 2005). More recently, it was shown that RAS/MAPK pathway inhibition using trametinib improved metabolic parameters, such as insulin resistance, in genetic and diet models of obesity in mice, (Banks *et al.*, 2015). Previously unpublished research showed that trametinib treatment of *Drosophila* increased lifespan and decreased the TAG content of flies fed a high sugar diet.

Initially, it was crucial to determine the efficacy of trametinib to inhibit RAS/MAPK pathway in response to high sugar diet and ageing. Western blot analysis was conducted on trametinib treated wild-type female flies with phosphorylation of ERK used as a read out for RAS/MAPK pathway activity. Trametinib treatment was shown to rescue both diet-induced and age-dependent increases in the phosphorylation of ERK. Furthermore, the extent to which trametinib decreased phosphorylation of ERK was consistent with the current literature, (Banks *et al.*, 2015; Urena *et al.*, 2024). This allowed for further investigations into the phenotypic effects of trametinib in response to diet-induced obesity and ageing.

Confocal microscopy analysis of lipid droplet size in the fat bodies of wild-type female flies revealed that trametinib also prevented both diet-induced and age-dependent increases in LD size. Interestingly, while trametinib reduced the size of LDs in flies fed with either normal or high sugar diets, the decrease in LD size was greater in response to high sugar diet. Furthermore, trametinib also decreased TAG storage both in response to high sugar diet and during normal ageing. These findings are consistent with previous studies which showed that knockdown of ERK2 in mouse fibroblast cells (NIH/3T3) also reduced LD number and TAG content (Andersson *et al.*, 2006) suggesting that these effects of ERK inhibition on lipid storage may be conserved.

One potential mechanism by which trametinib reduced the lipid content of flies could be through reduced feeding, yet feeding behaviour, analysed using the proboscis extension assay, did not show any significant differences in response to trametinib. Furthermore, trametinib did not affect glycogen storage in flies fed with a high sugar diet suggesting that the effects of trametinib are lipid specific.

Alternatively, trametinib could have exerted its effects on lipid storage by either enhancing lipolysis or inhibiting lipid synthesis. To explore these mechanisms, genetic manipulation of these two processes coupled with lifespan analysis was conducted. To investigate the effects of trametinib on enhanced lipolysis, a mutant of a canonical lipolytic enzyme, Brummer, was used. *brummer (bmm)* encodes an ortholog of mammalian ATGL, loss of which has been shown to increase lipid storage and lipid droplet size in flies and impair TAG mobilization through inhibition of lipolysis, (Gronke *et al.*, 2005).

Using a biochemical assay to measure TAG content in fly homogenates, trametinib treatment of *bmm* mutant flies on both normal and high sugar diets still resulted in decreased TAG stores. Furthermore, trametinib was still able to extend the lifespan of *bmm* mutant flies to a similar extent as wild-type control flies suggesting that the trametinib lifespan extending properties did not depend on *bmm*-mediated lipolysis. It is possible that an alternative lipolytic system may play a role in the effects of trametinib in *Drosophila*. This alternative lipolytic system depends on Akh signalling and controls the expression of one or more uncharacterized TAG lipases, which complement Bmm function, (Gronke *et al.*, 2007), therefore suggesting there may be systematic redundancy, (Heier and Kuhnlein, 2018).

On the other hand, in terms of lipogenesis, the main enzyme involved in de novo lipid synthesis is ACC, used as a proxy for enhanced lipid synthesis. Previous work showed that muscle-specific knockdown of acc prevented lifespan extension by dietary restriction. (Katewa et al., 2012), whereas overexpression of acc was shown to be pathogenic in a Drosophila model of cardiac dysfunction, (Lim et al., 2011). It was shown here that ubiquitous overexpression of acc in adult flies resulted in elevated TAG storage and reduced lifespan on a normal diet showing a pathological role for acc overexpression under control conditions. These effects of acc overexpression were further exacerbated on high sugar diet. Unexpectedly, trametinib treatment both decreased the TAG content and increased lifespan in acc overexpressing flies on both normal and high sugar diets. This suggests that acc-dependent enhanced lipogenesis is insufficient to block the effects of trametinib. However, it was plausible that acc overexpression was not sufficient to block the effects of trametinib on lipid storage and lifespan, (Wang et al., 2022) and so an alternative approach to modulate lipid synthesis was employed, through overexpression of a key upstream regulator of lipid synthesis, the transcriptional regulator of de novo lipogenesis, srebp. Srebp has an evolutionarily conserved function in controlling lipogenic gene expression, (Walker et al., 2011). Previously, it was shown that srebp null mutants lack fatty acids and do not survive development unless supplemented with dietary fatty acids, (Kinte et al., 2006). Also, ectopic expression or knockdown of srebp has been shown to reciprocally affect lipid stores in the midgut and body TAG levels, (Son *et al.,* 2014), suggesting that *srebp* overexpression may produce similar effects to *acc* overexpression on TAG storage. Again, trametinib extended lifespan and lowered TAG content in *srebp* overexpressing flies, similarly to *acc* overexpressing flies, showing that elevated lipid synthesis was not sufficient to block the ability of trametinib to reduce TAG storage and extend lifespan.

7.2 THE EFFECT OF TRAMETINIB ON THE LIPIDOME IN REPONSE TO DIET-INDUCED OBESITY

Previously in this study it was determined that trametinib has effects on the TAG content and the lipid droplet size in response to diet-induced obesity and during ageing. It was therefore intriguing to investigate whether other lipid species were also affected by trametinib treatment. Liquid chromatography coupled mass spectrometry was carried out to gain an insight into changes to lipid species that may explain the phenotypic effects of trametinib on high sugar diet. Analysis of the positive ion mode data found that trametinib decreased the abundance of a range of TAG species on both normal and high sugar diets while increasing the abundance of a range of phospholipid species. However, unexpectedly, an increase in overall abundance of TAGs was not observed in response to high sugar diet, as previously shown by Tuthill *et al.*, (2020) although there was an increasing trend. The Tuthill *et al.*, (2020) study investigated the abundance of even and odd chained TAGs separately, whereas both were combined in this study which perhaps explains the lack of significance. However, when investigating individual lipid species, trametinib did elicit a significant effect on individual TAG and phospholipid species.

High sugar diet was shown to decrease the abundance of odd-chained TAGs, previously shown by Tuthill *et al.*, (2020), yet an increase in abundance of even-chained TAGs was not observed. Trametinib significantly decreased the abundance of certain TAG species on normal and high sugar diets, which could explain the phenotypic effects of trametinib observed in this study.

On the other hand, analysis of lipids in the negative ion mode showed that high sugar diet significantly increased the abundance of phospholipid species, such as PS yet there was no significant change in the overall abundance of PS. On the contrary, Tuthill *et al.*, 2020 has shown the opposite trend in the abundance of PS species fed high sugar diet, yet the trend was not significant, (Tuthill *et al.*, 2020). Additionally, trametinib increased the abundance of other phospholipid species such as PG(32:2), PI(32:1), PI(34:2), PE(O-36:1), PE(36:2) and SM(35:1) however more in depth, targeted analyses is required to validate the role of these lipid species in facilitating the effects of trametinib treatment.

The role of PS has been previously implicated in maintenance of muscle mass in flies.

Together with the RNA-sequencing data exploration in this study, this suggests that perhaps trametinib prevents the deleterious effects of high sugar diet by maintaining muscle homeostasis in *Drosophila* by increasing the expression of genes involved in muscle maintenance as well as adapting the lipidome to support muscle maintenance, (Kim *et al.*, 2024).

Even though, the mass spectrometric analysis of the fly lipidome offered important insights into the effects of trametinib on lipid changes induced by a high sugar diet, a more targeted approach using a range of *Drosophila* tissues may increase the power and minimize the background noise of the data generated making the differentially abundant lipid species more apparent.

7.3 TRANSCRIPTIONAL EFFECTS OF TRAMETINIB IN RESPONSE TO DIET-INDUCED OBESITY

ERK is a key transcriptional effector of RAS/MAPK signalling and trametinib was demonstrated to prevent ERK phosphorylation in response to both high sugar and during ageing. Therefore, RNA-sequencing was carried out to analyse any transcriptional changes in response to trametinib that could possibly help to decipher the potential molecular mechanisms involved in the lifespan extending and lipid lowering effects of trametinib.

RNA-sequencing analysis was used as it is a deep, unbiased sequencing method, therefore it is commonly used in biological research to discover and quantify transcriptional changes in response to a stimulus, offering high sensitivity and accuracy, (Han *et al.*, 2015). The high sensitivity is essential as it allows for identification of differentially expressed genes that are lowly expressed, such as enzymes. Another advantage of using RNA-sequencing is the high reproducibility of data produced due to unique sequence mapping within the genome and lack of background noise, (Wang *et al.*, 2009b).

The RNA-sequencing analysis in this project showed good quality sequences across all samples spanning the whole genome with no bias nor mapping irregularities. There was some, albeit acceptable, variability between replicates within the same condition, making the data reproducible. Differentially expressed genes were determined using the DESeq2 toolkit, which is considered one of the best tools for identifying differentially expressed genes due to its high power, precision and sensitivity, (Love *et al.*, 2014). To validate the results observed, the sugar-responsive subset of differentially expressed genes was compared to a publicly available dataset generated by Dobson *et al.*, (2017). Even though the number of sugar-responsive genes identified by Dobson *et al.*, 2017 was considerably larger than that identified in this study, there was still a significant overlap

with 65.3% of the sugar-responsive genes identified in this study shared with Dobson *et al.*, 2017. The differences in the sizes of the two datasets could be due to flies being fed high sugar diet for 21-days in Dobson *et al.*, 2017 as compared to 10 days in this study. Certain transcriptional effects may be under-represented or undetected after a shorter exposure to high sugar diet. Additionally, quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was used to verify the differential expression of a gene of interest, *akh*. This gene was selected as Akh is known to regulate nutrient storage and energy homeostasis in *Drosophila* as well as being ERK-dependent on causing hyperglycaemia in response to high sugar feeding, (Galikova *et al.*, 2015; Li *et al.*, 2023). The fold change in expression of *a kh* from this study and Dobson *et al.*, 2017 was comparable between RNA-sequencing and qRT-PCR analysis.

Once the sugar-responsive genes were identified, a further subset of genes were identified of sugar-responsive genes that changed in the opposite direction in response to trametinib. Gene ontology and cluster analyses were then used to identify potential molecular mechanisms that could explain the phenotypic effects of trametinib in response to high sugar diet. Gene ontology analysis revealed enrichment in genes involved in neuropeptide metabolism, regulation of behaviour and biological quality. Further cluster analysis to find interacting genes generated 4 clusters of genes involved in neuropeptide production, fluid regulation, muscle maintenance and the electron transport chain.

One potential mechanism by which trametinib may elicit its effects is through transcription of neuropeptides. A gene of interest from cluster one affected by trametinib is *akh* itself, which encodes a metabolic neuropeptide involved in mobilization of energy substrates from the fat body in a range of insects. Recently, Akh signalling was shown to be dispensable for ontogenesis, locomotion, oogenesis and homeostasis of lipid and carbohydrate storage until the end of metamorphosis. However, during adulthood, Akh regulates fat body content and haemolymph sugar levels and nutritional responses, (Galikova *et al.*, 2015). More recently, it was shown that high sugar diet-induced hyperglycaemia is Akh-dependent via ERK translocation into peroxisomes to increase carbohydrate production in the fat body, (Li *et al.*, 2023). It is plausible that trametinib elicits its phenotypic effects by preventing Akh-dependent diet-induced hyperglycaemia.

Another plausible mechanism responsible for trametinib-induced phenotypes is through changes in *nos* expression, a gene of interest from cluster 2. *Nos* encodes an enzyme involved in the production of nitric oxide, a high concentration of which is an effector of killing pathogens during the insect immune response and functions as a messenger at low concentrations to regulate antimicrobial peptides, (Chen *et al.*, 2022). The role of the innate immune system in *Drosophila* has been shown to shift anabolic lipid metabolism from TAG storage to phospholipid synthesis to support the immune function, (Martinez *et*

al., 2020). The expression of *toll*, mammalian analogue of Toll-like receptor that activates the expression of antimicrobial peptides, has been shown to negatively regulate the expression of TAG synthesis enzymes and positively regulate phospholipid synthesis enzymes leading to an anabolic shift from TAG to phospholipid synthesis, (Martinez *et al.*, 2020). It may be that trametinib elicits its effects through regulation of antimicrobial peptide expression via *nos* leading to a shift in lipid anabolism and the subsequent lipid phenotype.

In the analysis of cluster 3, a gene of interest, *itp*, was discovered. Itp is a thirst-promoting and anti-diuretic hormone in *Drosophila* shown to be a functional analogue of human vasopressin and renin-angiotensin systems. Expression of *itp* is elevated by dehydration and the peptide increases thirst while repressing excretion, thereby promoting the conservation of water resources, (Galikova *et al.*, 2018). It was shown that *itp* regulates food intake because *itp* knockdown increased food intake whilst *itp* overexpression reduced food intake, (Galikova *et al.*, 2018). More recently, Galikova and Klepsatel showed that *itp* is a regulator of energy expenditure and transit of the meal through the digestive tract in *Drosophila*. Additionally, it was shown that *itp* regulates glycemia in an *akh* dependent manner, yet *itp*'s requirement for lipid and glycogen homeostasis were *akh* independent, (Galikova *et al.*, 2022). Itp was shown to act upstream of Akh and regulates transcription of the Akh receptor gene, *akhr*, (Galikova *et al.*, 2022). Trametinib may elicit its phenotypic effects through regulation of *itp* expression and *itp* associated genes such as *akh* and *akhr*, previously shown to be involved in regulation of nutrient status of *Drosophila*.

Lastly, analysis of cluster 4 showed interaction of genes involved in regulation of the electron transport chain and subsequently oxidative phosphorylation. Trametinib elicited changes in expression of genes encoding subunits of complex I and complex III of the electron transport chain. In Drosophila, mutations in complex I are associated with pathologies therefore complex I is recognized as a crucial regulator of animal longevity. Previous work showed that increased activation of complex I reduced ROS production and oxidative damage, leading to increased lifespan in *Drosophila*, (Tong et al., 2007). On the other hand, a diet high in fat has been shown to reduce the activity of complex I in Drosophila, leading to higher free radical leak, (Cormier et al., 2021). Studies investigating complex III are limited, yet it has been shown that complex III-derived ROS contributes to cellular responses to hypoxia and related cardioprotective ischemic preconditioning, (Chandel, 2010) and adipocyte differentiation, (Tormos et al., 2011). However, the investigation of complex I and complex III subunits in the context of dietinduced obesity and ageing are limited as overexpression of complex I and III to increase activity is currently technically impossible, as both complexes function as holoenzymes made of multiple subunits, some of which are encoded by the mitochondrial genome, making it difficult to overexpress the holoenzyme, (Carroll *et al.*, 2006). Yet, the cluster analysis suggests that perhaps trametinib elicits some of its phenotypic effects on high sugar diet via regulation of the genes involved in the electron transport chain, mainly complex I and complex III.

7.4 THE EFFECT OF TRAMETINIB ON PURINE METABOLISM IN RESPONSE TO HIGH SUGAR DIET

Lastly, the effects of a high sugar diet in Drosophila were not limited to the phenotypic effects of increased lipid content. Previous studies have reported that a high sugar diet leads to increased purine production, elevated uric acid levels and the formation of uric acid deposits within the Malpighian tubules, (van Dam *et al.*, 2020; Rani *et al.*, 2020). These effects were at least in part due to water imbalance and could be rescued by water supplementation, (van Dam et al., 2020; Rani et al., 2020). In this study, trametinib was shown to rescue elevated uric acid levels caused by high sugar diet and prevented the formation of uric acid deposits within the Malpighian tubules. Initial analysis investigated whether trametinib lowered uric acid content and the severity of Malpighian tubule deposits and by modulating the water homeostasis of flies. Interestingly, hydration status and water intake were previously associated with risks of kidney stones in humans, (Wang et al., 2022). However, the analysis of water content in flies fed high sugar diet in response to trametinib showed that trametinib increased the water content of flies fed a normal sugar diet, but had no significant effects on the water content of flies fed a high sugar diet. This suggested that trametinib prevented the formation of Malpighian tubule deposits on high sugar diet in a water-independent manner. Thus, the results presented in this study suggest that the effects of high sugar diet on water content and tubule deposits can be uncoupled because trametinib treatment prevented Malpighian tubule deposits in response to high sugar diet without affecting water content.

Next, it was investigated whether trametinib played a role in regulating *de novo* purine synthesis as the primary mechanism by which trametinib impacted uric acid production and tubule deposit formation. Supplementation of the fly diet with purines bypasses the rate limiting enzymes in the *de novo* purine synthesis pathway, thereby increasing uric acid production, and promoting the formation of Malpighian tubule deposits, (Lang *et al.*, 2019; van Dam *et al.*, 2020). Trametinib treatment alongside purine supplementation failed to have a significant effect on uric acid content and Malpighian tubule deposits was primarily dependent on inhibition of *de novo* purine synthesis rather than enhancing purine degradation. Interestingly, trametinib treatment in purine supplemented flies still extended their lifespan suggesting that the pro-longevity effects of trametinib are independent of its effects on purine synthesis. It is possible that trametinib plays a role in

other metabolic processes such as reduction of lipid droplets and TAG storage thereby limiting the metabolic burden on the fly. Also, an alternative explanation is that trametinib acts upstream of *de novo* purine synthesis to extend lifespan.

The RNA-sequencing exploration in this study showed that there were no sugarresponsive genes that were affected by trametinib that are involved in purine metabolism. Therefore, the effects of trametinib on purine metabolism may be independent of the transcriptional effects of ERK and may instead be dependent on phosphorylation mediated by ERK. Previously, it was shown that a key *de novo* purine synthesis enzyme, PFAS, is a direct phosphorylation substrate of ERK, (Ali *et al.*, 2020). It would be interesting to further investigate whether trametinib may act via PFAS, encoded by *ade2* in *Drosophila*, to elicit its effects on purine metabolism. Additionally, when looking at an archive of ERK phosphorylation targets, it was discovered that ERK phosphorylates two *de novo* purine synthesis enzymes, encoded in *Drosophila* by *ade2* and *ade5*, (Unal *et al.*, 2017), yet the roles of *ade2* and *ade5* in response to high sugar diet or trametinib have not yet been investigated. Assuming the conservation in ERK phosphorylation sites in *Drosophila* Ade2 and Ade5, trametinib treatment may, partly, elicit its effects on purine metabolism and lifespan by preventing ERK-dependent phosphorylation of both enzymes.

7.5 CONCLUSION

In conclusion, this project aimed to identify the metabolic effects of RAS/MAPK pathway inhibition in response to diet-induced obesity and during ageing. Previous research showed that genetic and pharmacological inhibition of RAS/MAPK pathway has metabolic effects associated with reduced lipid content, which was replicated here in *Drosophila* using the MEK specific inhibitor, trametinib. Furthermore, these metabolic effects of trametinib were associated with lifespan extension in response to diet-induced obesity and under normal conditions.

First, experiments on trametinib's efficacy showed that trametinib rescued the phosphorylation of ERK in response to diet-induced obesity and during ageing, showing a clear inhibition of the RAS/MAPK pathway. The characterization of trametinib's effects showed a TAG-specific reduction of lipid stores in response to high sugar diet and during ageing in *Drosophila*. The effects of trametinib were shown to also decrease the lipid droplet size, being the main energy storage site in animals. Initial work focusing on the effects of trametinib via lipid metabolism showed that trametinib elicits its effects independently of *bmm*-mediated lipolysis or lipid synthesis. Subsequently, a multiomics approach was employed comprised of RNA-sequencing and liquid chromatography coupled mass spectrometry to investigate the transcriptomic and lipid-metabolomic

effects of trametinib in response to high sugar diet. Surprisingly, analysis of differentially expressed genes showed no gene changes involved in lipid metabolism, yet further cluster analysis showed four prominent processes involved in neuropeptide processing, muscle maintenance, fluid regulation and the electron transport chain which could be potential molecular mechanisms involved in the phenotypic effects of trametinib in response to diet-induced obesity. Mass spectrometry analysis showed that trametinib affected the abundance of individual TAG and phospholipid species, yet the overall abundance of these lipid classes mostly showed trends.

A complementary effect of trametinib on purine metabolism was also investigated. Trametinib was shown to rescue uric acid content and Malpighian tubule deposits in response to high sugar diet which was determined to be dependent on inhibition of *de novo* purine synthesis, yet inhibition of *de novo* purine synthesis was dispensable for the pro-longevity effects of trametinib.

Future work should focus on exploiting the potential molecular mechanisms involved in the phenotypic effects of trametinib in response to diet-induced obesity. Additionally, a more targeted lipidomic approach should be employed to explore the effects of trametinib on individually isolated lipid classes in a range of lipid species and perhaps a range of timepoints. Furthermore, it would be crucial to utilize genetic manipulation of upstream targets of the *de novo* purine synthesis pathway to pinpoint the targets of trametinib in its effect on purine metabolism. Lastly, proteomics analysis of phosphorylation targets of ERK in response to trametinib treatment in a diet-induced obesity model of *Drosophila* would offer deeper insight into the post-translational effects of trametinib in response to diet-induced obesity which could uncover potential pharmacological targets. Altogether this study enabled a greater in-depth understanding of the metabolic effects of trametinib in a previously not well-studied area, allowing for identification of potential molecular mechanisms of action aiding in the search for potential therapeutic targets for treating metabolic disorders such as obesity and ageing.

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Reagent	Catalog number/product code	Manufacturer
0.2 mL PCR strip tubes, clear	12179770	Thermo Fisher Scientific
1.5 mL tubes, clear	11926955	Thermo Fisher Scientific
2 mL, screw cap tube, clear	E1420-2340	Starlab
96-well clear flat-bottomed microplates	10216341	Corning
Acetic acid	10492411	Thermo Fisher Scientific
Acetonitrile Optima	10055454	Thermo Fisher Scientific
Acrylamide / N,N'-Methylenebisacrylamide 37.5:1, 40% mix solution	10397511	Thermo Fisher Scientific
Active dry yeast	N/A	S.I. LeSaffre
Adenine	A8626	Merck
Agar	A7002	Merck
AmershamTM ProtranTM premium 0.45 µm nutrocellulose membrane	15269794	Cytiva
Ammonium formate	14517.3	Thermo Fisher Scientific
Avanti EquiSPLASH	330731-1EA	Croda International
Bovine serum albumin (BSA)	12737119	Thermo Fisher Scientific
Brewer's yeast	290331225	MP Biomedicals
Bright white real-time PCR 96-well plate for Roche Lightcycler 480	BW-96480	Primer Design
Chloroform	15406689	Thermo Fisher Scientific
Copper (II) sulphate pentahydrate	10604902	Thermo Fisher Scientific
Diethyl ether	11399047	Thermo Fisher Scientific
Dithiothreitol	10699530	Thermo Fisher Scientific
DMSO	11397938	Thermo Fisher Scientific
DNase-I	79254	QIAgen

Ethanol	12347163	Thermo Fisher Scientific
Experimental food vials	P1068/T	Regina Industries
Extra thick Blot Filter Paper, Precut, 7.5 x 10 cm	1703965	Biorad
Formaldehyde, 16%	11586711	Thermo Fisher Scientific
Glass beads	G8772	Merck
Glass collection vials	60000751CV	Waters
Glycine	10061073	Thermo Fisher Scientific
Goat anti-mouse antibody (HRP)	ab6789	Abcam
Goat anti-rabbit antibody (HRP)	ab6721	Abcam
Granulated sugar	N/A	Tate & Lyle
Guanine	G11950	Merck
Hexane	10147642	Thermo Fisher Scientific
Hydrogen peroxide	10386643	Thermo Fisher Scientific
Infinitiy Triglycerides reagent	TR22421	Thermo Fisher Scientific
Invitrogen dNTP set	10083252	Thermo Fisher Scientific
Invitrogen first strand buffer	10328062	Thermo Fisher Scientific
Invitrogen Oligo(dT)	11513127	Thermo Fisher Scientific
Isopropanol Optima	10783447	Thermo Fisher Scientific
Laemmli sodium dodecyl sulfate (SDS) sample buffer	15415809	Thermo Fisher Scientific
Leucine enkephalin	186006013	Waters
Luminata Forte Western HRP substrate	10394675	Merck
Methanol	11367996	Thermo Fisher Scientific
Methanol Optima	10031094	Thermo Fisher Scientific
Methyl tert-Butyl Ether	10772601	Thermo Fisher Scientific
Molybdenum blue spray reagent	M1942	Merck
Nile Red	415711000	Thermo Fisher Scientific

Oleic acid standard	75090	Merck
PageRuler plus prestained protein ladder	11832124	Thermo Fisher Scientific
Phosphate buffered saline	12821680	Thermo Fisher Scientific
Phosphaticylcholine standard	850457C	Croda International
Phosphatidic acid standard	840857C	Croda International
Phosphatidylethanolamine standard	850757C	Croda International
Pierce 660 nm protein assay reagent	10177723	Thermo Fisher Scientific
Pierce bovine serum albumin standard	10443834	Thermo Fisher Scientific
Pierce Ionic detergent compatibility reagent	10570044	Thermo Fisher Scientific
poly-L-lysine	P8920	Merck
Polymeric silica 60 matrix thin layer chromatography aluminium sheet	1.16484	Merck
PrecisionPLUS qPCR mastermix with SYBRgreen	Z-PPLUS-CL-20ML	Primer Design
QuantiChrom Uric Acid Assay Kit	DIUA-250	BioAssay Systems
Rabbit anti-tubulin antibody	2148S	Cell Signalling Technologies
Red grape juice	N/A	Ritchies
Rnase-OUT Rnase inhibitor	10777019	Thermo Fisher Scientific
Rneasy mini kit	74104	QIAgen
Skimmed milk powder	70166	Merck
Sodium Chloride	10112640	Thermo Fisher Scientific
Sodium dodecyl sulphate	15450685	Thermo Fisher Scientific
StarSeal advanced polyolefin film	E2796-9795	Starlab
Superscript-II RT enzyme	18064014	Thermo Fisher Scientific
Tegosept	FLY1136	Apex Bioresearch Products
Trametinib	T-8123	LC Laboratories
Trioleoylglycerol lipid standard	860903P	Croda International
Tris base	10376743	Thermo Fisher Scientific

Tris-HCL	10795104	Thermo Fisher Scientific
TRIzol	15596026	Thermo Fisher Scientific
Tween-20	10485733	Thermo Fisher Scientific
VECTASHIELD mounting medium containing DAPI	MBD0020	Merck
Water Optima	10095164	Thermo Fisher Scientific
Whatman filter paper	WHA1001918	Merck

Appendix 1: List of reagents used in this study.