Investigating the role of PDK-1 in longevity and healthy ageing using *Drosophila Melanogaster*

Doctoral thesis

for

the award of the doctoral degree

of the Faculty of Mathematics and Natural Sciences

of the University of Cologne

submitted by

Sophie Louise van Schelt

accepted in the year 2023

Table of Contents

Abbreviations

AMPK	Adenosine monophosphate-activated protein kinase
ACN	Acetonitrile solution
ADP	Adenosine diphosphate
AGC	cAMP-dependent protein kinase (PKA), the cGMP-dependent protein kinase (PKG) and the protein kinase C (PKC)
AGE-1	catalytic subunit ortholog to phosphoinositide 3-kinase
AHR	human aryl hydrocarbon receptor
AKH	glucagon-like-adipokinetic hormone
AKT	Protein kinase B
AMP	Adenosine monophosphate
AMP	Adenosine monophosphate
ANOVA	Analysis of variance
AOP	anterior open
ARD	Age related disease
AS160	AKT Substrate of 160kDa
ATG	autophagy-related proteins
ATP	Adenosine triphosphate
BCA	Bicinchoninic acid
BMM	brummer
bp	base pairs
C lobe	C- terminal lobe
c-Jun	Transcription factor Jun
C. elegans	Caenorhabditis elegans
CAA	chloroacetamide
CADD	Combined annotation-dependent depletion
CAT	Catalase
CI	climbing index
CO2	Carbon dioxide
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
CYP6W1	cytochrome P450 6w1
CYP6W1	cytochrome P450 enzymes
D.melanogaster	Drosophila melanogaster
daf-16	FOXO homologue C. elegnas
DAF-2	insulin-like growth factor 1
DAPI	4’,6-Diamidin-2-phenylindol
DDT	Dichlorodiphenyltrichloroethane
DFG motif	Asp-Phe-Gly motif
dILP	Drosophila insulin-like peptides

DNA	Deoxyribonucleic Acid
EB	entroblasts
Ecc15	Erwinia carotovora carotovora
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
EDTA	Ethylenediaminetetraacetic acid
eEF-2K	elongation factor 2 kinase
EEs	enteroendocrine cells
eIF4E	eukaryotic translation initiation factor 4E
EMA	European Medicine Agency
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinase
EtOH	Ethanol
FA	Formic acid
FBS	Fetal bovine serum
FDA	Food and Drug Administration
FHK	fork head
FOXO	Forkhead box protein O
G1	gap 1 phase
GAP	GTPase-activating proteins
GEF	Guanine nucleotide exchange factors
GEF	Guanine nucleotide exchange factors
GLUT1	Drosophila glucose transporter
GLUT4	insulin-sensitive glucose transporter
GPX	Glutathione peroxidase 1
GSK-3	Glycogen synthase kinase 3
GSK233	GSK2334470
GST	glutathione-S-transferases
GSV	storage vesicles
GTPase	guanosine triphosphatase
H2O2	Hydrogen peroxide
H3/H4	Histone 3/Histone 4
HCL	Hydrochloride
HIF-1α	Hypoxia-inducible factor 1 alpha subunit
HM	hydrophobic pocket
HRP	Horseradish peroxidase Hydrogen peroxide
IC50	50% inhibitory concentration
IIS	Insulin/insulin-like growth factor signalling
IND	Investigational New Drug
INR	Insulin Receptor
IRS	insulin receptor substrates

ISC	Intestinal stem cell
ISC	intestinal stem cells
KEGG	Kyoto Encyclopedia of Genes and Genomes
KOH	Potassium hydroxide
LB	Luria-Bertani
LC-MS/MS	Liquid Chromatography with tandem mass spectrometry
LIP4	lipase 4
LNK	Lymphocyte adapter protein
MAPK	Mitogen-activated protein kinase
Mkp3	mitogen-activated protein kinase phosphatase 3
mNSCs	median neurosecretory cells
MOA	mechanism of action
MPI Age	Max Planck Institute for Biology of Ageing
MS	Mass spectrometry
N lobe	N-terminal lobe
NaCL	Sodium chloride
NaCL	Sodium chloride
NF-κB	nuclear factor kappa B
NGR	negative geotaxis assay
NRF2	Nuclear factor erythroid 2-related factor 2 (
O.N.	over night
p.g.	per genotype
p16 INK4A	cyclin-dependent kinase inhibitor 2A
p53	tumor protein 53
PAK1	p21-activated kinase-1
PCR	polymerase chain reaction
PDK-1	3-phosphoinositide-dependent protein kinase-1
PDK-1 hum	PH domain humanized PDK-1
$PDK\text{-}1^{GR}$	PDK-1 mutant - genomic rescue
$PDK\text{-}1^{PH/null}$	PDK-1 mutant - PH domain with null allele
$PDK\text{-}1^{PH/PH}$	PDK-1 mutant - PH domain
$PDK\text{-}1^{PIF/null}$	PDK-1 mutant - PIF domain with null allele
$PDK\text{-}1^{PIF/PIF}$	PDK-1 mutant - PIF domain
PEPCK	phosphoenolpyruvate carboxykinase
PFA	Phosphate buffered saline
PGC-1 α	peroxisome proliferator-activated receptor coactivator-1 α
pH	potential hydrogen
PH domain	Pleckstrin homology domain
pH3	Phospho-histone 3
PI3K	Phosphoinositide 3-kinases

PIF pocket	PDK-1 interacting fragment pocket
PIP2	phosphatidylinositol (4,5)-bisphosphate
PIP3	Phospha-tidylinositol (3,4,5) triphosphates Phosphatidylinositol 3-kinase
PKA	protein kinase A
PKA	Protein kinase A
PKC	Protein Kinases C
PNT	pointed
PTB domain	phosphotyrosine-binding
PTEN	Phosphatase and tensin homolog
PTP-ER	protein tyrosine phosphatase-ERK/Enhancer of Ras1
PTP1B,	Protein tyrosine phosphatase 1B
RAF	rapidly accelerated fibrosarcoma
rapa	Rapamycin
RAS	Rat sarcoma
Rheb	Ras homolog enriched in brain
RIPA	Radioimmunoprecipitation assay buffer
RNA	Ribonucleic acid
ROS	Reactive Oxygen Spieces
RSK	$p90^{rsk}$
RTK	receptor tyrosine kinase
RTK	Receptor Tyrosine Kinases
S	Synthesis Phase
S6	Ribosomal Protein S6
S6K	p70 ribosomal protein S6 kinase
SDR	short chain dehydrogenases
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SGG	saggy
SGK	glucocorticoid-inducible kinase
SH2	Scr Homology 2
SIK3	salt-induced kinase 3
SMAD	Mothers against decapentaplegic homolog
SOD	superoxide dismutase
SYA	Sugar/yeast/agar
Syx13	Syntaxin 13
T loop	activation loop
TAME	Targeting Ageing with Metformin
TBST	Tris-buffered saline with Tween
TCA cycle	Tricarboxylic acid cycle
TCEP	Tris (2-carboxyethyl) phosphine
TMT	Tandem mass tag
TOR	Mechanistic target of rapamycin

tram	tramitinb
Tris-HCL	Tris hydrochloride
TSC1/2	tuberous sclerosis complex 1 and 2
UN	United nations
wDah	White Dahomey
WHO	World Health Organization
Wnt signalling	Wingless-related integration site
XTT	(2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2H-Tetrazolium-5-Carboxanilide)
2-O-Bn	2-*O*-benzyl-*myo*-inositol 1,3,4,5,6-pentakisphosphate
4E-BP	eIF4E-binding protein

Summary

We broadly define organismal ageing as progressive decline of physiological function, often associated with the onset and progression of age-related diseases (ARD). ARDs can severely impair quality of life and often lack appropriate pharmacological treatment, ultimately becoming fatal. Geroscience hypothesizes that many ARDs have common rather than unique underlying mechanisms. Understanding the signalling cascades involved in health- and life-span extension, would bring us one step closer to healthy humam ageing.

The evolutionarily conserved insulin/insulin-like growth factor signalling (IIS) pathway and the target of rapamycin (TOR) network are essential regulators of growth, metabolism and longevity. In Drosophila insulin-like peptides (dILPs) excretion by the median neurosecretory cells (MNCs) results in activation of the insulin-like receptor (INR), resulting in various downstream signalling cascades. A key upstream regulator within the IIS/TOR network is phosphoinositide-dependent kinase-1 (PDK-1). PDK-1 acts via two distinctive mechanisms of activation (MOA). MOA 1 depends on the pleckstrin homology (PH) domain which is involved in binding to phosphatidylinositol (PIP3) at the cell membrane, bringing PDK-1 into close proximity to protein kinase B (AKT). The PH-dependent AKT interaction with PIP3 prompts a conformational change that enables PDK-1 to phosphorylate the T-loop residue of AKTThr342. AKT activation results in many downstream signalling cascades, among other the activation of longevity transcription factor forkhead box O (FOXO) and central longevity node TORC1. MOA 2 depends on the functionality of the PDK-1 Interactive fragment (PIF) pocket and a priming phosphorylation in the hydrophobic motif of the substrate. The PIF dependent substrates of PDK-1 are diverse and are part of various cellular processes, for example P70-S6 kinase (S6K), protein kinase A (PKA), protein kinase C (PKC) and mitogen-activated protein kinase kinase (MEK). Most kinases activated in a PIF dependent manner are part of the AGC family (cAMP-dependent protein kinases A, cGMP-dependent protein kinases G, and phospholipid-dependent protein kinases C) and signalling dependent on PIF pocket interaction will be referred to as AGC signalling. By mutating each kinase domain, I investigated the PDK-1 dependent health and lifespan effect in Drosophila Melanogaster.

Down regulation of PH domain dependent signalling resulted in significant developmental delays, reduction in size and lowered fecundity. I hypothesize that these phenotypes are regulated non-cell autonomous due to reduced AKT-FOXO signalling in the MNCs, resulting in a lowered dILP excretion. Contradictory to the negative effects on early life phenotypes, I observed a significant increased resistance to starvation, oxidative stress, xenobiotic stress and a longevity phenotype in both sexes. Furthermore, intestinal over-proliferation and a reduction in climbing ability, both ARDs in Drosophila, were delayed in onset and significantly reduced in severity. Based on the vast body of knowledge about essential signalling pathways in health- and lifespan in Drosophila, both the AKT-TORC1 axis and the AKT-FOXO axis are predicted to play an essential role in PDK-1PH/PH longevity. Using pharmacological epistasis experiments, the role of TORC1 inhibition in PDK-1PH/PH signalling was accentuated.

Similarly, a reduced functionality of the PIF pocket resulted in developmental delays, strong reduction in size and severely lowered fecundity. Contradictory to the PDK-1PH/PH mutants, I hypothesize that this phenotype results form a cell autonomous reduction in signalling due to highted cell cycle activity. Lowered signalling in the AGC branch resulted in improved starvation resistance, oxidative stress

resistance and xenobiotic resistance in both sexes. Equally, lifespan and healthspan, measured by climbing ability, was significantly improved in females and males. Strikingly, the uncontrolled intestinal mitotic activity ARD was absent in the PDK-1PIF/PIF mutants. Seemingly conversely, the appropriate proliferative response was improved, implying a tighter regulation of proliferation signalling. As the AGC branch does not act via the conventional IIS/TOR longevity mechanisms, longevity must be regulated via distinctive signalling cascades. Signalling via MEK anterior open (AOP), PKA and PKC are predicted to play a role in the health and lifespan gain in the PDK-1PIF mutants.

Our current pharmacological paradigm is treating each disease individually, thereby leaving many ARDs untreated. Understanding the signalling cascades involved in ageing allows for the repurposing of existing FDA and EMA approved medication to treat ageing. By humanizing the Drosophila PDK-1 gene, I aimed to investigate the potential of the PH domain specific inhibitor 2-O-benzyl-myo-inositol 1,3,4,5,6-pentakisphosphate (2-O-Bn). PDK-1Hum/Hum flies are viable, despite developmental delays and a reduction in size. Female are severely reduced in fecundity and males are sterile. Taken together, humanization of PDK-1 significantly affected PDK-1 signalling due to unknown limitations in protein functioning. Surprisingly, neither viability nor lifespan was affected in the PDK-1Hum/Hum mutants. I have shown that humanization of PDK-1 is viable and resulted in a normal lifespan, despite it being a pivotal kinase in the IIS/TOR network. Unfortunately, no effect was observed upon treatment with 2-O-Bn. It is currently unknown if bio-availability or potency is the limiting factor in effective targeting of PDK-1Hum/Hum.

Treatment of ageing will require understanding the bigger picture of a kinase domain specific signalling. There are many theories trying to explain the principles behind the mechanisms of ageing. The hyperfunction theory implies that signalling cascades are optimised for early-life traits, therefore signalling at the wrong intensity for healthy ageing. Conversely, agonistic pleiotropy suggests that the same mechanisms which are selected to protect us until reproductive age, are protecting us at later in life but ultimately fail. My results show that both theories apply within a single kinase. While PH domain dependent signalling leans more towards the agonistic pleiotropy, PIF pocket signalling tends to the hyperfunction theory. In conclusion, PDK-1 has a crucial role in health and longevity, via a multitude of pathways, explained by two theories of ageing.

Zusammenfassung

Wir definieren das Altern des Organismus im weitesten Sinne als fortschreitende Verschlechterung der physiologischen Funktionen, die häufig mit dem Auftreten und Fortschreiten altersbedingter Krankheiten (engl.: age-related diseases, ARD) einhergeht. ARDs können die Lebensqualität stark beeinträchtigen und sind oft ohne geeignete pharmakologische Behandlung tödlich. Geroscience stellt die Hypothese auf, dass vielen ARDs eher gemeinsame als einzigartige Mechanismen zugrunde liegen. Ein Verständnis der Signalkaskaden, die an der Verlängerung der Gesundheits- und Lebensspanne beteiligt sind, würde uns dem gesunden Altern des Menschen einen Schritt näherbringen.
Der evolutionär konservierte Insulin/insulinähnliche Wachstumsfaktor-Signalweg (engl.: insulin/insulin-like growth factor signalway, IIS) und das TOR-Netzwerk (engl.: target of rapamycin) sind wesentliche Regulatoren von Wachstum, Stoffwechsel und Langlebigkeit. Bei Drosophila führt die Ausscheidung von Insulin-ähnlichen Peptiden (engl. insulin-like peptides, dILPs) durch die medianen neurosekretorischen Zellen (engl. median neurosecretory cells, MNCs) zu einer Aktivierung des

Insulin-ähnlichen Rezeptors (engl. insulin-like receptor, INR), was verschiedene nachgeschaltete Signalkaskaden zur Folge hat. Ein wichtiger vorgeschalteter Regulator innerhalb des IIS/TOR-Netzwerks ist die Phosphoinositid-abhängige Kinase-1 (engl. Phosphoinositide-dependent kinase-1, PDK-1). PDK-1 wirkt über zwei unterschiedliche Aktivierungsmechanismen (engl. mechanisms of activation, MOA). MOA 1 hängt von der Pleckstrin-Homologie (PH)-Domäne ab, die an der Bindung an Phosphatidylinositol (PIP3) an der Zellmembran beteiligt ist und PDK-1 in die Nähe der Proteinkinase B (engl. protei kinase B, AKT) bringt. Die PH-abhängige AKT-Interaktion mit PIP3 führt zu einer Konformationsänderung, die es PDK-1 ermöglicht, den Rest der T-Schleife von AKTThr342 zu phosphorylieren. Die AKT-Aktivierung führt zu zahlreichen nachgeschalteten Signalkaskaden, unter anderem zur Aktivierung des Transkriptionsfaktors für Langlebigkeit Forkhead Box O (FOXO) und des zentralen Knotenpunkts für Langlebigkeit TORC1. MOA 2 hängt von der Funktionalität der PDK-1 Interactive fragment (PIF)-Hülle und einer Priming-Phosphorylierung im hydrophoben Merkmal des Substrats ab. Die PIF-abhängigen Substrate von PDK-1 sind vielfältig und an verschiedenen zellulären Prozessen beteiligt, z. B. P70-S6-Kinase (S6K), Proteinkinase A (PKA), Proteinkinase C (PKC) und Mitogen-aktivierte Proteinkinase-Kinase (MEK). Die meisten Kinasen, die PIF-abhängig aktiviert werden, gehören zur AGC-Familie (cAMP-abhängige Proteinkinasen A, cGMP-abhängige Proteinkinasen G und Phospholipid-abhängige Proteinkinasen C), und die von der Interaktion mit der PIF-Tasche abhängige Signalgebung wird als AGC-Signalgebung bezeichnet. Durch Mutation der einzelnen Kinase-Domänen untersuchte ich die PDK-1-abhängigen Auswirkungen auf Gesundheit und Lebenserwartung in Drosophila Melanogaster.

Die Herabregulierung der von der PH-Domäne abhängigen Signalübertragung führte zu erheblichen Entwicklungsverzögerungen, einer Verringerung der Größe und einer verminderten Fruchtbarkeit. Ich stelle die Hypothese auf, dass diese Phänotypen nicht-zellautonom reguliert werden, da die AKT-FOXO-Signalübertragung in den MNCs reduziert ist, was zu einer verminderten dILP-Ausscheidung führt. Im Gegensatz zu den negativen Auswirkungen auf die Phänotypen im frühen Leben beobachtete ich bei beiden Geschlechtern eine signifikant erhöhte Resistenz gegenüber Hunger, oxidativem Stress, xenobiotischem Stress und einen Langlebigkeits-Phänotyp. Darüber hinaus traten übermäßige Darmproliferation und eine Verringerung der Kletterfähigkeit, beides ARDs bei Drosophila, verzögert auf und waren deutlich weniger schwerwiegend. Basierend auf dem umfangreichen Wissen über essentielle Signalwege für Gesundheit und Lebenserwartung in Drosophila, spielen sowohl die AKT-TORC1-Achse als auch die AKT-FOXO-Achse eine wesentliche Rolle für die Langlebigkeit von PDK-1PH/PH. In pharmakologischen Epistase-Experimenten wurde die Rolle der TORC1-Hemmung bei der PDK-1PH/PH-Signalgebung hervorgehoben.

In ähnlicher Weise führte eine reduzierte Funktionalität der PIF-Hülle zu Entwicklungsverzögerungen, starker Verkleinerung und stark verminderter Fruchtbarkeit. Im Gegensatz zu den PDK-1PH/PH-Mutanten stelle ich die Hypothese auf, dass dieser Phänotyp aus einer zellautonomen Verringerung der Signalübertragung aufgrund erhöhter Zellzyklusaktivität resultiert. Die verringerte Signalübertragung im AGC-Zweig führte bei beiden Geschlechtern zu einer verbesserten Resistenz gegen Hunger, oxidativen Stress und Xenobiotika. Auch die Lebens- und Gesundheitsspanne, gemessen an der Kletterfähigkeit, war bei Weibchen und Männchen deutlich verbessert. Auffallend ist, dass die unkontrollierte mitotische Darmaktivität ARD bei den PDK-1PIF/PIF-Mutanten nicht auftrat. Umgekehrt war die angemessene proliferative Reaktion scheinbar verbessert, was auf eine strengere Regulierung der Proliferations-Signalgebung hindeutet. Da der AGC-Zweig nicht über die herkömmlichen IIS/TOR-Langlebigkeitsmechanismen wirkt, muss die Langlebigkeit über andere Signalkaskaden reguliert werden. Es wird angenommen, dass die Signalübertragung über MEK

anterior open (AOP), PKA und PKC eine Rolle bei der Verbesserung der Gesundheit und der Lebensdauer der PDK-1PIF-Mutanten spielt.

Unser derzeitiges pharmakologisches Paradigma besteht darin, jede Krankheit einzeln zu behandeln, was dazu führt, dass eine Vielzahl von ARDs unbehandelt bleibt. Das Verständnis der Signalkaskaden, die an der Alterung beteiligt sind, ermöglicht es, bestehende, von der FDA und der EMA zugelassene Medikamente für die Behandlung der Alterung umzuwidmen. Durch die Humanisierung des Drosophila-PDK-1-Gens versuchte ich, das Potenzial des PH-Domänen-spezifischen Inhibitors 2-O-Benzyl-Myo-Inositol-1,3,4,5,6-Pentakisphosphat (2-O-Bn) zu untersuchen. PDK-1Hum/Hum-Fliegen sind lebensfähig, trotz Entwicklungsverzögerungen und Größenminderung. Die Weibchen sind in ihrer Fruchtbarkeit stark eingeschränkt und die Männchen sind steril. Insgesamt hat die Humanisierung von PDK-1 die PDK-1-Signalübertragung aufgrund unbekannter Einschränkungen der Proteinfunktion erheblich beeinträchtigt. Überraschenderweise war weder die Lebensfähigkeit noch die Lebensspanne der PDK-1Hum/Hum-Mutanten beeinträchtigt. Ich habe gezeigt, dass die Humanisierung von PDK-1 lebensfähig ist und zu einer normalen Lebenserwartung führt, obwohl es sich um eine zentrale Kinase im IIS/TOR-Netzwerk handelt. Leider wurde bei der Behandlung mit 2-O-Bn keine Wirkung beobachtet. Derzeit ist nicht bekannt, ob die biologische Verfügbarkeit oder die Potenz der begrenzende Faktor für eine wirksame Ausrichtung von PDK-1Hum/Hum ist.

Für die Behandlung des Alterns ist es erforderlich, das Gesamtbild einer Kinase spezifischen Signalübertragung zu verstehen. Es gibt viele Theorien, die versuchen, die Prinzipien hinter den Mechanismen der Alterung zu erklären. Die Hyperfunktionstheorie besagt, dass die Signalkaskaden für die Merkmale des frühen Lebens optimiert sind und daher für ein gesundes Altern in der falschen Intensität signalisieren. Die agonistische Pleiotropie hingegen besagt, dass dieselben Mechanismen, die ausgewählt wurden, um uns bis zum reproduktiven Alter zu schützen, uns auch im späteren Leben schützen, aber letztendlich versagen. Meine Ergebnisse zeigen, dass beide Theorien innerhalb einer einzigen Kinase zutreffen. Während die von der PH-Domäne abhängige Signalgebung eher zur agonistischen Pleiotropie neigt, tendiert die PIF-Hüllen-Signalgebung zur Hyperfunktionstheorie. Zusammenfassend lässt sich sagen, dass PDK-1 eine entscheidende Rolle für Gesundheit und Langlebigkeit spielt, und zwar über eine Vielzahl von Wegen, die durch zwei Theorien des Alterns erklärt werden.

1 Introduction

1.1 Ageing as disease

1.1.1 Ageing human population

The ageing population, defined as 60 years and above, will nearly double between 2015 and 2050 to 2.1 billion people (WHO, 2022). While this is a dramatic increase, ageing of the world population started around 1950 (**Figure 1**) (Roser, Ortiz-Ospina and Ritchie, 2019). With older age we accumulate conditions such as cardiovascular diseases, cancer, osteoarthritis, pulmonary disease, diabetes, depression and dementia, leading to a general loss of self-sufficiency. Additionally, ageing or age-related-diseases (ARDs) produce a heavy economic and psychological burden for patients, family and society as a whole (Atella *et al.*, 2019; Sheridan, Mair and Quinõnes, 2019). The need to act now to ensure a healthy global population is not unnoticed. The United Nations (UN) General Assembly declared 2021–2030 the UN Decade of Healthy Ageing. In a global collaboration between governments, international agencies, industry and academia, the aim is to foster longer and healthier life (United Nations, 2021). To ensure quality of life of this part of the population, understanding human ageing is crucial.

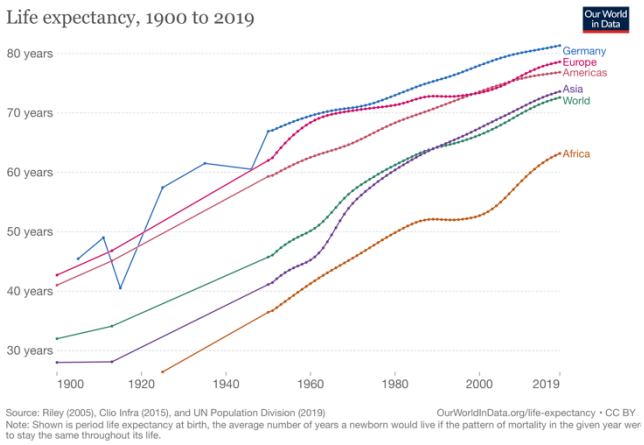

Figure 1: life expectancy in Germany, Europe, Americas, Asia, Africa and the world*. In the western world a steep incline in life expectancy can be observed from 1900 until 2019. A similar incline is measured in Africa, but has not reached western world life expectancy yet.*

1.1.2 Ageing as disease and FDA/EMA approval for anti-ageing pharmaceutics

We broadly define organismal ageing as progressive decline of physiological function. The decline is strongly correlated with increased susceptibility to death. While ageing and ARDs are often named together, they are distinct. During life an organism matures and eventually passes the line where maturation becomes ageing. In human ageing, onset of physical impairment and ARDs depend on individual lifestyle and genetics. Cardiovascular disease and cancer are still the main causes of death in Germany (2019) (**Figure 2**) (Hannah Ritchie, Fiona Spooner and Max Roser, 2019). Even before becoming fatal, ARDs can severely impair quality of life. Which ARD will eventually be deadly depends on the severity of the ARD and effectiveness of potential therapeutic treatment. Our current pharmacological paradigm is treating each ARD individually, assuming each has a unique underlying mechanism. Unfortunately, as most elderly people suffer from a range of ARDs, treatment of one ARD might only extend lifespan marginally. Geroscience hypothesizes that many ARDs have common underlying mechanisms. Suppressing these mechanisms, would slow the rate of ageing and thereby affect multiple ARDs (Gems, 2015). Onset and severity of multiple ARDs will simultaneously shift,

thereby prolonging high-quality life. In other words, healthspan would be extended and the time spend in a decrepit ageing phase limited. The notion that ageing is malleable and that life- and healthspan can be extended by pharmacological intervention introduced a new wave of ageing research (Partridge, 2010; Fontana, Kennedy and Longo, 2014).

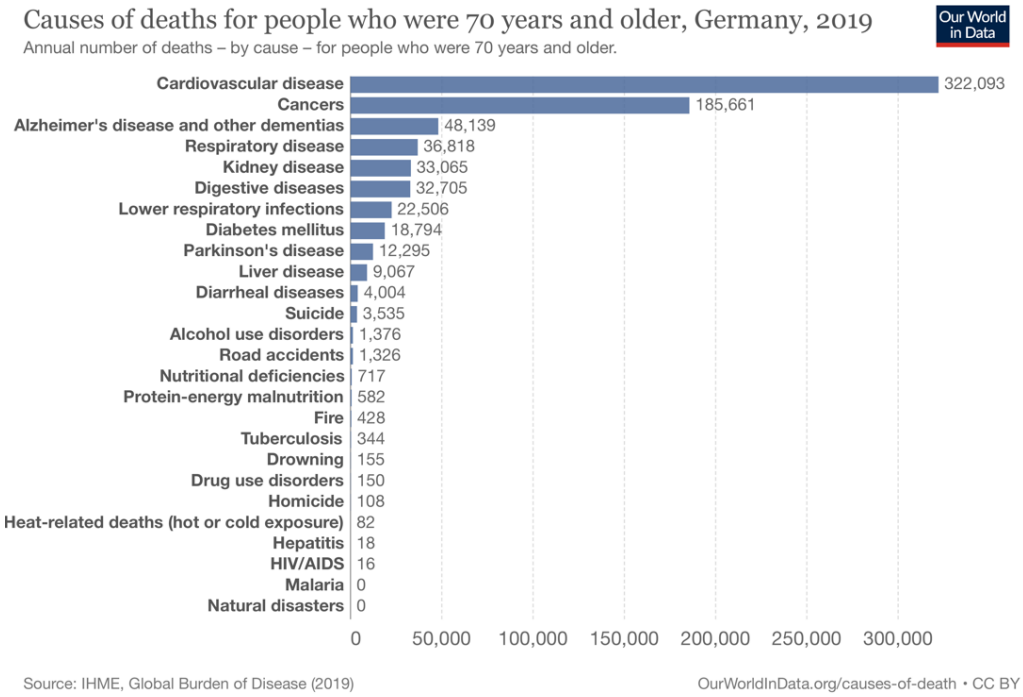

Figure 2: Causes of death for people who were 70 years and older in Germany (2019)

As mentioned above, ARDs are treated with targeted and single indication commercially available pharmaceuticals. These drugs are all registered and approved by the FDA (Food and Drug Administration) and the EMA (European Medicines Agency). Only when a disease indication is listed by the FDA and EMA can an Investigational New Drug (IND) application be filed (EMA, 2022; FDA, 2022c). When an IND is approved, clinical trials can be started and the drug eventually approved for human use. Drug approval is indication-specific and only in exceptional circumstances can a drug be used differently than indicated by the FDA/EMA (FDA, 2022b). Thus far, neither the FDA nor the EMA recognizes ageing as a disease. However, the understanding of the fundamental pathways involved in ageing across species has let to new clinical trials treating ARDs. For example, rapamycin, currently approved by the FDA as immunosuppressant, has been successfully used in a skin-ageing clinical trial. The placebo-controlled trial showed reduction in $p16^{INK4A}$, a well-known marker for cellular senescence and associated with skin ageing (Chung *et al.*, 2019). Additionally, a general improvement in clinical appearance after topical Rapamycin treatment was observed. A second, randomized, placebo controlled trial showed that new generation mTOR inhibitors significantly decreased infection and improved vaccination response (Mannick *et al.*, 2018). Both trials show that, even though ageing by itself is no indication for an IND, knowledge from geroscience is used in the development of new pharmaceuticals. When a study is not an official part of an IND, ageing by itself can be investigated. A new study investigates the effects of Rapamycin on biochemical and physiological ageing measurements, and general safety of taking Rapamycin intermittently for a prolonged time (12 months) (Watson and Zalzala, 2020). All three trials clearly show that understanding the fundamentals

of ageing can lead to successful treatment of ARDs. However, the heterogeneity of ageing in the human population, definition of beneficial outcomes/exclusion criteria and the timescale all make clinical trials targeting ageing as a disease highly complex and currently unfeasible (Kulkarni *et al.*, 2022).

Reducing the burden of ageing would have enormous psychological and financial benefits. It is not surprising that both big-data-mining and targeted approaches are used to explore anti-ageing medication (de Magalhães, Stevens and Thornton, 2017). Overall, a large number of potential anti-ageing pharmaceuticals have been identified (Tacutu *et al.*, 2018). Some of these are currently approved by the FDA and EMA for other indications. Repurposing of existing and approved drugs for a specialized geroscience FDA approval track has been suggested (Kulkarni *et al.*, 2022). Currently, the Targeting Ageing with Metformin (TAME) trail is the only planned series of clinical trials with the aim to develop an anti-ageing drug. Apart from funding and recruitment of participants, the indication ageing still needs to be approved by the FDA (afar, 2022). While society and regulatory institutions are getting ready to adopt new views on ageing, the academic world is discovering intricate pathways regulating ageing in humans and model organism.

1.1.3 Malleability of ageing in model organisms

The notion of plasticity and malleability of ageing by single genes is relatively new. It took flight after the discovery of the long-lived *age-1* and *daf-2 Caenorhabditis elegans (C. elegans*) mutants (**Figure 3**) (Friedman and Johnson, 1988; Kenyon *et al.*, 1993; Campisi *et al.*, 2019). While in the *age-1* paper longevity was attributed mainly to reduced fertility, Kenyon *et al.* ended the paper with a speculation which created a whole new research field; *"Perhaps a common underlying mechanism operates to extend the lifespan of these organisms."* Both *age-1* and *daf-2* are part of the insulin/insulin-like growth factor (IGF) signalling (IIS) pathway, the PI3K and insulin receptor (InR) respectively. It was followed by the discovery that *daf-2* longevity is dependent on the Forkhead Box-O (FoxO) worm ortholog, *daf-16* (Lin *et al.*, 1997). While the initial discoveries were assumed to be *C. elegans* specific, it became clear that these pathways were also conserved in *Drosophila*, mice and humans. With the discovery that *Drosophila flies* mutant for the insulin receptor substrate *chico* and mice lacking the insulin receptor were long-lived, the role of IIS in ageing was proven to be evolutionarily conserved (Clancy *et al.*, 2001; Blüher, Kahn and Kahn, 2003; Fontana, Partridge and Longo, 2010) The identification of the longevity allele of FOXO3A in humans pushed malleability of ageing into the human realm (Kuningas *et al.*, 2007; Willcox *et al.*, 2008; Pawlikowska *et al.*, 2009). Similarly to the discovery road of malleability of longevity through IIS, reduced activity in the TOR network was first associated with longevity in *C. elegans* (Vellai *et al.*, 2003). Evolutionary conservation of the role of the mTOR network in regulation of ageing was later shown by work in *Drosophila* (Kapahi *et al.*, 2004), and mammals (Harrison *et al.*, 2009; Evans *et al.*, 2011).

Thus far, a myriad of genes belonging to a few crucial pathways have been discovered to play a role in ageing. The hallmarks of ageing paper correlates these pathways with common denominators of ageing in multicellular organism; deregulated nutrient signalling, altered intracellular communication, mitochondrial dysfunction, genomic instability, telomere attrition, stem cell exhaustion, cellular senescence, epigenetic alterations and loss of proteostasis (López-Otín *et al.*, 2013). While the interplay between these hallmarks is not to be overlooked, this thesis will mostly focus on deregulated nutrient signalling.

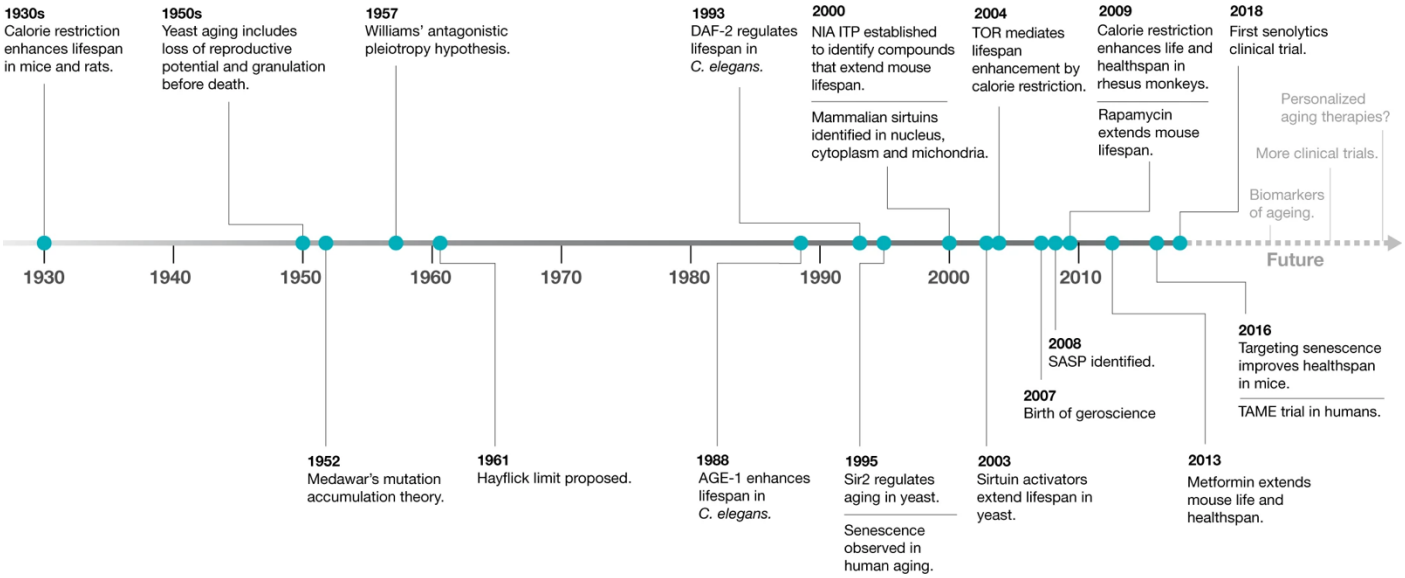

Figure 3:. Key discoveries in the geroscience over time. *From Campisi et al., 2019*

1.2 The IIS longevity network

1.2.1 Activation of IIS

The evolutionarily conserved insulin/insulin-like growth factor signalling (IIS) pathway and the Target of Rapamycin (TOR) network are essential regulators of growth and metabolism. While the TOR pathway directly senses nutritional status the IIS pathway acts via hormones. Insulin signalling in *Drosophila* starts with *Drosophila* insulin-like peptides (dILPs), encoded by 7 genes. dILP1, dILP2, dILP3 and dILP5 are produced by the median neurosecretory cells (mNSCs) in the brain in response to nutrient availability (Brogiolo *et al.*, 2001; Rulifson, Kim and Nusse, 2002; Broughton *et al.*, 2005a). There is partial functional redundancy between the dILPs, but slight differences in circulating sugar levels, energy storage and feeding preferences upon secretion have been observed (Grönke *et al.*, 2010; Post *et al.*, 2018; Semaniuk *et al.*, 2018, 2021). dILPs act on the insulin-like receptor (INR). The *Drosophila* INR mostly likely exists as a covalent disulphide-linked dimer even in the absence of its ligand, similarly to the human receptor tyrosine kinase (RTK) family (Meyts, 2016). Activation of the INR creates docking sites for insulin receptor substrates (IRS) to bind. IRS are cytoplasmic adaptor proteins transmitting the signal from the INR to elicit a cellular response. They often contain Pleckstrin homology (PH) or phosphotyrosine-binding (PTB) domains to locating them both close to receptors and their downstream signalling substrates. Phosphorylation of IRS does not lead to inherent activity as they do not possess an active kinase domain, but rather facilitate protein-protein interaction thereby organising downstream signalling. Different IRS have different binding domains for proteins, thereby providing specificity to IIS signalling. In *Drosophila* CHICO is the most well-known IRS, and its phosphorylation provides a binding site for p60, the regulatory subunit of PI3K, thereby recruiting Phosphoinositide 3-kinases (PI3K) to the plasma membrane. It also contains a binding domain for DRK, which in turn is an adaptor for Rat sarcoma (RAS) (Slack *et al.*, 2015). Another, more recently identified IRS is LNK, which regulates reproduction, cell cycle and lipid accumulation similarly as

CHICO. Both CHICO and LNK contain a PH domain and a PTB-binding motif. Uniquely, LNK possess an Scr Homology 2 (SH2) domain (Oldham *et al.*, 2002; Werz *et al.*, 2009). Both IRSs are needed for full activation of PI3K signalling and either provided enough for partial stimulation. Even at the highest levels of the signalling cascade of IIS/TOR network there is redundancy, showing the robustness of this network. Countering the action of the dILPs is the glucagon-like-adipokinetic hormone (AKH). AKH is secreted by the corpora cardiaca neuroendocrine cells, which are located near the esophagus. Healthy energy homeostasis is established via the interplay between dILPs, AKH and their downstream signalling cascades.

1.2.2 The PI3K-AKT branch of IIS

Upon binding dILP ligands, the intracellular part of the receptors *trans*-phosphorylate specific Tyr residues. These phosphorylated residues become binding sites for signalling partners CHICO and LNK, starting the signalling cascade. There are two canonical IIS cascades, either via PI3-kinase or via extracellular signal-regulated kinases (ERK). The INR activates lipid PI3K either directly or via CHICO. PI3K converts phosphatidylinositol (4,5)-bisphosphate (PIP_2) to phosphatidylinositol (1,4,5)-trisphosphate (PIP_3) (Leevers, Vanhaesebroeck and Waterfield, 1999). PIP_3 functions as secondary messenger, recruiting 3-phosphoinositide-dependent protein kinase-1 (PDK-1) and AKT. Close proximity between PDK-1 and AKT allows for activation of Protein kinase B, also known as AKT (Alessi *et al.*, 1998), which subsequently leads to the phosphorylation of downstream substrates, including TOR involved in regulation of protein synthesis (Harris and Lawrence, 2003); Glycogen synthase kinase 3 (GSK-3) (Cohen and Frame, 2001) involved in the inhibition of glycogen synthesis; Forkhead box O (FOXO), known for its effects on longevity and regulation of gluconeogenic and adipogenic genes (Accili and Arden, 2004; Willcox *et al.*, 2008); and Akt substrate of 160 kDa (AS160), crucial in the translocation of Glucose transporter 4 (GLUT4) (Sano *et al.*, 2003). AKT diversities signalling by affecting many different downstream signalling cascades and is thus a signalling node in the IIS/TOR pathway.

1.2.3 The AKT branch of the IIS signalling cascade

AKT regulates many cellular processes including metabolism, proliferation, cell survival, growth, and angiogenesis, by phosphorylation of serine/threonine residues on its targets. AKT is involved in the activation and inhibition of ~100 substrates (Manning and Cantley, 2007).
AKT stimulates TORC1 activity via phosphorylation of TSC2 and prolinerich Akt substrate 40 (PRAS40)(Dan *et al.*, 2014). Phosphorylation of TSC2 Ser939 and Thr^{1462} attenuated the inhibitory binding to Ras homolog enriched in brain (Rheb), thereby increasing TORC1 activation (Manning *et al.*, 2002; Zoncu, Efeyan and Sabatini, 2011). TORC1 consist of various components depending on activation status. PRAS40 inhibits TORC1 by preventing substrate interaction with the complex (Wang *et al.*, 2007). Phosphorylation by AKT reduces the interaction with TOR, enabling TORC1 to activate downstream targets (Haar *et al.*, 2007; Wang *et al.*, 2007).

Many substrates are involved in more than one process and most processes are also regulated by other substrates, showing the intricacy of the network. AKT recognizes the sequence Arg-Xaa-Arg-Xaa-Xaa-Ser/Thre followed by a hydrophobic residue (Alessi *et al.*, 1996). AKT promotes glycogen synthesis by inhibiting Glycogen synthase kinase-3 (GSK-3). Interestingly, not only AKT phosphorylated GSK-3,

protein kinase A (PKA) can phosphorylate similar residues. Inhibitory phosphorylation by AKT is modulated by the PI3K-PDK-1-AKT axis, while the PKA regulation dependents on hormonal stimulation or G protein coupled receptors that are linked to changes in intercellular cAMP levels (Fang et al., 2000; Frame, Cohen and Biondi, 2001). Influencing both metabolism and cell cycle progression, AKT synchronizes these key cellular processes by acting on GSK-3. Synchronization via a single kinase can be achieved due the multitude of signalling cascades GSK-3 is involved in. Apart from its role in metabolism, active GSK-3 promotes degradation of cyclins D1 and E, both driving G1/S phase transition, and transcription factors cellular ju-nana (c-Jun) and Myelocytomatosis (Myc). Ensuring that energy storage and cell cycle progression align.
Synchronizing another key cellular process with nutrient availability, AKT prevents apoptosis via inhibition of apoptosis signal related kinase (ASK1). Additionally, via positive regulation of nuclear factor kappa B (NF-κB) AKT stimulates pro-survival genes, thereby regulating both sides of the survival coin (Hers, Vincent and Tavaré, 2011).

In the same line, AKT regulates transcription factor forkhead box transcription factors O (FOXO). Inhibitory AKT-mediated phosphorylation of FOXO results in its transcriptional inactivity by preventing its translocation to the nucleus (Puig *et al.*, 2003). Therefore, when AKT signalling is blocked, non-phosphorylated FOXO shuttles to the nucleus where it activates pro-longevity genes (Puig *et al.*, 2003). Among those is the eukaryotic translation initiation factor 4E (eIF4E)-binding protein (4E-BP). 4E-BP is a master regulator of translation in response to stress and metabolomic changes (Carvalho et al., 2017). In addition to FOXO, 4E-BP is also directly regulated via phosphorylation by GSK-3, ERK and TOR, emphasizing the redundancy in the IIS/TOR network (Qin, Jiang and Zhang, 2016). FOXO influences metabolism, aging and longevity via transcriptional regulation of various key genes. For example, FOXO activates phosphoenolpyruvate carboxykinase (PEPCK), the rate-limiting enzyme of gluconeogenesis (Rulifson, Kim and Nusse, 2002; Broughton *et al.*, 2005a). FOXO influences the cell cycle by positive transcriptional regulation of the Cyclin G2, thereby blocking cell cycle progression and promoting growth (Svendsen *et al.*, 2014). FOXO teams up with GSK-3, regulating cell cycle by controling Myc mRNA levels in a tissue specific manner (Teleman et al., 2008). Lastly, FOXO itself is known to regulate *Inr* and *Lnk* expression, thus creating powerful feedback loops (Puig *et al.*, 2003; Slack *et al.*, 2010).

1.2.4 The role of IIS signalling in glucose transport

The prototypical effect of insulin is to stimulate glucose uptake into muscles and fat tissues. In mammals this is accomplished by translocation of the insulin-sensitive glucose transporter (GLUT4) to the cell membrane. GLUT4 functions independent of adenosine triphosphate (ATP) via a diffusion mechanism. In the absence of insulin stimulation, GLUT4 is present in storage vesicles (GSVs) located in the cytoplasm. Upon IIS activation, AKT phosphorylates AS160 and PDK-1 phosphorylates Atypical Protein Kinases C (PKCs). Via a currently only party understood mechanism, AS160 and PKCs stimulate the GSVs to fuse with the cell membrane, hence allowing glucose to be transported into the cell (Huang and Czech, 2007). In Drosophila the main glucose transporter is GLUT1, which is ubiquitously expressed (Escher and Rasmuson-Lestander, 1999; Chintapalli, Wang and Dow, 2007). Reduction in insulin signalling, by the combined lack of dILP2,3,5 increases extracellular glucose levels (Ugrankar *et al.*, 2015). The main mammalian PKC-dependent phosphorylation residues on the central loop and C-terminus of GLUT4, are conserved in the *Drosophila* GLUT1 protein (Escher and Rasmuson-Lestander,

1999). Additionally, when mammalian GLUT4 trafficking was monitored in Drosophila, it was clearly regulated in a hormonal response to insulin (Crivat *et al.*, 2013). In yeast, glycogen biosynthesis is regulated by PDK-1, showing that PDK-1 is not only involved in glucose uptake but also in storage of energy (Pastor-Flores *et al.*, 2011). Together with GSK-3 controlling glucose synthesis and PKC glucose uptake, both targets of PDK-1 activation, IIS affects most metabolic processes in the cell. Based on the shared physiology and phosphorylation sites glucose uptake in *Drosophila* is most probably regulated via a similar signalling cascade as in humans.

1.2.5 The RAS-MEK-ERK branch of IIS

INR activation also leads to activation of the RAS-MEK-ERK signalling cascade. Activation of CHICO results in activation of Ras, which is a small guanosine triphosphatase (GTPase). Ras oscillates between the inactive guanosine diphosphate (GDP)-bound state and the active GTP-bound state, a process controlled by Guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs). GTP binding results in conformational changes and translocating to interact with various secondary messengers, among those RAF (McKay and Morrison, 2007; Goitre *et al.*, 2014). RAF leads to activation of the Mitogen-activated protein kinase/ERK kinase (MEK) signalling cascade by phosphorylating two essential serine 218 and 222 residues in the activation loop of MEK (Alessi *et al.*, 1994). Not only RAF can phosphorylate these residues, PDK-1 is reported to phosphorylate residues Ser222 and Ser226 leading to activation (Williams *et al.*, 2000; Sato, Fujita and Tsuruo, 2004). MEK then phosphorylates Tyr and Thr residues in its sole substrate extracellular-signal-regulated kinase (ERK) (Shaul and Seger, 2007). Both residues are part of a highly conserved motif Thr-Glu-Tyr the activation loop present in all human ERK proteins (Katz, Amit and Yarden, 2007; Mehdizadeh *et al.*, 2016). Activation of RAS-RAF-MEK-ERK signalling regulates the activity of various transcription factors including the transcription factors p53, an infamous tumour suppressor (Milne *et al.*, 1994); *c-Jun,* a proto-oncogene regulating cell proliferation and apoptosis (Morton *et al.*, 2003); ETS (Yang *et al.*, 1996) and AOP, involved in the regulation of longevity in *Drosophila* (Slack *et al.*, 2015). Not only transcription factors are regulated by ERK, ribosomal s6 kinase (RSK) is phosphorylated by ERK. ERK dependent phosphorylation primes RSK for PDK-1 activation. Upon PDK-1 phosphorylation of the activation loop in Ser227, RSK regulates processes like transcription, translation, migration and proliferation (Jensen *et al.*, 1999; Frödin *et al.*, 2000; Anjum and Blenis, 2008). ERK results in a powerful downregulation of the pathway by inhibiting RAS, RAF and MEK, creating a strong feedback loop (Mendoza, Er and Blenis, 2011). ERK itself is downregulated by dephosphorylation at either the Thr or Tyr residue, a process mediated by two phosphatases, protein tyrosine phosphatase-ERK/Enhancer of Ras1 (PTP-ER) and mitogen-activated protein kinase phosphatase 3 (Mkp3) respectively (Shaul and Seger, 2007). Lastly, the transcription factor AOP is a negative regulator of the signalling cascade. Additionally, ERK signalling is involved in the regulation of glutathione-S-transferases (GSTs) and cellular detoxification genes (McElwee *et al.*, 2007a; Nässel, Liu and Luo, 2015). While the two signalling branches of the IIS, the PI3K-AKT-FOXO and the RAS-ERK-ETS, are mostly distinct, they regulate a partially overlapping set of genes and cellular processes (Alic *et al.*, 2014; Slack *et al.*, 2015).

1.2.6 IIS signalling is regulated via various negative feedback loops

There is an inverse correlation between INR prevalence at the cell membrane and long-term ambient insulin concentration (Wheeler and Yarden, 2015). Receptor endocytosis, a common negative

feedback mechanism in Receptor Tyrosine Kinases (RTK) family, results in either recycling or degradation, one of the most potent ways to regulate the activity of a signalling network (Meyts, 2016). Additionally, the INR can be inhibitorily phosphorylated by ERK, S6K and c-Jun-N-Terminal kinase (JNK) (Taniguchi, Emanuelli and Kahn, 2006). Note that all these kinases are activated by IIS/TOR network, thereby creating various negative feedback loops. Constitutively active phosphatases play a major role in insulin signalling attenuation. Two well studied phosphatases are PTP1B, the major phosphatase acting on the INR and phosphatase and tension homologue (PTEN), which dephosphorylates the 3' position of PIP_3 thereby reducing PDK-1 ability to phosphorylate AKT (Maehama and Dixon, 1999; Gao, Neufeld and Pan, 2000; Zhang, 2002).

1.3 The TOR network and its role in longevity

1.3.1 The TOR network integrates signalling from various stressors

TOR is a serine/threonine protein kinase of the PI3K-related (PIKKs) family, known for their reaction to stressors. TOR is a critical node in the cellular stress response and is, among others, regulated by DNA damage, reduction in energy levels, amino acid availability and oxygen (Sengupta, Peterson and Sabatini, 2010; Evans *et al.*, 2011). Activation of the TOR network integrates at least five different signalling components; PI3K signalling, adequate intracellular ATP levels, Wnt signalling, amino acid availability and hypoxia signalling (Inoki *et al.*, 2005). In response, TOR regulates growth by balancing anabolic processes, such as macromolecular synthesis and nutrient storage, and catabolic processes, including autophagy and utilization of energy.

1.3.2 The five signalling pathways leading to TOR activation

TOR functions as catalytic unit in two complexes, TORC1 and TORC2 (Loewith *et al.*, 2002). As mentioned above, PI3K signalling via AKT interacts with the TOR network, thereby connecting both signalling pathways. AKT inhibits tuberous sclerosis complex 1 and 2 (TSC1/2) an PRAS40 (Gao *et al.*, 2002; Sengupta, Peterson and Sabatini, 2010). Inhibition of TSC1/2 results in the positive regulation of the GTPase Rheb, an upstream kinase responsible for TORC1 activation (Zhang *et al.*, 2003; Kim *et al.*, 2008). AKT also inhibits GSK-3, which ultimately also leads to activation of the TOR pathway. GSK-3 can phosphorylate TSC, hence inhibit TOR, but the interaction depends on a priming phosphorylation by ATP sensor 5'-AMP-activated protein kinase (AMPK) (Bolster *et al.*, 2002; Kahn *et al.*, 2005). Correlating intracellular ATP levels and growth is essential, because growth is a highly ATP consuming process. Both AKT-dependent signalling cascades ultimately lead to activation of TORC1. GSK-3 is a central node for WNT signalling, crucial for cell fate determination and migration, thereby integrating nutrition signals and developmental signals and giving them on to the TOR network (Inoki *et al.*, 2006; Xu, Kim and Gumbiner, 2009)

Many cellular processes lead to an accumulation of AMP, for example a reduction in glycolysis or diminished mitochondrial oxidative phosphorylation. The accumulation of AMP is sensed by AMPK and signalled onwards to TOR. Hypoxia, due to insufficient oxygen supply, increases the cellular AMP/ATP ratio thereby feeding into the AMPK-TOR signalling branch. Additionally, and more directly, hypoxia activates signalling via Sima, the *Drosophila* homologue of Hypoxia-inducible factor 1 alpha

subunit (HIF-1α) (Arquier *et al.*, 2006; Dengler, 2020). HIF-1α promotes hypoxia related gene expression, among those an activator for Tsc1/2 (Reiling and Hafen, 2004). Both mechanisms inhibit TOR signalling, preventing growth in unfavourable energy and oxygen conditions.

The last component of TOR activation is the availability of amino acids. High amino acid levels promote the translocation of TORC1 to lysosomal membranes where its activator Rheb is located (Sancak *et al.*, 2008). So far, this translocation has been observed only in high amino acid conditions. Hence, amino acid availability is both required and essential to activate the TOR network. In contrast, amino acid deficiency leads to rapid inactivation of TOR signalling and dephosphorylation of the downstream targets S6K and 4E-BP (Beretta *et al.*, 1996; Wullschleger, Loewith and Hall, 2006).

1.3.3 Downstream targets of TOR

TORC1 directly phosphorylates and regulates several downstream targets including p70 ribosomal protein S6 kinase (S6K) (Terada *et al.*, 1994; Zhang *et al.*, 2000); eukaryotic translation initiation factor 4E-binding protein (4E-BP) (Beretta *et al.*, 1996); HIF-1α; Myc a regulator of metabolism, apoptosis and cell competition (Gallant *et al.*, 1996; Grifoni and Bellosta, 2015); autophagy-related protein 1 (ATG1) a key initiator of autophagy (Chang and Neufeld, 2009); peroxisome proliferator-activated receptor coactivator-1 α (PGC-1 α) regulating mitochondrial activity (Cunningham *et al.*, 2007).

S6K promotes translation initiation via phosphorylation of initiation factor 4B (eIF4B), small ribosomal subunit S6 and elongation factor 2 kinase (eEF-2K) (X. Wang *et al.*, 2001; Holz *et al.*, 2005). Additionally, TORC1 regulates translation of 5'-TOP mRNA transcripts. As many ribosomal proteins contain a TOP sequence, both S6 activation and increased translation of TOP mRNA results in an upregulation of the ribosomal machinery and, correspondingly, growth (Meyuhas, 2000). TOR dependent inhibition of 4E-BP results in a dissociation with cap-binding protein eukaryotic initiation factor 4E (eIF4E), thereby allowing eIF4E to form a pre-initiation complex and bind mRNAs, hence promoting initiation of translation (Gingras *et al.*, 1998; Shamji, Nghiem and Schreiber, 2003). By tightly regulating these processes, cell growth and proliferation are in tune with energy levels and nutrient availability.

TORC1 signalling activates Myc post-transcriptionally, leading to Myc/MAX heterodimers binding E-boxes in DNA (Gallant *et al.*, 1996). 90% of the TORC1-activated genes contain an E-box, suggesting that Myc might be a main mediator of TORC1 transcriptional activation (Teleman *et al.*, 2008). Myc controls ribosome biogenesis and protein synthesis, and hence the accumulation of cellular mass and size. In *Drosophila* the role of Myc is dependent on developmental stage and cell type, affecting either cell size, cell number or cell autonomous apoptosis (Gallant *et al.*, 1996; Gallant, 2013).

Depletion of ATP levels, sensed by TOR, makes the deactivation of anabolic processes and activation of catabolic processes crucial. Both adipogenesis, via an inhibitory 4E-BP signalling cascade (Zhang *et al.*, 2009), and lipogenesis, among other regulated by S6K and sterol regulatory element-binding proteins (SREBPs) (Peterson *et al.*, 2011), are downstream effectors of TORC1 signalling. Additionally, TOR promotes mitochondrial respiration and gene expression via PGC-1 α to increase ATP levels (Cunningham *et al.*, 2007).

1.3.4 *The role of the TOR network in growth and autophagy*

Autophagy functions in a range of physiological process, including differentiation, development, ageing and tumorigenesis (Mizushima and Levine, 2010). The autophagic pathway is regulated by a group of ATGs that control the generation of double-membraned autophagosomes. Upon stimulation they fuse with lysosomes and degrade their contents. Signal induction is initiated by the ATG1/ATG13 complex through the membrane nucleation of autophagosomes. Vesicle expansion is mediated by ATG8 and the ATG5-ATG12-ATG16 complex. Fusion with lysosomes is regulated by general docking proteins (Chang and Neufeld, 2010). While TOR's role in autophagy is undisputed, the exact machinery of autophagy activation is still ambiguous. Under nutrient rich conditions TORC1 inhibitory phosphorylates both ATG1 and ATG13, preventing them to initiate autophagy. During starvation conditions, TORC1 dependent phosphorylation is reduced, allowing ATG1 and ATG13 to initiate autophagy (Noda and Ohsumi, 1998; Kamada *et al.*, 2000). Over time, autophagy results in an increase in amino acids, thereby reactivating the TOR pathway, hence stopping autophagy and stimulating replenishment of the lysosomes.

1.3.5 *Interplay between the IIS and TOR network*

The IIS and TOR pathways are tightly interconnected in the regulation of growth, stress response and ageing. This includes inhibition of TSC1/2 by ERK and activation of TORC1 by RSK. S6K also creates a negative feedback loop to IIS, via the inhibitory phosphorylation of CHICO (Haruta *et al.*, 2000; Shah and Hunter, 2006). Additionally, IIS and TOR act on similar downstream substrates including, but not limited to, S6, GSK3, FOXO, 4E-BP and MYC (Puig *et al.*, 2003; Wullschleger, Loewith and Hall, 2006; Mendoza, Er and Blenis, 2011). The intertwined signalling of these two pathways will be referred to as the IIS/TOR network in the rest of this thesis.

1.3.6 *Down regulation of the IIS/TOR network extends lifespan in Drosophila and other model organisms*

As previously mentioned, the role of the IIS in lifespan became abundantly clear over the last 30 years. Genetic and pharmacological inhibition of IIS activity causes lifespan extension in *C. elegans, Drosophila* and mice (Kenyon *et al.*, 1993; Clancy *et al.*, 2001; Selman *et al.*, 2008; Bjedov *et al.*, 2010; Castillo-Quan *et al.*, 2019). Down-regulation of the IIS pathway by ablation of the MNCs or deletion of the *dILP 2,3 and 5* genes results in extended lifespan in *Drosophila* (Broughton *et al.*, 2005a; Grönke *et al.*, 2010). Furthermore, mutation of the insulin receptor (Tatar *et al.*, 2001), the insulin receptor substrate CHICO (Clancy *et al.*, 2001) or LNK (Slack *et al.*, 2010) all promote longevity. Additionally, increased expression of the negative regulator PTEN increases lifespan (Hwangbo *et al.*, 2004). The second important branch of ISS signalling, the RAS-MEK-ERK, is equally important in longevity. Attenuation of the signalling cascade, either genetically or pharmaceutically, increases lifespan in yeast due to a deletion of RAS, (Fabrizio *et al.*, 2003), in *Drosophila* by genetic inhibition of RAS and pharmacologically of MEK (Slack *et al.*, 2015) and in mice downregulated for RAS signalling (Borrás *et al.*, 2011). IIS-mediated longevity is not just a matter of extended slowed development. Post development, fat-body-specific over-expression of FOXO extends lifespan in *Drosophila* (Giannakou *et al.*, 2004; Hwangbo *et al.*, 2004). Longevity and delayed development can thus be uncoupled, showing longevity is more than different allocation of limited energy reserves.

Not surprisingly, reduction of TOR signalling via either TOR (Zhang *et al.*, 2000), TSC1/2 or S6K modulation (Kapahi *et al.*, 2004) extends lifespan in *Drosophila* (Evans *et al.*, 2011). Additionally, over-expression of 4E-BP^{active} results in longevity in nutrient rich conditions (Zid *et al.*, 2009). Rapamycin treatment also extends lifespan in a TORC1 dependent way (Harrison *et al.*, 2009; Bjedov *et al.*, 2010; Juricic *et al.*, 2022). Increased autophagy and reduced S6K activity are essential downstream mediators of TORC1 dependent longevity in *Drosophila* (Bjedov *et al.*, 2010; Lu *et al.*, 2021; Juricic *et al.*, 2022; Zhang *et al.*, 2022). Administration of rapamycin also increases lifespan of mice, showing the conservation of TOR mediated longevity (Harrison *et al.*, 2009).

While the role of IIS/TOR in longevity is undisputed, the geroscience field is still elucidating which are the best targets to positively regulate health and longevity. Down-regulation of the network by mutations that target essential upstream components has been successful, but also caused undesired side effects like reduced fertility, growth defects or impaired wound healing (Werz *et al.*, 2009; Weinreich *et al.*, 2011; Slack *et al.*, 2015). Furthermore, somatic genetic interventions to improve health during ageing are in general not an option for use in humans. Thus, the identification of novel drug targets within the IIS/TOR network to target healthy ageing is an important aspect in geroscience. As such, protein kinases are interesting candidates, as for many of them pharmaceutical inhibitors have already been developed in the context of cancer research or other ARD. Repurposing pharmaceuticals already approved by the FDA and EMA for treating ageing is a far more achievable goal then development of novel components.

1.4 PDK-1; a master regulator of the IIS network

1.4.1 PDK-1 acts on 23 substrates

PDK-1 is a key upstream regulator within the IIS/TOR network. PDK-1 is part, and simultaneously a master regulator, of the AGC (the cAMP-dependent protein kinase (PKA), the cGMP-dependent protein kinase (PKG) and the protein kinase C (PKC) families) kinase family. In mammals, PDK-1 regulates numerous substrates by phosphorylation of specific threonine or serine residues located in the T-loop of target molecules. In mammals, PDK-1 is thought to be involved in the activation of 23 of the 63 currently known ACG kinases, among them AKT, S6K, PKA, and atypical PKCs, p90 ribosomal S6 kinase (RSK), serum and glucocorticoid-inducible kinase (SGK), p21-activated kinase-1 (PAK1), protein kinase PKN (PKN1 and PKN2), Mothers against decapentaplegic homolog (SMAD) and Nuclear factor-κB (NF-κB), earning it the name master regulator (Alessi *et al.*, 1997; Currie *et al.*, 1999; Biondi *et al.*, 2001; Bayascas, 2008, 2010).

1.4.2 Typical and unique kinase domains of PDK-1

The human and the fly PDK-1 proteins are composed of 556 and 422 amino acids, respectively. The protein sequence is highly similar between flies and human (58.1% identity) (Alessi *et al.*, 1997; Rintelen *et al.*, 2001). PDK-1 is a typical kinase, in that is possesses 5 key domains spread out over 2 lobes which are responsible for the functionality of the kinase. The catalytic core possesses an N-terminal lobe consisting mainly of β-sheets and a predominantly α-helical C terminal lobe (**Figure 4**)

(Pearce, Komander and Alessi, 2010). The activation segment, containing the T-loop (also known as A loop) which has the DFG (Asp-Phe-Gly) motif, is found in the C-lobe. Activation by phosphorylation of Ser241 in the T-loop results in conformation changes in the αC helix, hence facilitating the formation of new hydrogen bonds between αC helix, N-lobe and the phosphate of a bound ATP (Komander *et al.*, 2005). Then Aspartate from the DFG motif interacts with bound ATP by shifting the T-loop from the DFG-out/inactive to the DFG-in/active conformation. The hydrophobic pocket of PDK-1 interacts with a hydrophobic motif resulting in the reinforcement of αC helix conformation. Only when the DFG-in conformation is stabilized is the T-loop ready for catalysis, and PDK-1 can phosphorylate its substrate (Pearce, Komander and Alessi, 2010; Fabbro, Cowan-Jacob and Moebitz, 2015). Lastly, PDK-1 possesses a C-terminal PH domain (Alessi *et al.*, 1997). While many kinases have a PH-domain, the human PH-domain of PDK-1 has an unusual "bud" in the structure (Komander *et al.*, 2004). There is an N-terminal extension and an unusually spacious ligand binding site. While all PDK-1 domains are important for its functionality, two domains play a crucial role in substrate interaction; the PH domain and the hydrophobic pocket.

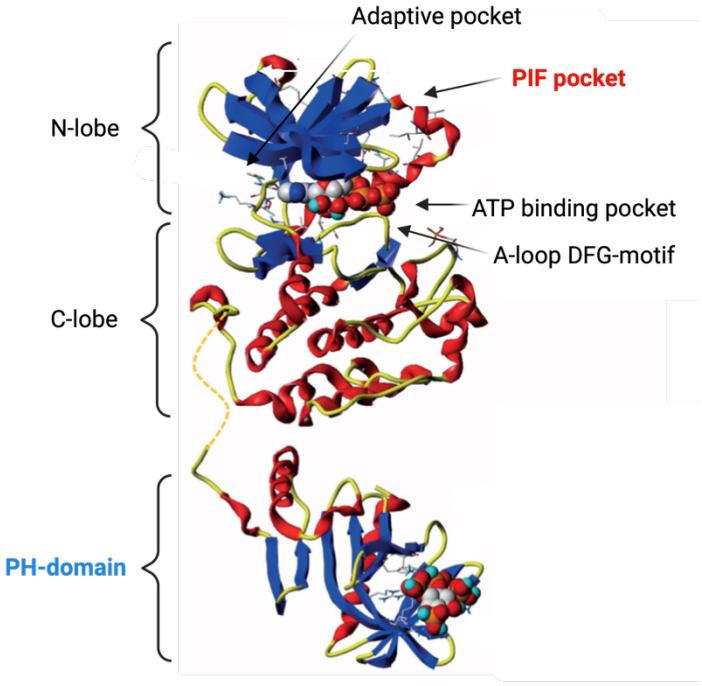

Figure 4: protein structure of PDK-1 (Human). *The N lobe contains the adaptive pocket, the ATP binding pocket and the PIF pocket. Between the C lobe and the N lobe is the A-loop with the DFG motif, currently in the DFG-out formation, hence the inactive form of the kinase. Lastly, the PH domain is attached to the C-lobe via a linker and it a separate unit by itself.*

1.4.3 Crucial residues in the PH domain and the PIF pocket

The PH domain consist of amino acids 459 to 550 in mammalian PDK-1. Two β-sheets, one α-helix and three loops form a bowl-like structure lined with positive charges where the PH domain interacts with PIP_3. The PH domain has a 15-fold higher affinity for PIP_3 compared to PIP_2. The characteristic PDK-1 bub is formed due to an additional two β-sheets and an α-helix, which fold over to form a hydrophobic

core. Mutating either Lys465 or Arg474 prevents interaction with PIP_3, while mutating Arg472, Tyr486, Lys495 or Arg521, Trp535 had only moderate effects (Anderson *et al.*, 1998; Currie *et al.*, 1999). Not surprisingly, Arg474 is crucial for AKT activation upon IIS stimulation (Komander *et al.*, 2004). Interestingly, mutating arginines 472, 473 and 474 in the PH domain resulted in embryonic lethality in mice (McManus et al., 2004). Taken together, a severe reduction in positive charge by mutating all arginines disrupts the interaction of the bowl with PIP_3, as does the mutation of the essential Arg474 residue.

The hydrophobic pocket in PDK-1 is called the PDK-1 interacting fragment (PIF) pocket due its use in substrate selection (Biondi *et al.*, 2000, 2001; Frödin *et al.*, 2000, 2002). The PIF pocket is formed by a conserved αC-helix, β-strands and an αB-helix in the N-lobe. Several residues are crucial for PIF pocket functionality, including L115, I119, T148, R131, Q150 and L155. Mutation of Lys115Ala, Ile119Ala or Gln150Ala reduced the ability of PDK-1 to interact with a PIFtide, a substrate mimic for PDK-1. Lys155 is essential for PIF dependent substrate interaction, as mutation completely abolished any interaction with the PIFtide (Biondi *et al.*, 2000, 2001). Phosphorylation of the PDK-1 substrates S6K and SGK was mildly reduced in Lys115Ala, Ile119Ala, Arg131Met and Gln150Ala mutants, but strongly affected in Lys155Glu (Biondi *et al.*, 2001; Frödin *et al.*, 2002). While all mutations affect the affinity of the PIF pocket to the HM, Lys155Glu destroys the pocket structure thereby abolishing any interaction. Of note, none of the mutations in the PIF pocket abolished or severely reduced AKT phosphorylation, underlying that the mechanism of activation are distinct and independent (Biondi *et al.*, 2001; Frödin *et al.*, 2002; Collins *et al.*, 2003).

1.4.4 Two models for PDK-1 dimerization

It has long been thought that PDK-1 is constitutively active and not under tight physiological regulation. Autophosphorylation of Ser241 in the activation loop is essential and sufficient for PDK-1 activation, resulting in a continuously active kinase (Casamayor, Morrice and Alessi, 1999; Wick *et al.*, 2003). Specificity of PDK-1 signalling might therefore be achieved by different means, including but not limited to: substrate availability, co-localization, allosteric activation or post-translational modifications of substrates. Taken together these factors ensuring specificity in PDK-1 signalling. Recently, the assumption that PDK-1 is constitutively active by autophosphorylation was brought in jeopardy, new models regulating PDK-1 activity via Ser241 phosphorylation have been proposed.

Both activation models require the formation of dimers relying on dimerization residue Tyr288. Most AGC kinases depend on a hydrophobic pocket and HM on the opposing kinase for dimerization. In PDK-1 the hydrophobic pocket involved in dimerization is the PIF pocket. When another PDK-1 protein is encountered Levina *et al.* imply that the PIF pocket associates with the HM present in the linker connecting the C-lobe and the PH domain (human: ^{383}Phe-Gly-Cys-Met^{386}) of the opposing PDK-1 kinase. As mentioned, a *trans*-interaction of a HM with a hydrophobic pocket is not unique in the AGC family, however the location of the HM in PDK-1 is (Levina *et al.*, 2022). Upon association of the HM with the PIF pocket, the activation loop conformation changes. In the new structure allows for *trans*-phosphorylation of Ser241, resulting in activation. A second model proposes that hydrophobic pocket is occupied by the αG helix in the C-lobe of an opposing PDK-1 protein, not a HM located near the PH domain. The interaction between the PIF pocket and the αG helix is reminiscent of AGC kinase interaction with its HM, but clearly distinct due to lack of hydrophobic residues (Biondi *et al.*, 2002;

Masters *et al.*, 2010). It must be noted that no matter where the HM responsible for the *trans*-autophosphorylation is located, the affinity is low compared to the binding of the PIF pocket with a PDK-1 substrate HM (Levina *et al.*, 2022). Expression levels, hence concentration in the cytoplasm, influences the likelihood of dimerization. Taken together, *trans*-autophosphorylation will most likely ensure high activity levels of PDK-1, previously mistaken as constitutive active.

1.4.5 Autoinhibition of PDK-1 by the PH domain

While the two models disagree on the exact domains involved in dimerization, the general requirement for dimerization is shared. Additionally, the model proposed by Levine *et al.* necessitates an additional step in PDK-1 activation. New evidence suggested that, in the absence of PIP_3, the PH domain binds to the catalytic cleft, obscuring both the substrate-binding helix and the dimerization surface (Levina *et al.*, 2022). This way the PH-domain would has an intramolecular inhibitory function. PI3K activation resulting in an highted concentration of PIP_3 at the plasma membrane. The interaction affinity between the PH domain and PIP_3 is very powerful (KD 1.6nM) (Currie *et al.*, 1999). The high affinity promotes dissociation of PH-domain with any previous interaction, in this case the catalytic cleft. While interacting with PIP_3 with the PH domain, PDK-1 can dimerize and activate. Via this mechanism PIP_3 acts as an on-switch for dimerization and autophosphorylation. PIP_3-dependent increase in PDK-1 substrate phosphorylation was previously observed, but thus far misunderstood (Masters *et al.*, 2010; Najafov, Shpiro and Alessi, 2012; Levina *et al.*, 2022). Not only PDK-1 depends on PIP_3 binding for *trans*-autophosphorylation, but also both AKT, PKC and SGK-3 have similar mechanisms (Leonard *et al.*, 2011; Pokorny *et al.*, 2021; Truebestein *et al.*, 2021). If this model is true, then PDK-1 is not constitutively active in cells, but instead dependent on insulin signalling for activation, contradictory to the Masters *et al.* model solely relying on dimization for *trans*-autophosphorylation.

1.4.6 Final activation by substrates

The final part of PDK-1 activation is also unique in the AGC family. Normally, the hydrophobic motif in the C-terminal of the kinase wraps around the N-lobe to insert two aromatic residues into the hydrophobic pocket. This results in stabilization of the αC helix and readies the kinase for phosphorylation. As stated above, unlike most AGC kinases, PDK-1 does not have a HM in the C-terminal. Nonetheless, PDK-1 possesses a hydrophobic pocket kinase domain requiring an interaction. Since PDK-1 lacks its own HM, the hydrophobic pocket associates with HM of the substrate, thereby earning the name PDK-1 Interacting Fragment (PIF) pocket (Schulze *et al.*, 2016). The priming phosphorylation on the substrate promotes the association of the PIF pocket and the HM of the substrate (Biondi *et al.*, 2000; Levina *et al.*, 2022). The association between PIF pocket and HM results in the desired conformational change and higher ATP affinity in PDK-1. While the interaction of the PIF pocket with its substrate has been known for a long time, lately it has been proposed to be part of the activation of PDK-1. According to the model, it is thus the substrate itself which finalizes PDK-1 activation allosterically (Levina *et al.*, 2022).

1.4.7 PH domain dependent activation of AKT

The PH domain is involved in binding to PIP_3 at the cell membrane, bringing PDK-1 into close proximity to AKT, which also contains a PH domain. The PH-dependent AKT interaction with PIP_3 prompts a conformational change that enables PDK-1 to phosphorylate the T-loop residue of AKT, Thr342 in *Drosophila* and Thr308 in mammals (Alessi *et al.*, 1997; Stokoe *et al.*, 1997; Currie *et al.*, 1999; Milburn *et al.*, 2003). The binding of PDK-1 to PIP_3 does not induce any conformational changes, it just brings the kinase and substrate into close proximity (Bayascas *et al.*, 2008). An additional phosphorylation in the C terminal of the catalytic domain on Ser473 by TORC2 is needed for full activation of AKT. Activation of AKT results in the canonical AKT/FOXO branch activation (Pearce, Komander and Alessi, 2010). As mentioned before, the interaction affinity between the PH domain and PIP_3 is very high (Currie *et al.*, 1999). The strength of this interaction makes the PH-domain mechanism of activation (MOA) hard to block upon IIS activation (Najafov *et al.*, 2011a).

1.4.8 The PIF pocket dependent activation of substrates

In contrast to the PH domain driven interaction at the plasma membrane, most PDK-1 substrate interactions take place independently of the PH domain. A priming phosphorylation of a serine/threonine located in the C terminal of the catalytic domain of the substrate, close to the hydrophobic motif, is needed for PDK-1 to identify a substrate (See Table 1) (Frödin *et al.*, 2002; Pearce, Komander and Alessi, 2010). When the hydrophobic motif of the substrate is phosphorylated, there is a PIF pocket driven interaction stabilizing the active conformation of PDK-1 resulting in phosphorylation of the substrate. The PIF dependent substrates of PDK-1 are diverse and are part of various cellular processes. It is the priming phosphorylation which allows for specificity in PDK-1 signalling. The kinases responsible for the priming phosphorylation of the most prominent PDK-1 substrates are listed in the table below.

Table 1: Phosphorylation sites of PDK-1 substrates. PDK-1 dependent phosphorylation, priming phosphorylation or other mode of activation. Red coloured substrates are PIF pocket dependent substrates, blue coloured substrates are PH domain dependent substrates.

Substrate	PDK-1 dependent residue	Priming residue	Reference
S6K	T loop Thr229	HM Thr389 by TORC1	(Pullen *et al.*, 1998; Biondi *et al.*, 2001; Radimerski *et al.*, 2002; Mora *et al.*, 2005)
SGK	T loop Thr256	C terminal HM Ser422 by TORC2	(Kobayashi and Cohen, 1999; Biondi *et al.*, 2001; García-Martínez and Alessi, 2008)
RSK	N terminal kinase domain Ser227	ERK Linker Ser368 by Erk T loop Thr577 by Erk Autophosphorylation of ser386	(Jensen *et al.*, 1999; Frödin *et al.*, 2000)
PKA	Activation loop Thr197		(Cheng *et al.*, 1998; Biondi *et al.*, 2000)
PKCα and PKCβII (conventional)	Activation loop Thr500	Thr641 and Ser660 by autophosphorylation	(Edwards and Newton, 1997; Dutil, Toker and Newton, 1998)
MEK	Activation loop Ser222 and Ser226	Ser218 by Raf	(Williams *et al.*, 2000; Sato, Fujita and Tsuruo, 2004)
PKCδ (novel)	Activation loop Thr505 PIF pocket		(Le Good *et al.*, 1998)
PKCζ (atypical)	Activation loop Thr410 PIF pocket		(Chou *et al.*, 1998; Balendran *et al.*, 2000)
AKT	T loop Thr308 in PH domain dependent manner		(Stokoe *et al.*, 1997; Milburn *et al.*, 2003; Sarbassov *et al.*, 2005)

1.4.9 The AKT and the AGC branch of PDK-1 signalling

Overall, we can distinguish two mechanisms of activation by PDK-1; one PH domain dependent and the other PIF dependent (**Figure 5**). In this thesis I will refer to PH-dependent branch, where AKT is the main substrate of interest, as the AKT branch of IIS/TOR signalling. The PIF-dependent mode of activation affects most AGC kinases involved in various signalling cascades, of primary interest for longevity are S6K and MEK. I will therefore refer to these signalling cascades as the AGC branch of PKD-1 dependent signalling. By modulating these different downstream branches, PDK-1 regulates physiological processes related to cell growth, proliferation, survival and metabolism (Cho *et al.*, 2001; Bayascas, 2010).

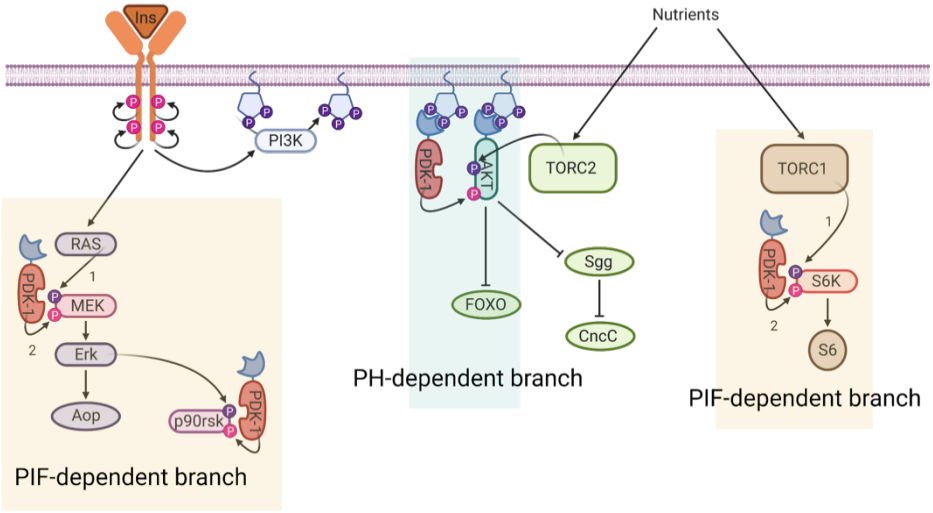

Figure 5: Simplified activation of PDK-1 substrates. *Phosphorylations performed by PDK-1 are pink. The AKT branch is dependent on PH-domain dependent interaction with PIP_3. Translocation of both PDK-1 and AKT allows for phosphorylation of AKT. The PIF-mediated activation of S6K, RSK and MEK are dependent on a priming phosphorylation by other kinases than PDK-1. These substrate interactions are part of the AGC branch of IIS/TOR network signalling.*

1.4.10 Lethality in PDK-1 mutants

As discussed above, PDK-1 occupies a key position in the IIS/TOR signalling network controlling numerous downstream cellular processes, including but not limited to cell survival, proliferation, differentiation, protein synthesis and metabolism. It is not surprising that complete loss of function of PDK-1 is lethal in both *Drosophila* (Cho *et al.*, 2001) and mice (Lawlor *et al.*, 2002). PDK-$1^{-/-}$ mice show embryonic abnormalities from E8,5 onwards and do not survive pass day E9,5. Tissue specific PKD-1 knock-out is not embryonically lethal, but often leads to premature deaths due to organ failure. PDK-$1^{-/-}$ liver mutants (L-PDK-$1^{-/-}$) die of liver failure at 4-16 weeks (Mora *et al.*, 2005). Heart-specific loss of PDK-1 results in heart failure 5 to10 weeks later (Di *et al.*, 2010). Loss of PDK-1 function in ovaries results in premature ovarian failure leading to infertility, but does not affect mortality (Reddy *et al.*, 2009). L-PDK-$1^{-/-}$ mice respond to insulin stimulation and have normal blood glucose levels in homeostatic conditions. However, a big dose of glucose by injection causes short term hyperglycaemia indicating failure to rapidly and maximally activate the IIS/TOR signalling network (Mora *et al.*, 2005).

Single point mutations in PDK-1 can also be fatal. PDK-1^{R131M} PIF pocket mutant mice develop embryonic abnormalities in size and developmental patterns and do not survive past E19.5 (Collins *et al.*, 2003). The mutation specifically reduces the functionality of the PIF pocket by preventing an interaction with the HM of the substrate. Indeed, there is a reduction of phosphorylation of S6K, SGK and RSK, while p-AKT^{Thr389} remains unchanged (Frödin *et al.*, 2002). Mutation of PIF pocket residue Lys155 resulted in even earlier lethality at E11.5 (Collins *et al.*, 2003). As previously discussed, PDK-1^{K155E} did not phosphorylate S6K nor RSK, rending the AGC signalling branch defective. Not only the PIF pocket mutation are lethal, mutating three consecutive arginines in the PH domain resulted in lethality at E10,5 (McManus *et al.*, 2004). In summary, in mammals both PH- and PIF-pocket-dependent signalling cascades are required for survival beyond the embryonic stage.

1.4.11 The effects of PDK-1 down-regulation in model organisms

Remarkably, expression of a PDK-$1^{fl/fl}$ hypermorph ensuring 50% reduction in PDK-1 expression is viable in mice. These mice are smaller and have a reduced body weight, viability and fertility (Lawlor *et al.*, 2002). A similar diminution in body size is observed in hypermorph PDK-1^{K465E} affecting PH-domain functionality (Bayascas *et al.*, 2008). Both PDK-$1^{fl/fl}$ and PDK-1^{K465E} mutant mice have reduced body mass due to smaller cell size rather than a reduction in cell count. While PDK-$1^{fl/fl}$ reduces expression and PDK-1^{K465E} reduces functionality, phenotypically they are comparable. Generally, the signalling cascade is down-regulated in both mutants, with some crucial differences. PDK-$1^{fl/fl}$ mutants can respond to insulin stimulation by activation of AKT, S6K and RSK (Lawlor *et al.*, 2002). Strikingly, PH-domain mutants do phosphorylate AKT^{Thr308} upon insulin stimulation, but the response has a 3-5-fold lower maximum and is more transient than in wild type litter mates. This finding reenforces the idea that, while the PH domain affinity for PIP_3 is reduced, residual interaction remains. Unsurprisingly, TORC1 dependent p-AKT^{Ser473} was unchanged. Diminished AKT^{Thr308} phosphorylation reduces AKT downstream signalling, as observed by a reduction in $S6K^{Thr299}$ and $S6K^{Thr389}$ phosphorylation (Bayascas *et al.*, 2008). The priming p-$S6K^{Thr389}$ requires TORC1 signalling (Pullen *et al.*, 1998). The low and transient nature of the AKT activation might not be sufficient to cascade the signal down to TORC1. Without the priming phosphorylation, PDK-1 does not recognize S6K as a substrate and won't interact. Absence of S6K activity was also shown by strong reduction in S6 phosphorylation. Interestingly, when food was used to stimulate fasted mice, phosphorylation of both S6K phosphorylation sites was close to wild type (Bayascas *et al.*, 2008). The stimulation of TORC1 by nutrient availability was sufficient for activation, hence priming p-$S6K^{Thr389}$ and subsequent recognition by PDK-1. The observed effects on the signalling cascades indicate that, while the mutation is PH domain specific, both the AKT and the AGC branch are affected.

In contrast, a slight loss of function PKD-1^{sa680} *C. elegans* mutant showed an increased lifespan by almost two-fold. Both DAF-2 (IGF) and AGE-1 (PI3K) are required for the signal propagation and it is DAF-16 (FOXO) dependent (Paradis *et al.*, 1999). The PDK-1 *C. elegans* mutants have a low fertility and are unable to lay eggs, leading to hatching offspring within the mother. Additionally, they have phenotypically long body (Lon) and do not spread out in search of food. Over-active AKT-1 (mg144) expression rescues fertility but not the egg laying, abnormal phenotypical body or absence of food seeking phenotypes. This indicates the observed phenotypes are regulated by different branches of PDK-1 signalling in *C. elegans.*

All things considered, reducing PDK-1 functionality must be done in moderation, as severe impairment of either the PH domain or PIF pocket causes lethality or organ failure. While key residues for both kinase domains are slowly identified, no viable PIF pocket mutation has been identified. Our current understanding of longevity, and the promising results in *C. elegans,* raises the question of PDK-1 dependent longevity in *Drosophila.*

1.5 Aims of the thesis

Despite, the extensive gain in knowledge about PDK-1 PH-domain and PIF pocket dependent phosphorylation, hardly anything is known about the role of PDK-1 on the organismal level. Explorations in this area have been limited by the lethality in embryonic stage for most mice models, with the exception of PDK-1^{K465E} mice. While some phenotyping data has been gathered, this has been rather limited.

In my PhD thesis I have uses the fruit fly *Drosophila melanogaster,* to characterize the effect of mutations in the PDK-1$^{PH\text{-}domain}$ and PDK-1$^{PIF\text{-}pocket}$ domain on health- and longevity. As there are specific inhibitors targeting both domains individually, it is important to test which downstream branch contributes more to the regulation of longevity. Furthermore, I investigate downstream mechanisms and organ-specific functions of PDK-1, to identify which organs may underlie the observed phenotypes. Lastly, I performed phosphor-proteomics to characterize the insulin signalling cascade in both the PDK-1^{PH} and PDK-1^{PIF} mutants.

Additionally, I examine the possibility of using a *Drosophila* model to investigate PDK-1 as a putative anti-ageing drug target. Despite significant homology between *Drosophila* and mammals, mammalian inhibitors are not likely to display high affinity for the *Drosophila* PDK-1 protein. Therefore, I generated a humanized *Drosophila* PDK-1 fly line, in which parts or the whole *Drosophila* PDK1 gene are replaced by the human PDK1 sequence. The aim was to use the humanized fly model to study the anti-ageing properties of existing PDK-1 inhibitors without the need of performing studies in higher organisms.

2 Methods

2.1 General Fly methods

2.1.1 Maintenance of Flies

Fly stocks are kept at either 25°C or 18°C on a 12/12 hours light/dark cycle with 65% humidity. During all experimental procedures the flies are fed the standard SYA (sugar/yeast/agar) diet unless specified otherwise (Bass et al., 2007) (Table 1). All experimental flies are reared at 25°C. Synchronized hatching and larval density is controlled using the squirting technique. In population cages the P0 generation lays eggs for 16h on juice plates containing fresh yeast paste. Eggs are collected in phosphate-buffered saline (PBS), washed and 20µ of liquid including eggs is transferred into fresh bottle of SYA food. To allow for different developmental times squirts are set up in consecutive days (Table 2). 9 Days after the white Dahomey (w^{Dah}) squirt, hatching flies are collected in fresh bottles. The flies are allowed to mate for 48h. Flies are sorted under CO2 anaesthesia and maintained in experimental vials containing 20 flies of one sex (unless specified otherwise).

Table 2: ***SYA diet ingredients***

Ingredient	Percentage
Sugar	5%
Yeast	10%
Agar	1.5%
Nipagen (10% in EtOH)	0.3%
Propionic Acid	0.03%

Table 3: ***Squirting set up scheme***

Genotype	Day 1	Day 2	Day 3	Day 4
w^{Dah}			x	
PDK-1^{GR}				x
PDK-$1^{PH/PH}$		x		
PDK-$1^{PH/null}$	x			
PDK-$1^{PIF/PIF}$		x		
PDK-$1^{PIF/null}$	x			
PDK-$1^{WT/null}$			x	

2.1.2 Transgenic flies used in this study

The following transgenic flies are used in this study (Table 3). Transgenic flies were backcrossed for at least 6 generation into the w^{Dah} outbred wild-type strain. The wild-type population contained the endosymbiontic bacterium *Wolbachia pipientis*. See generation of transgenic flies and humanization of PDK-1 for information about the generation of new transgenic fly lines.

2.1.3 Lifespan analysis

After rearing as specified, 10 independent biological replicates per condition were created (i.e., n=200 flies per genotype and treatment). Flies were tipped to fresh vial every other day. While tipping deaths

in each vial are scored. Data is plotted as survival over time and analysis is performed using a long-rank test.

Table 4: ***Strains used in this thesis***

Strain name	source
W^{Dah}	Gronke et al. 2010
PDK-1^{GR}	Partridge lab
PDK-$1^{PH/PH}$	Partridge lab
PDK-$1^{PIF/PIF}$	Partridge lab
PDK-$1^{WT/null}$	Partridge lab
PDK-1^{hum}	This study
Injection line	Founder line as specificized in creation of mutants
Tm3b balancer	Partridge lab

2.1.4 Developmental assay

Five virgin females where continuously mated with 3 males. Every day flies are transferred to fresh SYA vials. Either eggs are frozen O.N. and then counted to determine fecundity of the females. Or eggs are counted on the same day and allowed to develop at 25°C. Development of pupea is scored twice a day during day 4/5/6/7 depending on the genotype. Hatching is scored twice times a day during day 8/9/10/11 depending on the genotype. Viable adult flies are used to calculate egg-to-adult viability. 6 till 8 replicates per condition are used. Fecundity and viability statistics are performed with a one-way ANOVA. Pupation and hatching are split up per day and compared with one-way ANOVA.

2.1.5 Stress assay

After normal rearing and mating, 6-8 vials with once-mated flies are aged on standard SYA food for 10 days. At day 10 flies are transferred to stress food (diets below). Depending on the motility window, flies are scored one or thrice times a day.

Starvation stress: 1% agar in ddH_2O
Oxidative stress: 20 mM Paraquat in standard SYA food
Xenobiotic stress: 0.03% w/v DDT in standard SYA food

2.1.6 Climbing assay

After normal rearing and mating, 6-8 vials with once-mated flies are aged on standard SYA food for 7, 15, 20, 25, 30 or 35 days. Before the assay flies are placed in plastic tubes used in the assay for acclimation. During the assay flies are placed into vial 1 out of a six-compartment climbing apparatus. After tapping all flies to the bottom, flies are allowed to climb for 30 seconds to reach the top of the second vial, directly placed above the first original vial. Upon 30 seconds the top vials are move across and flies are tapped down, meaning that flies who climbed till the second above the first vial, will be tapped down in vial two next to the original vial one. This procedure was repeated 5 times and the

resulting spread of flies in the 6 vials is used to calculate the Climbing Index (CI); lowest = 0 = all flies in vial 1, highest = 1 = all flies in vial 5). One-way ANOVA was used to calculate difference per day.

2.1.7 Feeding assay

After normal rearing and mating, 6-8 vials with once-mated flies are aged on standard SYA food for 10 days. 5 flies per vial were used for the feeding assay (=proboscis extension assay). 24 h before the assay, flies were acclimatized to the room and tipped to SYA and starvation food. Depending on the condition, flies where tipped again to SYA food or starvation food. Number of flies extending their proboscis to the food was scored as "feeding". Measurement was taken over a set time period depending on the assay.

2.1.8 Refeeding assay

After normal rearing and mating, vials with once-mated flies are aged on standard SYA food for 10 days. Depending on the conditions they were tipped to either SYA food or starvation food. After 24h starvation or normal feeding, flies were tipped once more to either SYA or starvation food and allowed to feed for a fixed amount of time points: 5 min, 10 min, 20 min, 30 min, 40 min, 1 hour, 1.5 hour, 2 hours, 4 hours, 8 hours, 12 hours and 24 hours. The three conditions are defined by:

FED:	10 days SYA food – SYA food 24h – SYA food for 30 min
Starvation:	10 days SYA food – starvation food 24h – starvation food for 30 min
Refed:	10 days SYA food – starvation food 24h – SYA food for defined amount of time.

Flies are either used for phosphoproteomics or western blots (see methods sections).

2.2 Molecular biology methods

2.2.1 Generation of transgenic fly lines

The PDK-1 mutants where created previously by Andrew Finlayson. He created the PDK-1 founder line and the vector expressing the rescue construct. Both genetic tools are used to generate the humanized PDK-1 mutants.

The pGE-attB-GMR vector expressing the rescue construct was transfected into the Chemically competent OneShot TOP10 Escherichia coli (LifeTechnologies) according to the manufacturer's instructions. An appropriate antibiotic was used for positive selection of the transformants, e.g. 100 µg/mL ampicillin in Luria-Bertani (LB) plates were used. LB medium contained 5 g yeast extract, 10 g tryptone, 10 g NaCl 10 g (Sigma), and 1 l deionized water. The solution was adjusted to pH 7.0 and autoclaved before usage. QIAprep Miniprep or Midiprep kits (Qiagen) were used to purify the plasmids from the bacteria.

Using alignment via DNA star alignment tool, overlapping kinase domain between *Drosophila* and human PDK-1 gene are identified. The region of interest in the *Drosphila PDK-1* gene (isoform D) was replaced by the corresponding cDNA of the human *PDPK-1* gene (hPDK1-001). The edge between fly

and human sequence was in a region of shared amino acid sequence. The amino acid sequence was reverse translated to cDNA and codon optimised (Kuzusa.org.jp). The resulting cDNA sequence was ordered from Sanger Sequencing at Eurofins Genomics (Germany). The pGE-attB-GMR vector containing the rescue construct was restricted at the at XhoI to AatII to create sticky ends, as was the ordered cDNA sequence. All restriction digestion reactions were performed with the enzymes provided by NEB according to their manufacturer's instructions. T4 DNA ligase (NEB) was used for the ligation reactions. The resulting vector was transfected into OneShot TOP10 Escherichia coli and 4 single colony were selected for sequencing after a MidiPrep. The success of the cloning was verified by Sanger sequencing at Eurofins Genomics (Germany) using the primers: AGCCCCAACCATATCTG; ACCGACGACTTATCGCC; AACTCTCCCTCTTTACTCT; AGTTGCCAAGAGCTGTTTCCG; CACGCTAATCGCCGACTTCG; CAGACGCTCCGTCAACAAACC; to target the whole rescue region.

Primers targeting just the kinase domain of interest: Primers for PIF pocket domain mutation:
F: AGTTGCCAAGAGCTGTTTCCG - R: TTCGTCGTCGCCGTCGTC. Primers for PH pocket domain mutation:
F: ACCTGAACGATGCCGAGAAG - R: CTACGCCATCGCTTCGTTTG.

All constructs were injected in house by Jacqueline. Embryos were injected with 400 ng/µL DNA. Constructs were inserted into the fly genome using the phiC31 and attP/attB site-directed integration system (Bischof et al. 2007). To create the PDK-1$^{\text{fully Hum}}$ and PDK-1$^{\text{PH Hum}}$ the created vector was injected into the founder line previously created by dr. Finlayson.

2.2.2 Fly genotyping

Single-fly gDNA preparation: a single fly was homongenized using a pipette containing 50 µl squishing buffer (Tris-HCl (pH 8.2) 20 mM, EDTA 1mM, NaCl 25mM) with freshly added proteinase K (final concentration 200µg/mL). The fly homogenate was incubated at 37°C for 30 minutes. Proteinase K was then inactivated at 95 °C for 3 min. The solution was centrifuged at 16,000 x g for 15 min. The supernatant containing gDNA was transferred to a new tube and stored at 4 °C.

For the PCRs to genotype, the HotStarTag Plus master mix of Qiagen was used following the manufactures instructions. PCR were run on a Ventri 96-well thermal cycler (AppliedBiosystems).

F: CACGGCAGCGGAAAAGAGAAAC
R: TTAAATTTCCCAAGCGATAGAGC
W^{Dah}: band of 337 bp
Mutants: band of 460 bp

To analyse the size of DNA fragments or plasmids, 1x TAE-buffered agarose gel electrophoresis was performed to separate the DNA (TAE 50x TrhisBase 242g, Acetic acid 57,1 ml, EDTA 0,5 M 100 ml, ddH_2O to a 1000 ml). DNA was stained with 10 µl GelRED DNA Gel Stain (Invitrogen) per 100 ml agarose gel. Electrophoresis was performed using Sub Cell GT horizontal electrophoresis cells (BioRad) for 40-60 min at 120-150 V. Hyperladder 50 bp or hyperladder 1 kb (Bioline) served as DNA fragment size markers. For cloning approaches, DNA fragments were eluted from the agarose gel using the QIAquick Gel Elution Kit.

2.2.3 *RNA extraction*

Total RNA was extracted with Trizol (Invitrogen) according to the manufacturer's instructions. To further minimise DNA contamination, the RNA pellets were treated with DNase I (ThermoFisher) according to the manufacturer's instructions. RNA concentration was measured using the Qubit BR RNA assay (ThermoFisher). cDNA was generated using the SuperScript III first-strand synthesis kit (Invitrogen) and random hexamers according to the manufacturer's instructions. 600 ng of total RNA was used to create cDNA.

2.2.4 *Quantitative Real-Time Polymerase chain reaction*

For q-RT-PCR of mRNA TaqMan Universal Real-Time PCR MasterMix (LifeTechnologies) was used according to the manufacturers manual, the Taqman probes used in this thesis are in Table 5. q-RT-PCR was performed with a 7900HT real-time PCR system (Applied Biosystems) or with a QuantStudio7 (ThermoFisher). Relative expression (fold induction) was calculated using the ΔΔCT method and Rpl32 (for mRNAs) as a normalization control.

Table 5: ***Taqman probes used in this thesis***

Target gene	Taqman Probe number
PDK-1	4351371
Bmm	4351372
FOXO	4331182
Lip4	4331182
Cyp6w1	4351372

2.3 Biochemistry

2.3.1 *Western blotting*

Proteins from mechanically separated heads (20x) or whole female flies (5x) were extracted with 100 – 300 µl RIPA buffer (10 ml RIPA Buffer (Thermo Fisher) 1 Complete mini protease inhibitor without EDTA and 1 PhosSTOP tablet (Roche)). Tissues are homongenized in RIPA buffer using a hand homogenizer. Proteins were quantified using the BCA Protein Assay Kit (ThermoFisher), and diluted accordingly. Laemmli Buffer (100 mM Tris pH 6.8, 20% glycerol, 4% SDS, (Carl Roth)) and 5% β-mercaptoethanol (Sigma) were added and then boiled at 95°C for 5 min. 20 -30ug protein was loaded into stained SDS-PAGE gels (Bio-Rad). The Gel was run at 80 volt for 10 minutes to stack the protein and then 120 Volt for 60-90 min depending on protein of interest. Proteins were transferred to 0.45-µm nitrocellulose membranes (GE Healthcare), with wet transfer for 60 min. Non-specific binding was blocked with 1 hour shaking in 5 % BSA in TBST. Primary antibodies were diluted mouse anti p-AKT^{Thr342} (1:1000); mouse anti p-AKT^{thr308} (1:1000), mouse anti S6K (1:1000), mouse anti actin (1:0000). Incubation was done overnight at 4 °C. Following three washes in TBST, membranes were probed with HRP-conjugated anti-mouse (1:10,000, ThermoFischer, AB_2536527) or anti-rabbit (1:10,000, ThermoFischer, AB_2536530) secondary antibodies for 1 h at RT. Signal development was performed

using ECL Western Blotting Detection reagents (GE Healthcare) and the ChemiDocImager (BioRad). Imager was subsequently used for band intensity quantifications.

2.3.2 Triglyceride assay

Five flies per sample were homogenised in 500 µl PBST (PBS + 0.05% Tween) using the fly gun. An additional 500 µl was added and the homogenisate was incubated at 70°C for 5 minutes and centrifuged at 14000 rpm for 5 minutes. The supernatant was diluted 1:10 and 50 µl was incubated with 200 µl of pre-warmed Thermo Infinity Triglyceride solution (ThermoScientific) for 5 minutes at 37°C. Using a plate reader Infinite 200 (Tecan) the absorbance at 550 nm was measured. Absolute values were calculated using a triglyceride standard ranging from 0 - 0.88 µg/µL triglycerides (Cayman Chemical). The triglyceride values were normalised to the protein concentration.

2.3.3 Immunostaining of fly tissues

Fly dissections are carried out in PBS and fixed for 30 min 4% PFA (Paraformaldehyde; Carl Roth) in PBS (Sigma) at room temperature while shaking. Tissues were washed 3 x 10 min in PBT with 0.5% Triton X-100 (PBST; Sigma) at room temperature. Then blocked in PBST with 5% bovine serum albumin and 0.01% sodium azide (Carl Roth) for 1 hour at R.T. The antibody anti-pH3 (Cell Signaling) was used in a 1:1000 dilution and incubated overnight at 4 °C. Following 3 washes of 10 min each in PBT, tissues were incubated with a Alexa568 goat anti mouse (ThermoFischer, catalog #A11031, 1:10000 diluted in blocking buffer) for 1 hour at room temperature. Tissues were mounted on a microscope slide in VectaShield Antifade Mounting Medium with DAPI (Vectorlabs). Imaging was done using a Leica SP5-X confocal microscope. Positive pH3+ cells were counted using the 10x magnification using a hand counter. One-way ANOVA was performed to test for significance.

2.3.4 Lysotracker staining

Fly dissections were performed in cold PBS (Sigma) and stained with 1 mM Lysotracker DND-99 (Invitrogen) 1:2000 and 1 mg/ml Hoechst 33342 1:1000 (Sigma) for 3 min. After rinsing for 3x 5min in PBS (Sigma), guts were mounted with VectaShield antifade mounting medium without DAPI (Vectorlabs) and immediately imaged on a Leica upright SP8-X confocal microscope. For each gut, three images were taken proximal to the proventriculus gut region. Analysis is performed by IMARIS. One-way ANOVA was performed to test for significance.

2.4 Cell culture methods

2.4.1 Culture and maintenance of $S2^+$ cells

$S2^+$ cells were cultured in fresh Schneiderís Drosophila Medium (ThermoFisher; 21720024) complemented with 10% heat-inactivated fetal bovine serum (FBS) (ThermoFisher; 26140079), 1% Pen-Strep and 1% L-Glutamate (ThermoFisher ; 10378016). Cells are grown in T75 flasks as a semi-adherent monolayer at and seeded at a density of of $2 \times 10^6 – 4 \times 10^6$ cells/mL with 28°C with ambient CO_2. Cells are split with 1:5 dilution into new culture vessels every 2/3 days. For all experiments, cells

were collected, counted with an automatic cell counter (Anvajo fluidlab R-300) and seeded in desired numbers.
For experimental procedures cells are plated at 1 x 10^6 cells/ml and left to grow overnight. Using a cell-scraper cells are disassociated from the plate, collected and centrifuged at 100 g for 3 minutes. The supernatant was removed and the cells are resuspended in medium or starvation medium (medium without FBS). Cells are either plated in 6 well-plate at a density of 4 × 10^6 cells/mL and then treated either with control of treatment. Cells are collected after the designated time frame by using the cellscraper and collecting the medium containing the cells in Eppendorf's. Cells are centrifuged at 1000 g for 3 minutes, and washed with PBS twice, before freezing in liquid nitrogen.

Cells collected with insulin stimulation are prepared slightly differently. Cells are collected, medium is removed and washed with PBS. Cells are distributed into Eppendorf's at a concentration of 1 x 10^7 in 30 µl. Depending on treatment group, either treated with 2 µl insulin (5ng/µl) and then snap lysed in 10 µl 5X RIPA buffer used for western blots of 5X Guadinium chloride buffer used for Phosphoproteomics. Lysis is allowed for 1 minutes with vertexing and then the samples are frozen in liquid nitrogen.

Freezing cells shortly after stimulation required some alterations to normal cell freezing protocols. While normally cells are washed after treatment, the 3-minute centrifugation step would annihilate the delicate time sequence. Thus, the cells are washed with PBS before treatment of insulin. Insulin treatment was performed in a small volume (30 µl). Samples where "snap" lysed with high concentration lysis buffer (10 µl) and then frozen in liquid nitrogen

2.4.2 XTT assay

The XTT cell viability assay (Cell signaling; 9095) asses cell proliferation and viability was used to investigate the potency of the PDK-1 inhibitors. Cells were plated at a confluence of 2 x 10^6 cells and 2 x 10^6 cells /96-well in quadruplicates. Drugs or control was added in appropriate concentration and cells were left for O.N. to settle. The next day XTT reagent was added according to the manufacturer's instructions and the the spectrophotometric absorbance was measured at 450 nm with a plate reader Infinite 200 (Tecan) after 1 hours, 2 hours and 5 hours. Cell viability was normalised to the respective untreated controls.

2.5 Phosphoproteomics

2.5.1 Phosphoproteomics Fly sample preparation

Samples were prepared according to the standard protocol of the Proteomics Facility of the Max Planck Institute for Biology of Ageing (version April 2022). Flies or cells are reared with standard conditions. 4 biological replicates per condition were used. 100 heads, 80 abdomen or 10 whole flies were collected and homogenised using the tissue lyser (Qiagen). 250 µl of lysis buffer (Guadinium chloride 6M, TCEP (Tris(2-carboxyethyl)phosphine) 2.5 mM, CAA (chloroacetamide) 10 mM, Tris-HCl 100 mM) was added to the tissue. Samples are vortexed and incubated at 95 °C for 10 minutes. Metal balls which are used in the tissue lyser are removed. Samples are then sonicated with the Bioruptor

(Diagenode) (30 seconds sonication, 30 seconds pause, 10 cycles, high power). Samples were then centrifuged at 20000 g at RT for 20 minutes. The supernatant was transferred to new tubes, and protein concentration was measured using the NanoDrop (ThermoFisher) . Of note, the NanoDrop is inaccurate when measuring fly heads with red eyes, when processing mutants, this step was skipped and uniform protein concentration was assumed. 1.5mg of protein lysate was diluted 10-fold with 20 mM Tris and 1 µL trypsin (Promega, mass spectrometry grade) was added. Trypsin digestion was performed overnight at 37 °C. Digestion was stopped by adding 50% formic acid (FA) to 1% final concentration.

Peptide clean-up was performed using the Sep-Pak C18 (Waters,3 cc Vac Cartridge, 200 mg Sorbent per Cartridge, 55 - 105 µm). Sep-Pak was wetted by adding 800 µL methanol, then 40% acetonitrile/0.1% FA and equilibrated with 0.1% FA. The protein digest was added and vacuum was used to get the sample into the Sep-Pak. The sample was washed 3 times with 0.1% FA and the final elution was done with 100µl of 40%ACN/0.1% FA using a plastic syringe to push the liquid through. Elution was performed twice. The peptides were air-dried using a SpeedVac and the pellet resuspended in 60 µl 0.1 % FA. After peptide quantification by NanoDrop, 30 µg of peptide was used for tandem mass labelling (TMT) and dried in a SpeedVac.

2.5.2 TMTPro Labeling

80 µg of dried clean peptides were reconstituted in 20 µL of 0.1M TEAB. Tandem mass tag (TMTpro, Thermo Fisher Scientific cat. No A44522) labeling was carried out according to manufacturer's instruction with the following changes: 0.5 mg of TMTPro reagent was re-suspended with 30 µL of anhydrous acetonitrile. 10 µl of TMTPro reagent in acetonitrile was added to 20 µL of clean peptides in 0.1M TEAB. The final ACN concentration was 33.3% and the ratio of peptides to TMTPro reagent was about 1:2. After 60 min of incubation on a shaker at 500 rpm the reaction was quenched with 5 µL of 5% hydroxylamine. Labelled peptides were pooled and dried in Speed-Vac.

Dried pooled TMTpro-labeled peptides were resuspended in 1% TFA, 5% ACN and desalted using Sep-Pak C18 3 cc Vac Cartridge, 200 mg Sorbent (Waters™ WAT054945) manually according to manufacturer's instruction.

2.5.3 TMT-labeled Phosphopeptides Enrichment

The cleaned TMT-labeled peptides were subjected to the Thermo Scientific™ Pierce™ HiSelect TiO2 phosphopeptide enrichment kit (PN#A32993). The TiO2 eluent was kept for Mass Spec analysis.
The TiO2 flow-through (FT) and wash fractions were pooled and subjected to the Thermo Scientific™ Pierce™ HiSelect Fe-NTA phosphopeptide enrichment kit (PN#32992) to enrich the phosphopeptides further. The Fe-NTA eluent was dried with Speed-Vac, resuspended with 0.1% FA and desalted with home-made StageTips.

2.5.4 *LC-MS/MS analysis*

Peptides enriched with HiSelect TiO2 and HiSelect Fe-NTA were separated on a 40 cm, 75 µm internal diameter packed emitter column (Coann emitter from MS Wil, Poroshell EC C18 2.7micron medium from Agilent) using an EASY-nLC 1200 (Thermo Fisher Scientific). The column was maintained at 50°C. Buffer A and B were 0.1% formic acid in water and 0.1% formic acid in 80% acetonitrile, respectively. Peptides were separated on a segmented gradient from 6% to 31% buffer B for 90 min at 300 nl / min, followed by a higher organic wash. Eluting peptides were analyzed on a Orbitrap Fusion LUMOS Tribrid mass spectrometer equipped with a FAIMS Pro interface (Thermo Fisher Scientific). The FAIMS device was operated in two compensation voltages, -50 V and -70 V. Synchronous precursor selection based MS3 was used for the acquisition of the TMTPro reporter ion signals. Raw files were split based on the FAIMS compensation voltage using FreeStyle (Thermo Fisher Scientific).

2.5.5 *Protein identification and quantification*

Proteomics data was analyzed using MaxQuant (Cox and Mann, 2008), version 1.6.17.0. Peptide fragmentation spectra were searched against the canonical sequences of the fly reference proteome (proteome ID UP000000803, downloaded September 2018 from UniProt). Methionine oxidation, protein N-terminal acetylation, and phosphorylation of Serine, Threonine, and Tyrosine, were set as variable modifications; cysteine carbamidomethylation was set as fixed modification. The digestion parameters were set to “specific” and “Trypsin/P”. Quantification was set to “Reporter ion MS3”. The isotope purity correction factors, provided by the manufacturer, were imported and included in the analysis. The minimum number of peptides and razor peptides for protein identification was 1; the minimum number of unique peptides was 0. Protein identification was performed at a peptide spectrum matches and protein false discovery rate of 0.01. The “second peptide” option was on. Differential expression analysis was performed using limma (Ritchie *et al.*, 2015) version 3.34.9 in R version 3.4.3 (R Core Team, 2017). TMTPro reporter intensity values for singly and multiply modified peptides were added together to derive the reporter intensity values per phosphor site. Only phosphor sites with localization probability equal to or higher than 0.5 were included in the analysis.

2.6 Statistical analysis

Statistical analysis was performed using GraphPad Prism. Individual statistical tests are indicated in the figure legends. For multiple comparison testing, One-way and Two-way ANOVA were used. When more than two pairs of groups were compared, the Tukey test was used. When only two groups where compared the student t-test was used. Lifespan and stress assays are analysed using a log-rank test. P values < 0.05 were considered significant: *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001.

3 Results

3.1 Generation of PH- and PIF-specific PDK1 mutants

Homozygous loss of function mutants of PDK-1 are lethal in *Drosophila* (Lawlor *et al.*, 2002), and are therefore not suited to study the role of PDK-1 in longevity. Therefore, we generated hypomorphic PDK-1 alleles by separately mutating the PH and PIF domains, which should affect different downstream signalling pathways. The PH domain interacts with PIP_3 to phosphorylate and activate AKT signalling. Additionally, there seems to be a role for the PH domain in *trans*-autophosphorylation. The hydrophobic PIF pocket binds and interacts with downstream targets in the AGC branch of PDK-1 signalling.

In order to identify amino acid residues crucial for PH domain and PIF pocket function of the *Drosophila* PDK-1 protein, we aligned the *Drosophila* and human PDK-1 sequence. Not only does *Drosophila* PDK-1 function overlap with mice PDK-1 functionality, but their protein structure is highly similar (58.1% identity) (Alessi *et al.*, 1997; Rintelen *et al.*, 2001). Previous research in mice showed that Lysine 465 is crucial for PH-domain-dependent interaction with PIP_3 (Bayascas, 2008). Lysine 465 is part of the positively charged bowl like structure of the PH domain, and mutation results in a slight drop in positive charge but does not affect the stability of the PH domain. PDK-1^{K465E} is evolutionarily conserved and corresponds to Lysine 601 (K601) in the *Drosophila* PDK-1 protein (Figure 6A_.

While the key residues of the PIF pocket have been identified, so far no viable mutant has been generated. The placement of the interacting residues in the PIF pocket, Lys115, Ile119, Gln150, **Leu155** and Lys198, are regulated via conformational changes of various helixes and strands. These residues interact with two conserved phenylalanines in the hydrophobic motif of PDK-1 substrates (Biondi *et al.*, 2000, 2001). Lethality in mice was observed by mutating residues that abolish the interaction with the phenylalanines and disrupt the pocket structure. Therefore we chose to mutate Lys115, a residue which when mutated diminishes activation of AGC substrates in cells but does not disrupt the pocket (Biondi *et al.*, 2000, 2001). PDK-1^{K115} is evolutionarily conserved from flies to mammals, and corresponds to lysine 198 (K198) in the *Drosophila* PDK-1 protein (Figure 6B).

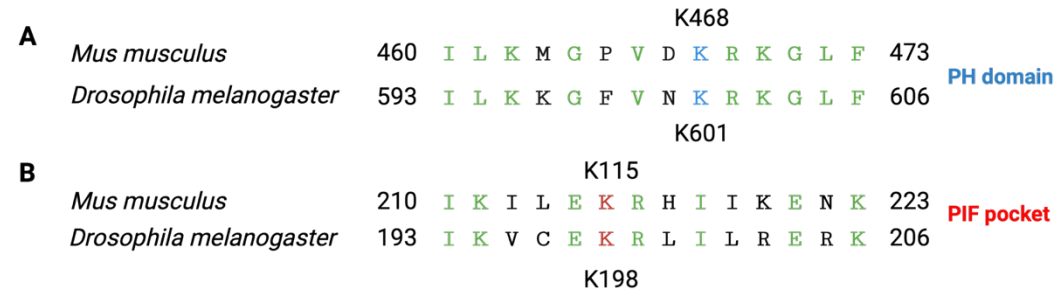

Figure 6: Sequence homology of the PDK-1 protein between Mus musculus and Drosophila melanogaster in the PIF pocket and the PH domain. Amino acid residues identical between flies and mice are indicated in green. Mutated amino acids in the PIF and PH domain mutants are indicated in red and blue, respectively.

Mutant fly lines were generated by Dr Andrew Finlayson using genomic engineering (Huang *et al.*, 2009). Therefore, in the first step, ends out homologous recombination was used to replace the genomic sequence of the endogenous PDK1 gene corresponding to exons 9 to 18 by an attP site and a *white*$^+$ marker gene flanked by two loxP sites (Figure 7A.1) (Kiehart, Crawford and Montague, 2000). In the next step, the two loxP sites were used to excise the *white*$^+$ marker gene by introducing a Cre

recombinase. The resulting PDK-1 founder fly line is a PDK1 null mutant and will be referred to as PDK-1^{null} (Figure 7A.2-3). PDK-1^{null} flies were homozygous lethal, consistent with the phenotype of PDK-$1^{-/-}$ mutants in other organisms (Cho *et al.*, 2001; Lawlor *et al.*, 2002). To generate a wild type control, a PDK-1 rescue construct containing the deleted region was introduced via ϕ C31 mediated recombination using the introduced attP site (Figure 7A.4). PH and PIF mutants were generated in the same way by mutating K601 and K198 in the rescue construct to glutamic acid and alanine, respectively. To facility easy tracking of the mutants, the mini *white^{+} marker* gene was left in. The PDK-1^{K601E} allele will be referred to as PDK-1^{PH} and the PDK-1^{K198A} allele will be referred to as PDK-1^{PIF}. Both mutant fly lines were homozygous viable and were back crossed into the outbred, wild type, white Dahomey fly strain.

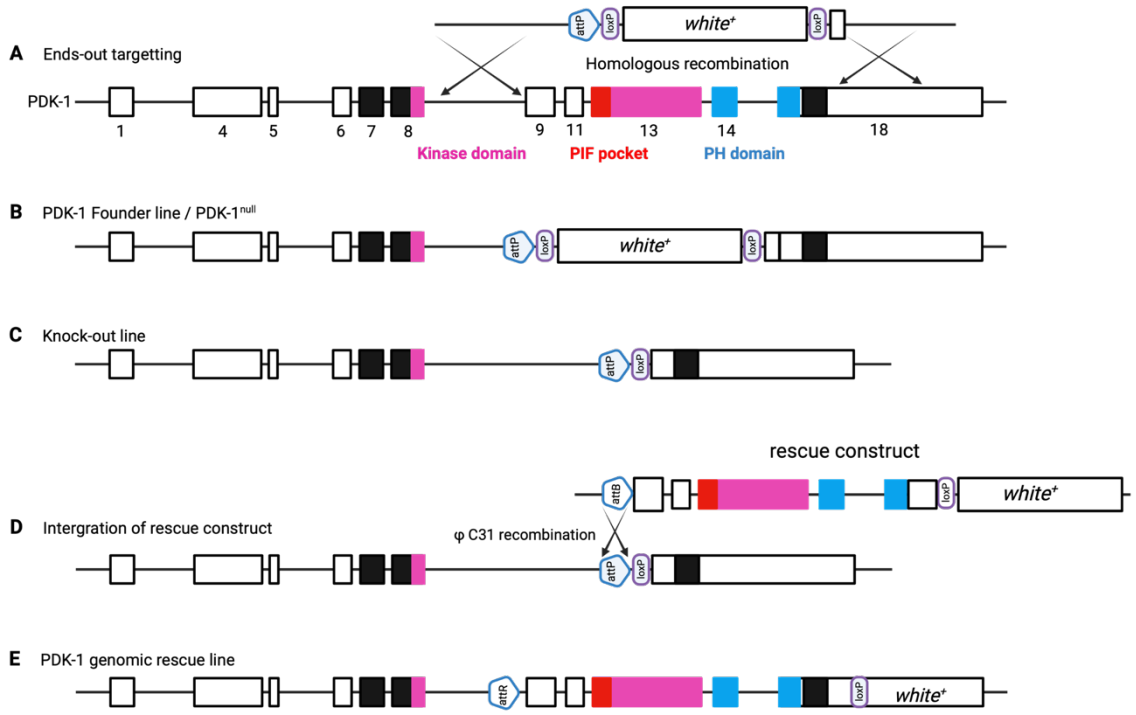

Figure 7 : Genomic engineering of the PDK-1 gene locus to generate PH- and PIF-specific PDK1 mutants*. The PIF pocket (Red) in exon 13 and PH domain (Blue) in exon 18 are indicated.*
A+B: Homologous recombination was used to replace the sequence in the PDK-1 gene corresponding to exons 8-18 with an attP, LoxP and white gene.
C: The PDK-1 founder line was generated by excision of the white+ marker gene using Cre recombinase. D+E: Genomic rescue (GR) control and PH and PIF mutant lines were generated by inserting the corresponding constructs into the attP site using φ C31-mediated recombination.

By mutating both the PH domain and the PIF pocket we reduced the functionality of PDK-1 in a kinase-domain-specific manner. To investigate the role of both mechanisms of activation further, we combined the homozygous PDK-1 mutants with the PDK-$1^{null/Tm3b}$ founder line, creating PDK-$1^{PH/null}$ and PDK-$1^{PIF/null}$. These transheterozygous PDK-1 mutants have not only reduced functionality but should also have reduced expression due to the absence of one allele, thereby making them stronger hypomorphs. When we compared PDK-1 expression via qPCR we indeed observed a reduction in expression in the PDK-$1^{PH/null}$ and the PDK-$1^{PIF/null}$ hypermorphs compared to their homozygous counterparts (data from Gödderz (MPI AGE)). Genomic engineering altered the expression of PDK-1

in *Drosophila* slightly compared to w^{Dah}. There was a mild upregulation in PDK-1 expression in the homozygous mutants similarly to PDK-1^{GR}. Taken together, we know that expression levels are heightened in the homozygous mutants and reduced in their stronger hypomorphs. Using the PH domain and the PIF pocket mutant the downstream mechanism of PDK-1 signalling, and their effects in longevity and health can be dissected.

3.2 Phenotypic characterization of PDK-1PH and PDK-1PIF mutations

3.2.1 PDK-1 mutants have slowed development

PDK-1 occupies a key position in the signalling network, controlling numerous downstream cellular processes, including but not limited to proliferation, differentiation and metabolism. IIS mutants affecting upstream pathway components, including *dilp2-3,5* and *chico* mutants, show reduced size and delayed development (Böhni *et al.*, 1999; Clancy *et al.*, 2001; Grönke *et al.*, 2010). Downregulation of IIS affects developmental timing, and increased IIS signalling advances metamorphosis, emphasizing the important function of IIS during development (Walkiewicz and Stern, 2009). Both the AKT and the AGC branch have been implicated in the regulation of growth. The AKT branch regulates growth primarily via regulation of cell cycle progression, glycogen syntheses and apoptosis. In contrast, the AGC branch regulates growth via initiation of translation and control over transcription.

To address how mutations of the PH and PIF domains of PDK1 affected development, I first measured egg-to-adult viability. There was no significant difference in egg-to-adult viability between PDK-1^{GR} control and PDK-$1^{PH/PH}$ and PDK-$1^{PIF/PIF}$ flies. However, egg-to-adult viability of PDK-$1^{PH/null}$ and PDK-$1^{PIF/null}$ flies was significantly reduced by about 50% (Figure 8A), suggesting that both branches need to be strongly inhibited during development to affect viability in flies. Next, body weight of freshly emerged adult flies was measured, as a proxy for growth. While female PDK-$1^{PH/PH}$ mutants showed no reduction in body weight, PDK-$1^{PH/null}$ flies weighted significantly less than PDK-1^{GR} control flies, suggesting that while the AKT branch regulates growth in females, a severe inhibition of the pathway is required to do so (Figure 8B). In male flies both mild inhibition of the AKT branch in PDK-$1^{PH/PH}$ and the stronger inhibition in the PDK-$1^{PH/null}$ was sufficient to affect growth. The involvement of the AKT branch in growth is consistent with findings in heterozygous PDK-1^{K465E} mice (Bayascas, 2008). In contrast to female PDK-$1^{PH/PH}$ mutants, PDK-$1^{PIF/PIF}$ mutants had a significantly reduced body weight, which was further reduced in PDK-$1^{PIF/null}$ flies (Figure 8B). A similar effect was observed in the male PIF mutants. Thus, even a mild downregulation of the AGC branch resulted in reduced body weight in both sexes.

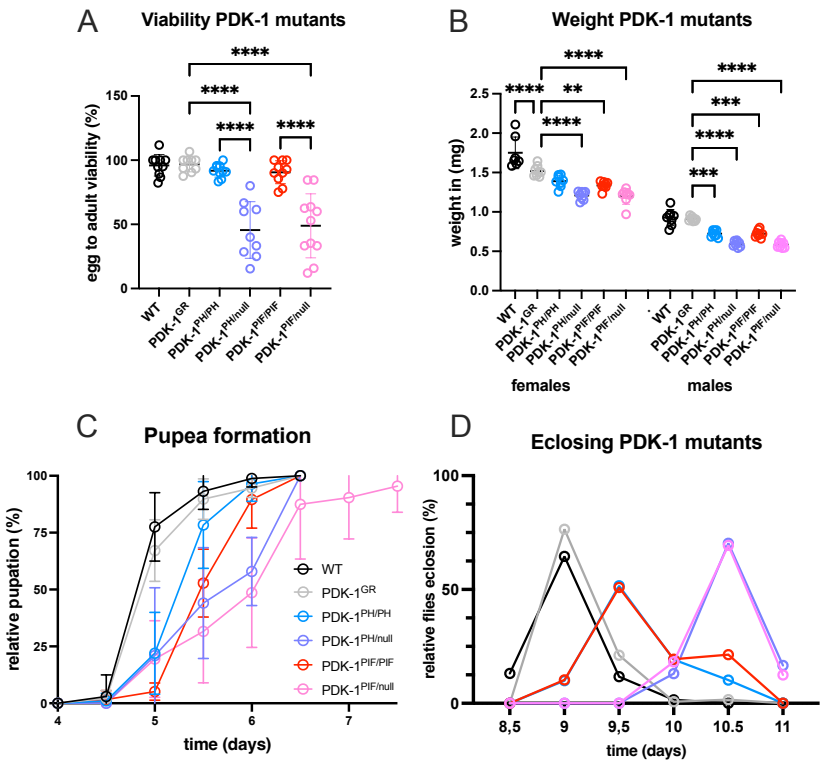

Figure 8: Reproduction and development in PDK-1^{PH} and PDK-1^{PIF}

A: Viability was assessed by counting the number of enclosed flies in comparison to eggs (N = 11 vials p.g.). w^{Dah} vs PDK-1^{GR} p= n.s.; PDK-1^{GR} vs PDK-1$^{PH/PH}$ p= n.s.; PDK-1^{GR} vs PDK-1$^{PH/null}$ p<0.0001; PDK-1^{GR} vs PDK-1^{PIFPIF} p= n.s.; PDK-1^{GR} vs PDK-1$^{PIF/null}$ p<0.0001. one-way ANOVA.

B: Weight at day 1 (N=16 flies p.g) Females: wDah vs PDK-1^{GR} p<0.01; PDK-1^{GR} vs PDK-1$^{PH/PH}$ = ns; PDK-1^{GR} vs PDK-1$^{PH/null}$ p<0.0001; PDK-1^{GR} vs PDK-1$^{PIF/PIF}$ p<0.05; PDK-1^{GR} vs PDK-1$^{PIF/null}$ p<0.0001. Males: wDah vs PDK-1^{GR} p=ns; PDK-1^{GR} vs PDK-1$^{PH/PH}$ p=0.001; PDK-1^{GR} vs PDK-1$^{PH/null}$ p<0.0001; PDK-1^{GR} vs PDK-1$^{PIF/PIF}$ p<0.001; PDK-1^{GR} vs PDK-1$^{PIF/null}$ p<0.0001. one-way ANOVA.

C: Pupation was counted twice a day till all larvae pupated (N=11 vials p.g.).

D: Fly eclosion was measured over a time span of 8.5 till 11 days after egg laying (N=10 vials p.g.). All time points w^{Dah} vs PDK-1^{GR} p= n.s.; d9 PDK-1^{GR} vs PDK-1$^{PH/PH}$ p<0.0001; PDK-1^{GR} vs PDK-1$^{PIF/PIF}$ p<0.0001; d9,5 PDK-1^{GR} vs PDK-1$^{PH/PH}$ p<0.0001; PDK-1^{GR} vs PDK-1$^{PIF/PIF}$ p<0.0001; d10,5 PDK-1^{GR} vs PDK-1$^{PIF/null}$ p<0.0001. one-way ANOVA.

** = p<0.05, ** = p<0.01, *** = p<0.001, **** = p<0.0001*

Development is often strongly prolonged in IIS mutants (Böhni *et al.*, 1999; Clancy *et al.*, 2001; Grönke *et al.*, 2010). Consistently, there was a clear delay (±1day) in pupae formation in PDK-1$^{PH/PH}$ and PDK-1$^{PIF/PIF}$ mutants compared to PDK-1^{GR} controls (Figure 8C). The delay observed in PDK-1$^{PH/null}$ and PDK-1$^{PIF/null}$ was more pronounced, roughly 1.5 days. As pupae formation and eclosion of adult flies are linked, it is not surprising to see a similar delay in the eclosion pattern. Both the PDK-1$^{PH/PH}$ and PDK-1$^{PIF/PIF}$ showed a peak in eclosion at day 9.5 rather than day 9 when control flies eclosed (Figure 8D). Eclosion of the stronger hypermorphs PDK-1$^{PH/null}$ and PDK-1$^{PIF/null}$ was more severely delayed, and occurred mainly at day 10.5. In conclusion, both the AKT and the AGC branch affect developmental timing. While no big differences were observed between the two branches, the strength of reduction in signalling did affect developmental timing.

Since there was a tendency for PDK-1 expression to be upregulated in PDK-1^{GR} control flies compared to wDah flies used for backcrossing, we also compared development between the two control strains. While there was no change in egg-to-adult viability or developmental timing, female PDK-1^{GR} flies

showed a slightly reduced body weight compared to wDah flies. Thus, slight overexpression of PDK-1 during development might reduce body weight. Notably, this was only observed in females. However, given that PDK-1^{GR} and PDK-1$^{PH/PH}$ and PDK-1$^{PIF/PIF}$ in contrast to wDah flies all carry a mini white gene, which by itself can affect physiology (Sasaki *et al.*, 2021), we consider the PDK-1^{GR} strain as the more appropriate control.

3.2.2 Both the AKT and the AGC branch regulate lifespan downstream of PDK1 in Drosophila

Downregulation of IIS or TOR network activity upstream in the signalling cascade can increase lifespan (Clancy *et al.*, 2001; Bjedov *et al.*, 2010; Grönke *et al.*, 2010). Thus, we next measured survival of PDK1^{PH} and PDK1^{PIF} mutant flies on standard SYA food. Flies homozygous for the PDK1^{PH} mutation were significantly longer lived than PDK-1^{GR} control flies, with a median lifespan extension of 19% and 15% and a maximum lifespan extension of 14% and 13% in females and males, respectively (Figure 9A, B, E, F). Thus, down regulation of AKT signalling downstream of PDK1 was sufficient to extend lifespan in *Drosophila*. Females that carried the PDK1^{PH} mutation in trans to the PDK1^{null} allele (PDK-1$^{PH/null}$) were even longer lived than homozygous PDK1$^{PH/PH}$ mutant females, with an extension in median and maximum lifespan extension of 35% and 22%, respectively (Figure 9A, E,F). In contrast, transheterozygous PDK-1$^{PH/null}$ males were no longer lived than homozygous PDK-1^{PH} mutant males and only showed an extension of 10% and 5% in median and maximum lifespan, respectively, compared to PDK-1^{GR} control flies. Thus, reduced PDK-1 expression in combination with loss-of PH function caused additional lifespan extension in females but not in males, indicating a sex-specific effect of reduced AKT signalling downstream of PDK1 on lifespan.

Flies homozygous for the PDK1^{PIF} mutation were significantly longer-lived than PDK-1^{GR} control flies, with a median lifespan extension of 24% and 25% and a maximum lifespan extension of 14% and 17% in females and males, respectively (Figure 9C, D, E, F). Thus, compared to PDK-1^{PH} flies, mutation of the PIF pocked caused a slightly bigger lifespan extension, which was especially obvious in male flies. In contrast to the PDK-1^{PH} mutation, only a small additional lifespan extension of 2% and no extension in maximum lifespan was observed when both expression level and functionally of the PIF pocket were reduced in PDK-1$^{PIF/null}$ mutant female flies. PDK-1$^{PIF/null}$ males had a slightly shorter median lifespan, 20%, while the maximum lifespan was not changed compared to homozygous PDK1^{PIF} males. Taken together, suppression of both the AKT and the AGC branches increases longevity, showing a sex specific pattern in the suppression of AKT and ACG signalling that maximised the increase.

Noteworthy, male PDK-1^{GR} control flies were slightly shorter lived than wDah control flies (-10% males), suggesting that mild overexpression of PDK-1 negatively affects male survival (Figure 9A-F). While a mild reduction was also observed in female median lifespan, this was not significant. Interestingly, female PDK-1^{GR} had a slightly reduced maximum lifespan, -6%, while males had a slightly increased maximum lifespan, 4%. In summary, mutation of both the PH and the PIF domain of PDK-1 extended lifespan in *Drosophila,* with the strongest lifespan extension in PDK-1$^{PH/null}$ females. Thus, in females a strong down regulation of AKT signalling extended lifespan more than either mild intervention or reduction in AGC signalling. As previously suggested in the introduction,

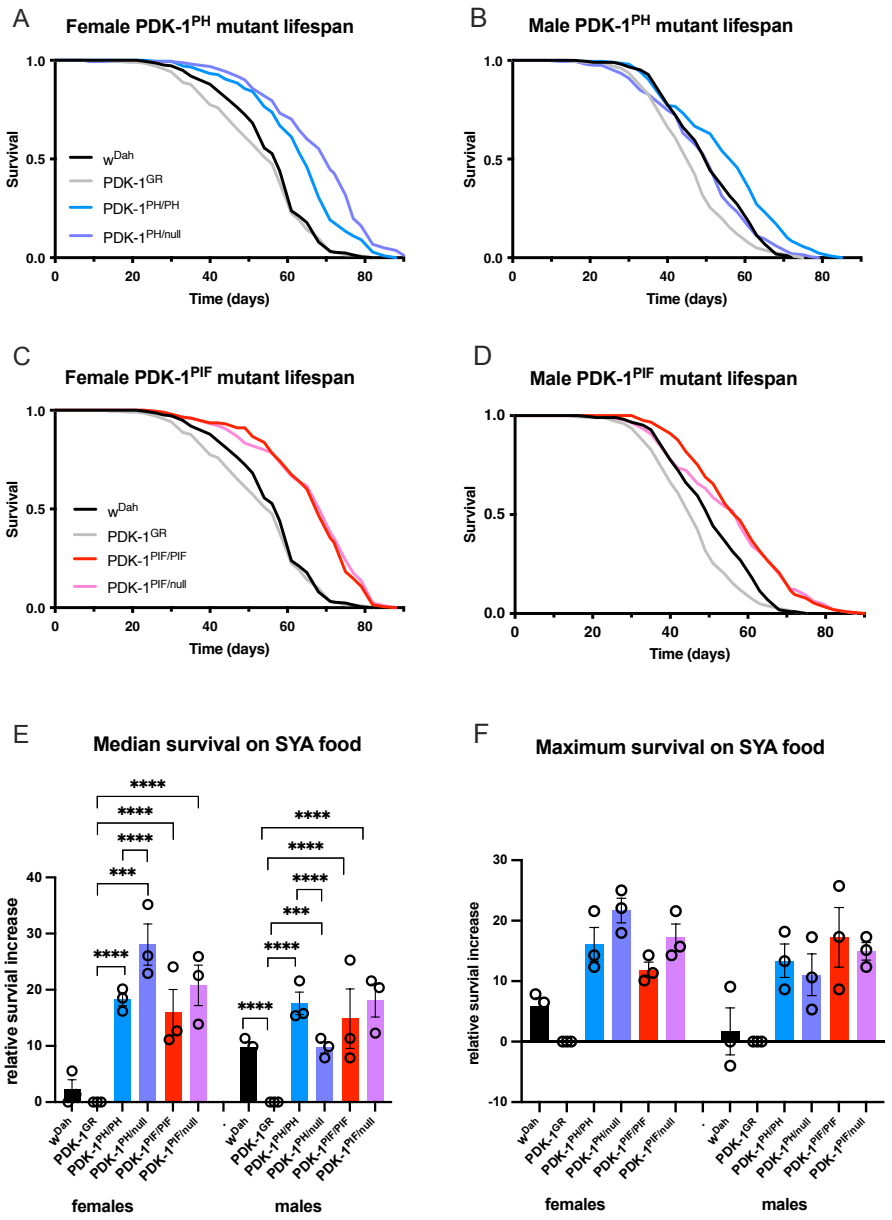

Figure 9: Lifespan of PDK-1^{PH} and PDK-1^{PIF} mutants. *Survival curves of PDK-1^{PH} female (A) and male (B) mutants. Survival curves of PDK1^{PIF}female (C) and male (D) mutants. For better comparison the same wDah and PDK^{GR} control survival curves are shown in A+C and B+D (N=200 p.g.).*

A: Females PDK-1^{PH}: w^{Dah} vs PDK-1^{GR} median 3.8% p= n.s.; PDK-1^{GR} vs PDK-1$^{PH/PH}$ median +17.3% p<0.0001; PDK-1^{GR} vs PDK-1$^{PH/null}$ median +33.6% p<0.0001; PDK-1$^{PH/PH}$ vs PDK-1$^{PH/null}$ median +16.3% p<0.0001. Log rank test.

B: Males PDK-1^{PH}: w^{Dah} vs PDK-1^{GR} median 9.8% p<0.0001.; PDK-1^{GR} vs PDK-1$^{PH/PH}$ median +15.4% p<0.0001; PDK-1^{GR} vs PDK-1$^{PH/null}$ median +9.8% p<0.001; PDK-1$^{PH/PH}$ vs PDK-1$^{PH/null}$ median 5.6% p<0.0001. Log rank test.

C: Females PDK-1^{PIF}: w^{Dah} vs PDK-1^{GR} median -3.8% p = n.s.; PDK-1^{GR} vs PDK-1$^{PIF/PIF}$ median +24% p<0.0001; PDK-1^{GR} vs PDK-1$^{PIF/PIF}$ +25.9% p<0.0001; PDK-1$^{PIF/PIF}$ vs PDK-1$^{PIF/null}$ median +1.9% p=n.s. log rank test.

D: Males PDK-1^{PIF}: w^{Dah} vs PDK-1^{GR} median 9.8% p<0.0001; PDK-1^{GR} vs PDK-1$^{PIF/PIF}$ median +25.7% p<0.0001; PDK-1^{GR} vs PDK-1$^{PIF/PIF}$ +20.3% p<0.0001; PDK-1$^{PIF/PIF}$ vs PDK-1$^{PIF/null}$ median +5.4% p=n.s. log rank test.

E: Relative median survival compared to PDK-1^{GR} on SYA food in 3 independent trails. Performed by van Schelt (MPI AGE), Ureña (UCL) and Gödderz (MPI AGE). Stars indicate p values from the trails depicted in A-D.

E: Relative maximum lifespan compared to PDK-1^{GR} on SYA food in 3 independent trails. Performed by van Schelt (MPI AGE), Ureña (UCL) and Gödderz (MPI AGE).

** = p<0.05, ** = p<0.01, *** = p<0.001, **** = p<0.0001*

strong inhibition of the AKT branch can result in indirect downregulation of the TOR pathway, a main part of the AGC branch. The longevity of the PDK-1$^{PH/null}$ flies might therefore not be purely dependent on the AKT branch signalling, but may also be caused by reduced activity of the AGC branch.

Increased longevity is often associated with reduced female fecundity, and this is also the case upon reduced IIS signalling. Furthermore, results from other model organisms directly implicate PDK1 function in the control of fertility and reproduction. *C. elegans* worms carrying the hypomorphic PDK-1^{sa680} allele showed reduced fertility, and this was also observed in PDK-1$^{fl/fl}$ and PDK-1^{K465E} mutant mice. Furthermore, complete loss-of function of PDK1 specifically in the ovary caused sterility in mice (Paradis *et al.*, 1999; Lawlor *et al.*, 2002; Bayascas, 2008; Reddy *et al.*, 2009). Thus, I measured egg laying of PDK1 PH and PIF mutant flies. Interestingly, there was no significant difference in egg laying between PDK-1$^{PH/PH}$ mutants and PDK-1^{GR} control flies, indicating that the lifespan extension of PDK-1$^{PH/PH}$ females is not caused by reduced fecundity and that mild reduction of AKT signalling has no effect on fecundity (Figure 10). In contrast, PDK-1$^{PH/null}$ mutant females laid significantly fewer eggs than PDK-1^{GR} control flies, a reduction of about 50%, which might suggest that stronger reduction of the AKT branch also affects fecundity, or alternatively, as discussed above, that additional downregulation of the AGC branch in these flies might underlie the reduction in egg laying. Consistent with this hypothesis, PDK-1$^{PIF/PIF}$ mutants had a 50% reduction in fecundity, and there was only a small additional reduction of fecundity in PDK-1$^{PIF/null}$ females. Thus, these results suggest that in *Drosophila* reduced fecundity upon reduced IIS signalling is manly driven by inhibition of the AGC branch but not the AKT branch of the network. Consistent with this assumption, ovarian failure upon loss of PDK-1 activity has been attributed to insufficient activation of S6K and S6 in mice (Reddy *et al.*, 2009), and rapamycin treatment strongly abolished egg laying in flies (Bjedov *et al.*, 2010).

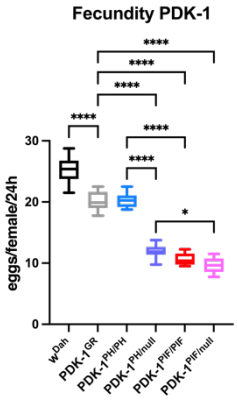

Figure 10: Fecundity in PDK-1 mutants at day 5. *Fecundity was measured at day 5 by allowing 4 flies to lay eggs for 24h on SYA food. (N= 40 flies p.g.) wDah vs PDK-1^{GR} p<0.001; PDK-1^{GR} vs PDK-1$^{PH/PH}$ <0.0001; PDK-1^{GR} vs PDK-1$^{PH/null}$ p<0.0001; PDK-1^{GR} vs PDK-1$^{PIF/PIF}$ p<0.0001; PDK-1^{GR} vs PDK-1$^{PIF/null}$ p<0.0001; PDk-1$^{PH/PH}$ vs PDk-1$^{PH/null}$ p<0.0001, PDK-1$^{PH/PH}$ vs PDK-1$^{PIF/PIF}$ p<0.0001, PDK-1$^{PH/nu;;}$ vs PDK-1$^{PIF/null}$ p<0.05. one-way ANOVA. * = p<0.05, **** = p<0.0001*

In summary, Increased longevity due to reduction in either the PH kinase domain or the PIF pocket kinase domain indicates a pivotal role for PDK-1 in ageing. To better understand which branch-specific pro-longevity mechanisms downstream of PDK1 might be involved in this regulation, I performed a more in-depth phenotyping of these flies addressing energy homeostasis and stress response.

3.2.3 PDK1 regulates starvation resistance both via the AKT and the AGC branch

The IIS/ TOR network is a key regulator of metabolism and energy storage. For example, dilp2 mutant and mNSC-ablated flies have increased starvation resistance (Broughton *et al.*, 2005b, 2008). Furthermore, PDK-1 has also been directly linked to metabolism of energy reserves in yeast (Pastor-Flores *et al.*, 2011). In order to test whether PDK-1 affects energy homeostasis in *Drosophila*, I measured starvation resistance of these flies. PDK-$1^{PH/PH}$ mutant flies were significantly longer-lived than PDK-1^{GR} control flies under starvation conditions and showed a 36% and 19% increased median survival for females and males, respectively (Figure 11A, D). There was only a mild additive effect on starvation resistance in female PDK-$1^{PH/null}$ flies, and no effect in males. Thus, reduced activity of the AKT branch downstream of PDK-1 increased starvation resistance. PDK-$1^{PIF/PIF}$ mutant flies also showed increased starvation resistance with an increase in median survival of 43% and 22% in females and males, respectively. Females of the stronger hypermorph PDK-$1^{PIF/null}$ were also starvation-resistant, but showed a decrease of 9% in survival compared to homozygous PDK-$1^{PIF/PIF}$ females (Figure 11B). PDK-$1^{PIF/null}$ mutant males were also starvation resistant and there was no difference compared to PDK-$1^{PIF/PIF}$ males. In summary, these results indicate that both branches were involved in starvation resistance in males and females, but that the genotype that the most resistant genotypes were sex specific.

Survival under starvation is mainly dependent on the ability of the organism to store and mobilize fat stores and increased lipid storage can be linked to starvation resistance in a dose-dependent manner in *Drosophila* (Teixeira *et al.*, 2003; Grönke *et al.*, 2005). Therefore, to address whether the difference in survival of the PDK1 mutant flies under starvation might be caused by differences in lipid storage, I measured their triacylglycerol (TAG) levels prior to starvation. TAG storage of PDK-$1^{PH/PH}$, PDK-$1^{PH/null}$, PDK-$1^{PIF/PIF}$ and PDK-$1^{PIF/null}$ mutant flies was significantly increased compared to PDK-1^{GR} control in females (Figure 11C). There, was no further increase in TAG content in PDK-$1^{PH/null}$ or decrease in the PDK-$1^{PIF/null}$ flies, to explain the slightly different starvation resistance. In males we observed an increase in TAG levels in all genotypes, including PDK-$1^{PH/null}$ and PDK-$1^{PIF/null}$ which did not show an increase in survival compared to PDK-1^{GR}. Thus, despite higher TAG levels these flies were not longer lived, suggesting that they might have problems in mobilizing their lipid stores during starvation.

Increased lipid storage might be caused by increased lipid synthesis or decreased lipid mobilization. The evolutionarily conserved TAG lipase Brummer (BMM) is the key rate-limiting enzyme for lipid mobilization in *Drosophila* and combined lack of BMM and AKH signalling prevents lipid mobilization in flies (Grönke et al., 2005, 2007). *Bmm* expression is regulated both by the AKT branch of the IIS/TOR network via FOXO and AGC via the TOR pathway (Luong *et al.*, 2006; Wang *et al.*, 2011). Thus, I measured the expression level of *bmm* via Q-RT-PCR. However, *bmm* expression was not significantly changed in PDK-$1^{PH/PH}$ and PDK-$1^{PIF/PIF}$ mutant female flies (Figure 11D). LIP4 is another lipase involved in intracellular hydrolysis of TAG, that is regulated in a FOXO dependent manner (Vihervaara and Puig, 2008). Q-RT-PCR analysis showed that *Lip4* expression was unaffected in PDK-$1^{PH/PH}$, PDK-$1^{PIF/PIF}$, PDK-$1^{PH/null}$ and PDK-$1^{PIF/null}$ mutant flies. In summary, PDK-1 regulates lipid storage and starvation resistance both via the Akt and AGC branch of IIS/TOR signalling, but not via transcriptional regulation of the key lipases *bmm* and *lip4.*

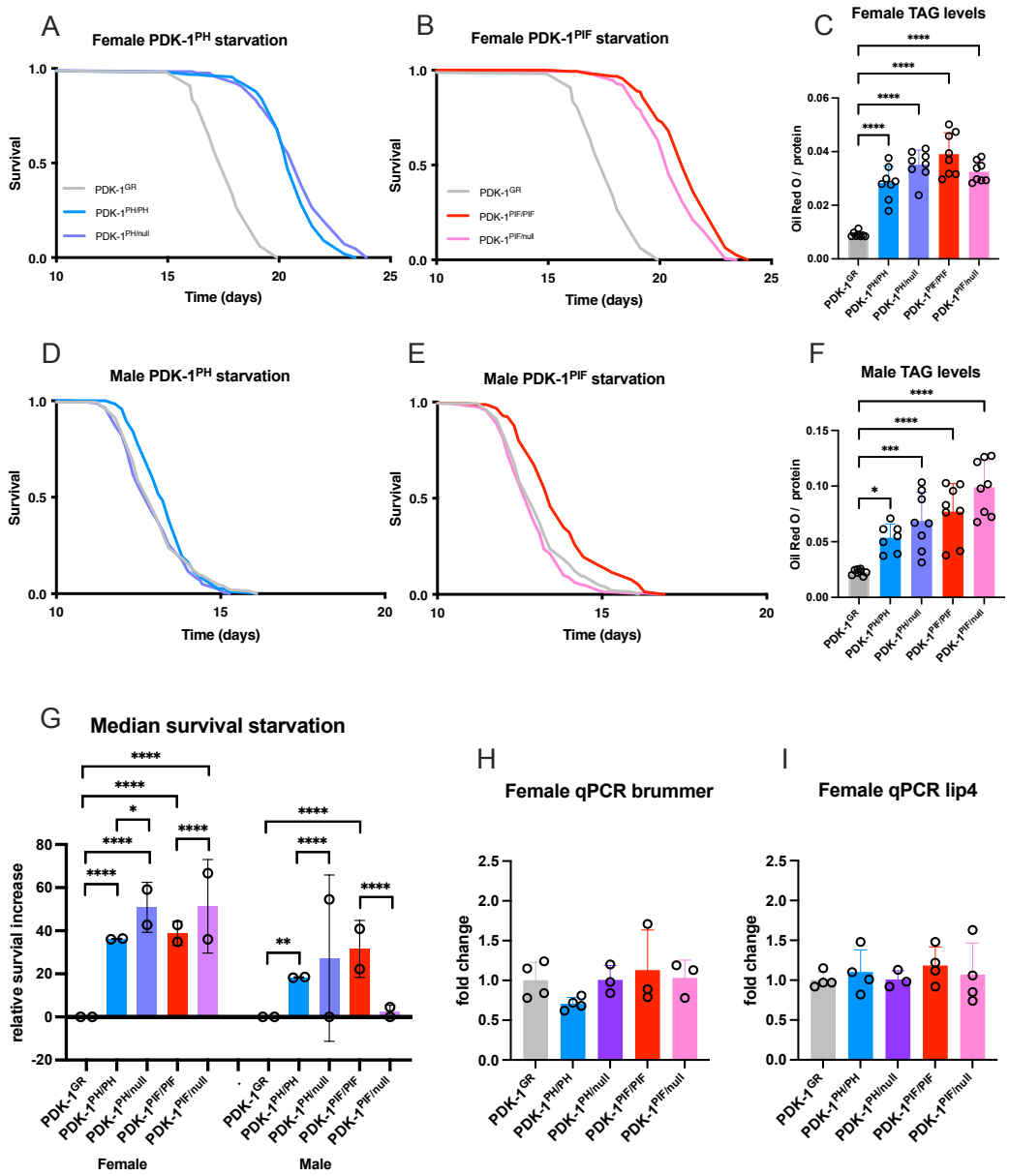

Figure 11: **Reduced activity of the AKT and AGC branch increased starvation resistance and lipid storage.** A,B,D,E: Flies of each genotype were placed on 1% Agar starvation medium after 10 days on SYA food (n=160 p.g.). For better comparison identical PDK-1^{GR} is plotted in graphs A+B and D+E.

A: PDK-1^{PH} females: *PDK-1^{GR} vs PDK-$1^{PH/PH}$ median +36% p<0.0001; PDK-1^{GR} vs PDK-$1^{PH/null}$ median +42,7 p<0.0001; PDK-$1^{PH/PH}$ vs PDK-$1^{PH/null}$ median +6,7% p<0.05. log rank test.*

B: PDK-1^{PIF} females: PDK-1^{GR} vs PDK-$1^{PIF/PIF}$ median +42,7% p<0.0001; PDK-1^{GR} vs PDK-$1^{PIF/null}$ median +36% p<0.0001; PDK-$1^{PIF/PIF}$ vs PDK-$1^{PIF/null}$ median -6,7% p<0.0001. log rank test

C: Whole body TAG content of 10 days old female flies (n=8 p.g.). *PDK-1^{GR} vs PDK-$1^{PH/PH}$ 3.1 fold p<0.0001; PDK-1^{GR} vs PDK-$1^{PH/null}$ 3.9 fold p<0.0001; PDK-1^{GR} vs PDK-$1^{PIF/PIF}$ 4.3 fold p<0.0001; PDK-1^{GR} vs PDK-$1^{PIF/null}$ 3.6 fold p<0.0001. one-way ANOVA.*

D: PDK-1^{PH} males: *PDK-1^{GR} vs PDK-$1^{PH/PH}$ median +18.5% p<0.0001; PDK-1^{GR} vs PDK-$1^{PH/null}$ p= n.s.; PDK-$1^{PH/PH}$ vs PDK-$1^{PH/null}$ median -18.5% p<0.0001. log rank test.*

E: PDK-1^{PIF} males: PDK-1^{GR} vs PDK-$1^{PIF/PIF}$ median +22.2% p<0.0001; PDK-1^{GR} vs PDK-$1^{PIF/null}$ p= n.s.; PDK-$1^{PIF/PIF}$ vs PDK-$1^{PIF/null}$ median -22.2% p<0.0001. log rank test

F: Whole body TAG content of 10 days old male flies (n=8 p.g.). *PDK-1^{GR} vs PDK-$1^{PH/PH}$ 2.4 fold p<0.05; PDK-1^{GR} vs PDK-$1^{PH/null}$ 3 fold p<0.0001; PDK-1^{GR} vs PDK-$1^{PIF/PIF}$ 3.3 fold p<0.0001; PDK-1^{GR} vs PDK-$1^{PIF/null}$ 4.3 fold p<0.0001. one-way ANOVA.*

E: Relative medium survival compared to PDK-1^{GR} on starvation food. Significant stars are based on the graphs A,B,D and E. 2 independent trails performed by van Schelt (MPI AGE) and Gödderz (MPI AGE).

H+I: whole female fly qPCR brummer and lipase 4 gene at day 10. No significant differences. one-way ANOVA.

* = p<0.05, ** = p<0.01, *** = p<0.001, **** = p<0.0001

3.2.4 Reduced PDK-1 signalling protects against oxidative stress

Oxidative and xenobiotic stress resistance is often associated with longevity. Long-lived IIS mutants, like *dilp2-3,5* mutant flies show an increased resistance to both oxidative and xenobiotic stress (Grönke *et al.*, 2010). Thus, I measured oxidative stress resistance of PDK-1 mutant flies by feeding them with paraquat (20 mM in SYA) or hydrogen peroxide (H_2O_2) (5 w/v% in a 1% Agar/10%sucrose diet). Paraquat induces oxidative stress primarily through dysregulation of the redox cycle and through the production of superoxide free radicals. Upon entering the cell, it affects a variety of enzymes including nicotinamide adenine dinucleotide phosphate (NADPH) and CYPs reductase in the redox reaction. Additionally, it produces a large number of reactive oxygen free radicals, participates in several other reactions yielding a variety of reactive oxygen species (ROS) and reactive nitrogen species (RNS). SODs accelerate the reaction of the superoxide anion ($O_2^{\bullet-}$) with itself to form H_2O_2 and oxygen ($2O_2^{\bullet-} + 2H^+ \rightarrow H_2O_2 + O_2$). By controlling $O_2^{\bullet-}$, SODs also control the concentrations of ROS and RNS (Wang *et al.*, 2018). Administration of paraquat unbalances the functioning redox cycle and as mitochondria, ER and peroxisome membrane (Chen *et al.*, 2021). In contrast to paraquats produced oxygen free radical, H_2O_2 is relatively stable. Catalase (CAT), glutathione peroxidases (GPXs), and peroxiredoxins (PRXs) convert H_2O_2 into water. High levels of H_2O_2 can results in complete occupation of all CAT, allowing H_2O_2 to interact with iron, generating the harmful and reactive hydroxy radical ($OH^{\bullet}$) through the Fenton/Haber-Weiss reaction. However, this reaction only happens at extremely high concentrations of H_2O_2 (Ransy *et al.*, 2020).

As paraquat induces the most upstream free radicals, oxidative stress resistance with paraquat treatment was tested first. Homozygous PDK-$1^{PH/PH}$ mutant females survived significantly longer upon paraquat treatment than PDK-$1^{GR/GR}$ control flies with a median increase in survival of 31%. Survival was further increased in PDK-$1^{PH/null}$ mutant flies, which showed an increase of 54% compared to PDK-$1^{GR/GR}$ control flies (Figure 12A). Oxidative stress resistance was also increased in PDK-$1^{PIF/\ PIF}$ mutant flies, 31% median gain, but not further increased in PDK-$1^{PIF/null}$ mutant flies (Figure 12B). Thus, both the AKT and the AGC branch are involved in oxidative stress resistance in females, but have a slightly different optimal signalling window. Strong downregulation of the AKT branch provided the most effective protection from oxidative stress.

In males neither the AKT nor the AGC branch positively affected survival on paraquat-induced oxidative stress. While the PDK-$1^{PH/PH}$ mutation did not affect survival, PDK-$1^{PH/null}$, PDK-$1^{PIF/PIF}$ and PDK-$1^{PIF/null}$ males showed reduced survival by -25%, -33% and -25%, respectively (Figure 12C+D). Thus, the effect of reduced AKT or AGC signalling on paraquat induced oxidative stress is sex-specific, being protective in females and detrimental in males.

To further investigated the difference between the sexes, males were exposed to H_2O_2. While paraquat is made harmless by SOD, H_2O_2 is counteracted by CAT, GPXs and PRXs. Surprisingly, we observed an improvement in survival in all PDK-1 male mutants. Moderate downregulation of AKT signalling in PDK-$1^{PH/PH\ males}$ resulted in an increased survival of 25%, while strong downregulation diminished the survival extension to 12.5% (Figure 12E). While mild reduction in AKT signalling improved survival on H_2O_2, severe downregulation of the pathway reduced median survival. There was no distinction in survival between strong or moderate downregulation in the AGC mutants, with both having 29% gain in median survival (Figure 12F). While reduction in AKT signalling resulted in the

most robust decline of median survival on paraquat, it resulted in the most consistent improvement on H_2O_2.

Taken together, both the AKT and the AGC affect the ability to handle oxidative stress in both females and males. However, there is a distinctive difference between the sexes. Female PDK-1 mutants withstand oxidative stressed induced by paraquat and H_2O_2 (female data not shown, performed by Gödderz (MPI AGE)) better than PDK-1^{GR}. It is therefore implied that reduced signalling in both branches upregulates SOD, CAT, GPX and PRX counter act the free oxygen radicals and reduce them to harmless components. The male data implies that reduced signalling in AKT or AGC branch results in a diminished function of SOD, as shown by the reduced survival on paraquat. Especially a reduction in the AGC branch signalling, hampered the ability to counteract $O_2^{\bullet -}$. However, the ability to convert H_2O_2 into water, thereby increasing survival in the H_2O_2 stress assay, did increase in all PDK-1 mutants. While the role of both the AKT and the AGC branch in oxidative stress is undisputed, effects are both sex- and enzyme-specific.

3.2.5 Reduced PDK-1 signalling protects against xenobiotic stress

Reduced IIS signalling is often associated with increased xenobiotic stress resistance (Grönke *et al.*, 2010). Thus, I measured xenobiotic stress resistance of PDK-1 mutant flies by exposing them to dichlorodiphenyltrichloroethane (DDT, 0.03% w/v) in normal SYA food. PDK-$1^{PH/PH}$ mutant females showed increased resistance towards DDT with an increase in median survival of 11% (Figure 13A). DDT resistance was further increased in PDK-$1^{PH/null}$ mutant females, which showed a 44% increase in median survival (Figure 13A). PDK-$1^{PIF/PIF}$ mutant females were also significantly more resistant to DDT treatment and there was a slight additive effect in PDK-$1^{PIF/null}$ mutant females, showing median lifespan gains of 28% and 56% respectively (Figure 13B). In summary, PDK-1 dependent signalling via the AKT and ACG branch regulate resistance towards xenobiotic stress in females. In both branches stronger downregulation was more protective to survive DDT xenobiotic stress.

In males, the effects of the PDK-1 mutations on DDT resistance were not as strong . While homozygous PDK-$1^{PH/PH}$ nor the PDK-$1^{PIF/PIF}$ did not show increased resistance to DDT, there was a clear increase only in PDK-$1^{PH/null}$ and PDK-$1^{PIF/}$null mutant males (Figure 13C+D). Thus, both the AKT and the AGC branch are involved in DDT resistance, but unlike in females a strong reduction in signalling is needed to increase survival on DDT.

Cytochrome p450 enzymes play an important role in cellular detoxication and DDT resistance. For example, overexpression of *Cyp6g1* or *Cyp6w1* genes, cytochrome p450 enzymes, improves DDT resistance (Daborn *et al.*, 2002; Pedra *et al.*, 2004; Schmidt *et al.*, 2017). Thus, I measured the expression of *cyp6w1* by Q-RT-PCR in female PDK-1 mutant flies. Surprisingly, cyp6w1 levels were reduced in both the PDK-1PH/null and the PDK-1PIF/null mutants (Figure 13F). Counter intuitively, the most resistant mutants showed the lowest expression of the *cyp6w1* gene. Thus, differences in cyp6w1 expression do not explain the increased DDT resistance of PDK-1 mutant flies.

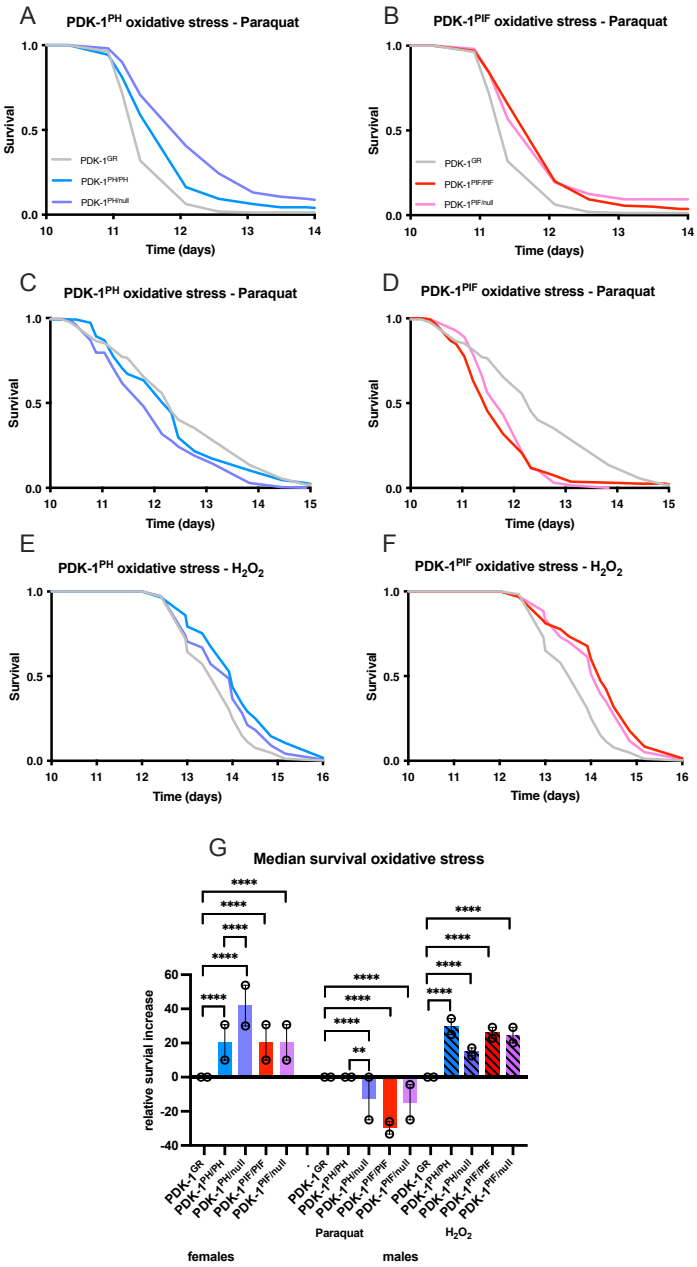

Figure 12: Both the AKT and AGC branch protect against oxidative stress. *A+B: Oxidative stress assay based on 20mM Paraquat (SYA diet) or 5 w/v% H_2O_2 (1% Agar / 10% sucrose diet) after 10 days on SYA food. (n=160). For better comparison identical PDK-1^{GR} is plotted in graphs A+B, C+D and E+F.*

A: Females PDK-1^{PH} : PDK-1^{GR} vs PDK-$1^{PH/PH}$ median 30.8% p<0.0001; PDK-1^{GR} vs PDK-$1^{PH/null}$ median 53.8% p<0.0001; PDK-$1^{PH/PH}$ vs PDK-$1^{PH/null}$ median 23.8% p<0.0001. Log rank test.

B: Females PDK-1^{PIF} : PDK-1^{GR} vs PDK-$1^{PIF/PIF}$ median 30.8% p<0.0001; PDK-1^{GR} vs PDK-$1^{PIF/null}$ median 30.8% p<0.0001; PDK-$1^{PIF/PIF}$ vs PDK-$1^{PIF/null}$ p= n.s. log rank test.

C: Males paraquat PDK-1^{PH} : PDK-1^{GR} vs PDK-$1^{PH/PH}$ p = n.s.; PDK-1^{GR} vs PDK-$1^{PH/null}$ median -25% p<0.0001; PDK-$1^{PH/PH}$ vs PDK-$1^{PH/null}$ median -25% p<0.01. Log rank test.

D: Males paraquat PDK-1^{PIF} : PDK-1^{GR} vs PDK-$1^{PIF/PIF}$ median-33.3 % p<0.0001; PDK-1^{GR} vs PDK-$1^{PIF/null}$ median -25% p<0.0001; PDK-$1^{PIF/PIF}$ vs PDK-$1^{PIF/null}$ p = n.s.. log rank test.

E: Males H_2O_2 PDK-1^{PH} : PDK-1^{GR} vs PDK-$1^{PH/PH}$ median 25% p<0.0001; PDK-1^{GR} vs PDK-$1^{PH/null}$ median 12.5% p<0.0001; PDK-$1^{PH/PH}$ vs PDK-$1^{PH/null}$ median -12.5% p<0.0001. Log rank test.

F: Males H_2O_2 PDK-1^{PIF} : PDK-1^{GR} vs PDK-$1^{PIF/PIF}$ median 29.2% p<0.0001; PDK-1^{GR} vs PDK-$1^{PIF/null}$ median 29.17% p<0.0001; PDK-$1^{PIF/PIF}$ vs PDK-$1^{PIF/null}$ p = n.s. log rank test.

G: Relative medium survival compared to PDK-1^{GR} on stress food. Significant stars are based on the graphs A-F. Experiments with H_2O_2 are performed *by van Schelt (MPI AGE) and Gödderz (MPI AGE).*

** = p<0.05, ** = p<0.01, *** = p<0.001, **** = p<0.0001*

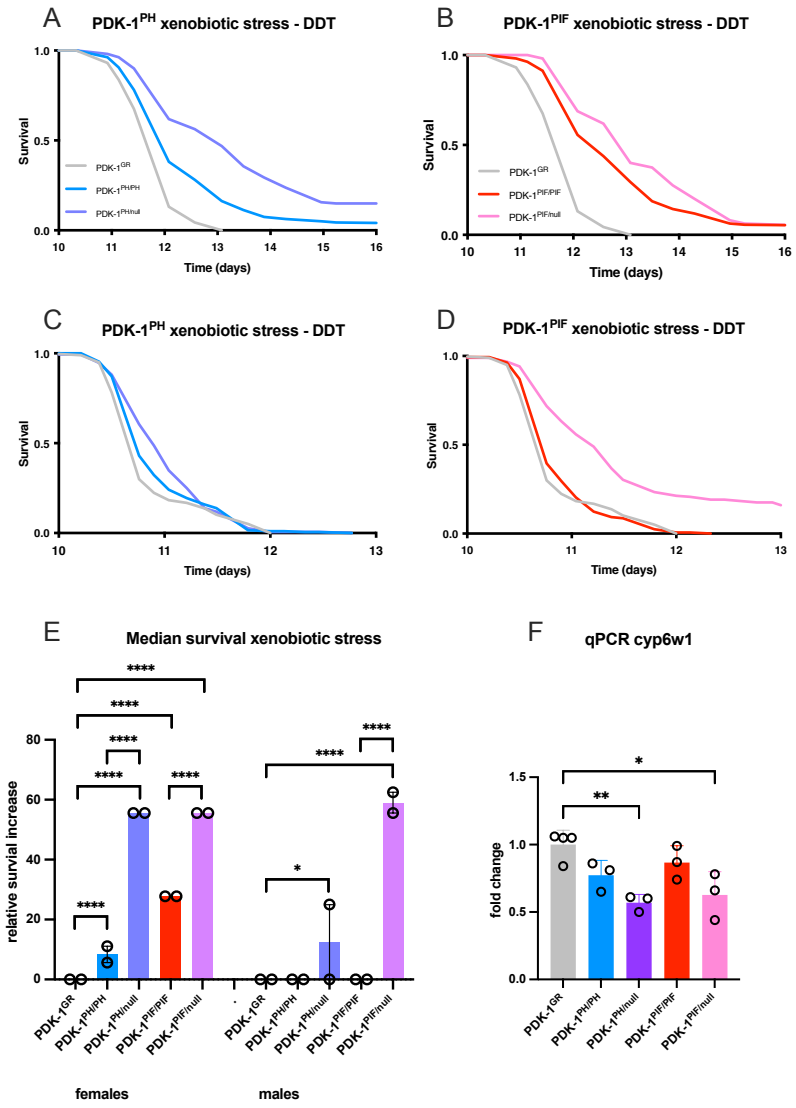

Figure 13: Both the AKT and AGC branch protect against xenobiotic stress. *A-D: Xenobiotic stress assays with DDT 0.03% w/v after 10 days on SYA food. (n=160 p.g.). For better comparison identical PDK-1^{GR} is plotted in graphs A+B and C+D.*
A: Female PDK-1^{PH}: PDK-1^{GR} vs PDK-1$^{PH/PH}$ median % p<0.0001; PDK-1^{GR} vs PDK-1$^{PH/null}$ median %p<0.0001; PDK-1$^{PH/PH}$ vs PDK-1$^{PH/null}$ median % p<0.0001. log rank test.
B: Female PDK-1^{PIF}: PDK-1^{GR} vs PDK-1$^{PIF/PIF}$ median % p<0.0001; PDK-1^{GR} vs PDK-1$^{PIF/null}$ median %p<0.0001; PDK-1$^{PIF/PIF}$ vs PDK-1$^{PIF/null}$ median % p<0.0001. log rank test.
C: Male PDK-1^{PH}: PDK-1^{GR} vs PDK-1$^{PH/PH}$ p= n.s.; PDK-1^{GR} vs PDK-1$^{PH/null}$ median 25% p<0.05; PDK-1$^{PH/PH}$ vs PDK-1$^{PH/null}$ p= n.s.. log rank test.
D: Male PDK-1^{PIF}: PDK-1^{GR} vs PDK-1$^{PIF/PIF}$ p= n.s.; PDK-1^{GR} vs PDK-1$^{PIF/null}$ median 63% p<0.0001; PDK-1$^{PIF/PIF}$ vs PDK-1$^{PIF/null}$ median 63% p<0.0001. log rank test.
E: Relative median survival compared to PDK-1^{GR} control flies on DDT. Significant stars are based on the graphs A-D.
F: Female whole fly qPCR of cyp6w1 at day 10. PDK-1^{GR} vs PDK-1$^{PH/null}$ p<0.01; PDK-1^{GR} vs PDK-1$^{PIF/null}$ p<0.05. one-way ANVOVA.
** = p<0.05, ** = p<0.01, *** = p<0.001, **** = p<0.0001*

3.2.6 PDK-1 mutations ameliorate age-related decline in climbing ability

While lifespan is an important read out in ageing research, the health span of an organism is equally important. In *Drosophila*, climbing ability is used as health span indicator (Ratliff *et al.*, 2015; Cochemé *et al.*, 2019). As in humans, with greater age a progressive decline of motor coordination and muscle

function is observed in flies (Minois, Khazaeli and Curtsinger, 2001; Goddeeris et al., 2003; Gargano et al., 2005; Jang and Van Remmen, 2011). Additionally, *Drosophila* exhibits an age-related deterioration in motor-neuron function reducing muscle function (Beramendi et al., 2007). Thus, in order to address the health span of PDK-1 mutant flies, I measured age-related changes in climbing ability by performing a negative geotaxis assay (NGR) using female flies (Gargano *et al.*, 2005). For quantification, a climbing index (CI) based on repeated climbing ability in 30 seconds was calculated.

There was no difference in the CI between female and male PDK-1 mutants and PDK-1^{GR} control flies at young age (d7), indicating that effects of PDK-1 on development do not interfere with climbing ability of the adult flies (Figure 14A-D). As previously reported, I observed a clear age-related decline in the climbing ability of PDK-1^{GR} control flies in both sexes (Figure 14). At day 20 the reduction in climbing ability was significant between young and old PDK-1^{GR} control flies. Thus, in both sexes the onset of this age-related phenotype is at middle adulthood in wild-type-like *Drosophila*. The decline in age-related climbing ability was ameliorated in both the PDK-1 PH domain and PIF pocket female mutant flies. PDK-$1^{PH/PH}$ and PDK-$1^{PH/null}$ females had a significantly higher CI than PDK-1^{GR} females at day 25 (Figure 14A). PDK-$1^{PIF/PIF}$ and PDK-$1^{PIF/null}$ females had a significantly higher CI than PDK-1^{GR} control females at day 30 (Figure 14B). Therefore, both the AKT and the AGC branch positively affected climbing ability in female flies. While the reduction in AKT branch signalling resulted in improved climbing at a slightly earlier age than PDK-1^{PIF} mutants, both the PDK-1^{PH} and the PDK-1^{PIF} mutants retained better climbing ability with age.

Not only the female PDK-1^{PH} mutants showed improvement in climbing, significant gain in CI was detected from day 20 and 25 for the male PDK-$1^{PH/PH}$ and PDK-$1^{PH/null}$ respectively (Figure 14C). Likewise, both the male PDK-$1^{PIF/PIF}$ and PDK-$1^{PIF/null}$ showed improved climbing from day 25 and 20 onwards (Figure 14D). Improvements were detected from day 20 onwards and once present remained till the end of the experiment at day 35. Thus, as in females, reduced activity of both the ATK and the AGC signalling branch positively affected age-related climbing ability of male flies.

While a delay in onset of an ARD is desirable, equally important is the severity of the ARD. After all, a delayed onset with increased acceleration of the phenotype is suboptimal. While the PDK-1^{GR} only retained 10% and 18% of the young climbing capacity in females and males respectively, the PDK-1 mutants performed much better. PDK-$1^{PH/PH}$ retained 42/47%, PDK-$1^{PH/null}$ only 56/51%, PDK-$1^{PIF/PIF}$ 41/39% and PDK-$1^{PIF/null}$ 50/57% for females and males respectively. To further analyse the progression of the ARD, a linear regression analysis was performed. The analysis revealed statistically significant differences for all genotypes compared to PDK-1^{GR}, indicating that the functional decline in climbing was dampened due to reduced signalling in either the AKT or the ACG branch (Figure 14E+F). There was no additional effect on age-related climbing ability in PDK-$1^{PH/null}$ and PDK-$1^{PIF/null}$ mutant flies compared to their homozygous mutants (Figure 14B). Taken together, this analysis indicates that both the AKT and the AGC branch delayed the onset of this age-related phenotype in a quite similar manner in both sexes. While the precise underlying mechanism are unclear, both the AKT and the AGC branch are mostly likely involved in the maintenance of healthy muscles and motor neuron connection.

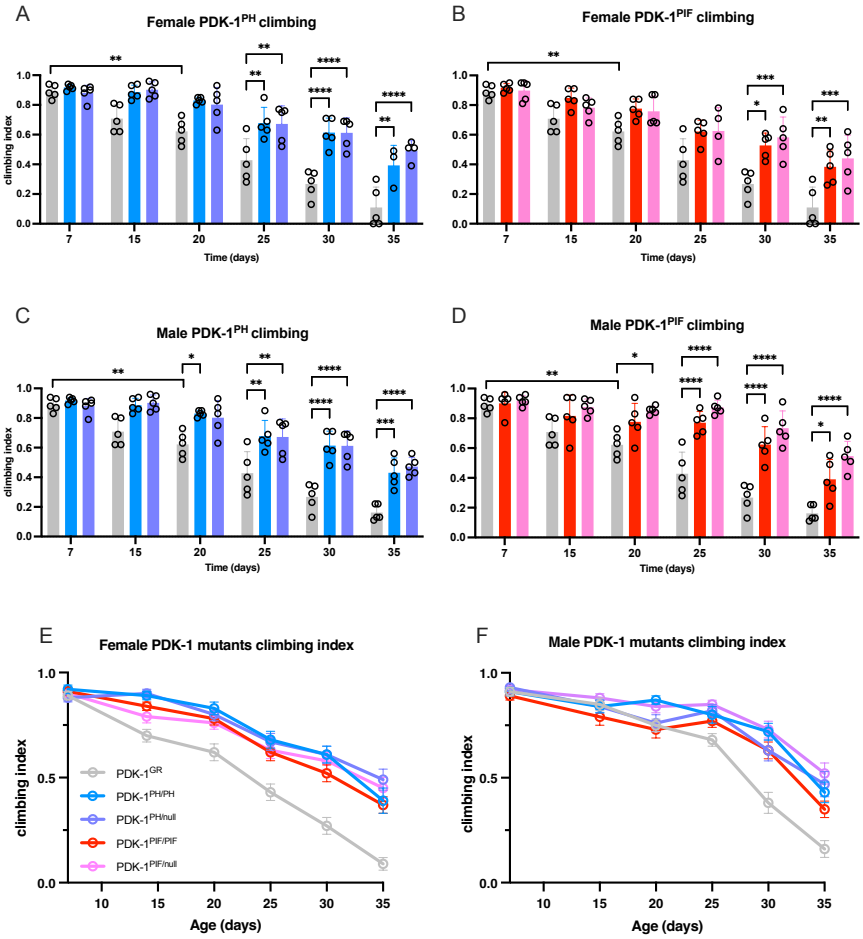

Figure 14: Both the AKT and the AGC branch ameliorate the age-related decline in climbing ability. *Negative geotaxis assay over time (N = 80 flies p.g. per time point). For better comparison identical PDK-1^{GR} is plotted in graphs A+B and C+D.*
Only significant comparisons are listed.
A+B: Female PDK-1^{GR} per day: d7 vs d20 p<0.01; d7 vs d25 p<0.001; d7 vs d30 p<0.0001.
C+D: Male PDK-1^{GR} per day: d7 vs d20 p<0.001; d7 vs d25 p<0.0001; d7 vs d30 p<0.0001.
A: Female PDK-1^{PH}: d25 PDK-1^{GR} vs PDK-1$^{PH/PH}$ p<0.01; d30 PDK-1^{GR} vs PDK-1$^{PH/PH}$ p<0.0001; d35 PDK-1^{GR} vs PDK-1$^{PH/PH}$ p<0.01; d25 PDK-1^{GR} vs PDK-1$^{PH/null}$ p<0.01; d30 PDK-1^{GR} vs PDK-1$^{PH/null}$ p<0.0001; d35 PDK-1^{GR} vs PDK-1$^{PH/null}$ p<0.0001. Longitudinal: PDK-1$^{PH/PH}$ d7 vs d25 p<0.05; PDK-1$^{PH/PH}$ d7 vs d30 p<0.01; PDK-1$^{PH/PH}$ d7 vs d35 p<0.0001; PDK-1$^{PH/null}$ d7 vs d25 p<0.05; PDK-1$^{PH/null}$ d7 vs d30 p<0.01; PDK-1$^{PH/null}$ d7 vs d35 p<0.0001. one-way ANOVA
B: Female PDK-1^{PIF}: d30 PDK-1$^{GR/GR}$ vs PDK-1$^{PIF/PIF}$ p<0.05; d35 PDK-1^{GR} vs PDK-1$^{PIF/PIF}$ p<0.01; d30 PDK-1^{GR} vs PDK-1$^{PIF/null}$ p<0.001; d35 PDK-1^{GR} vs PDK-1$^{PIF/null}$ p<0.001. Longitudinal: PDK-1$^{PIF/PIF}$ d7 vs d25 p<0.01, PDK-1$^{PIF/PIF}$ d7 vs d30 p<0.0001; PDK-1$^{PIF/PIF}$ d7 vs d35 p<0.0001; PDK-1$^{PIF/PIF}$ d7 vs d25 p<0.05; PDK-1$^{PIF/null}$ d7 vs d30 p<0.01; PDK-1$^{PIF/null}$ d7 vs d35 p<0.0001. one-way ANOVA.
C: Male PDK-1^{PH}: d20 PDK-1^{GR} vs PDK-1$^{PH/PH}$ p<0.05; d25 PDK-1^{GR} vs PDK-1$^{PH/PH}$ p<0.01; d30 PDK-1^{GR} vs PDK-1$^{PH/PH}$ p<0.0001; d35 PDK-1^{GR} vs PDK-1$^{PH/PH}$ p<0.001; d25 PDK-1^{GR} vs PDK-1$^{PH/null}$ p<0.01; d30 PDK-1^{GR} vs PDK-1$^{PH/null}$ p<0.0001; d35 PDK-1^{GR} vs PDK-1$^{PH/null}$ p<0.0001. Longitudinal: PDK-1$^{PH/PH}$ d7 vs d25 p<0.01; PDK-1$^{PH/PH}$ d7 vs d30 p<0.0001; PDK-1$^{PH/PH}$ d7 vs d35 p<0.0001; PDK-1$^{PH/null}$ d7 vs d25 p<0.05; PDK-1$^{PH/null}$ d7 vs d30 p<0.001; PDK-1$^{PH/null}$ d7 vs d35 p<0.0001. one-way ANOVA
D: Male PDK-1^{PIF}: d25 PDK-1$^{GR/GR}$ vs PDK-1$^{PIF/PIF}$ p<0.0001: d30 PDK-1$^{GR/GR}$ vs PDK-1$^{PIF/PIF}$ p<0.0001; d35 PDK-1^{GR} vs PDK-1$^{PIF/PIF}$ p<0.05; d20 PDK-1^{GR} vs PDK-1$^{PIF/null}$ p<0.05; d25 PDK-1^{GR} vs PDK-1$^{PIF/null}$ p<0.0001; d30 PDK-1^{GR} vs PDK-1$^{PIF/null}$ p<0.0001; d35 PDK-1^{GR} vs PDK-1$^{PIF/null}$ p<0.0001. Longitudinal: PDK-1$^{PIF/PIF}$ d7 vs d30 p<0.01, PDK-1$^{PIF/PIF}$ d7 vs d35 p<0.0001; PDK-1$^{PIF/null}$ d7 vs d35 p<0.0001. one-way ANOVA.
E: Females: PDK-1$^{PH/PH}$ p<0.0001, PDK-1$^{PH/null}$ p<0.0001, PDK-1$^{PIF/PIF}$ P<0.0001, PDK-1$^{PIF/null}$ p<0.0001.
F: Males: PDK-1$^{PH/PH}$ p<0.0001, PDK-1$^{PH/null}$ p<0.0001, PDK-1$^{PIF/PIF}$ P<0.0001, PDK-1$^{PIF/null}$ p<0.0001. Linear regression model.
** = p<0.05, ** = p<0.01, *** = p<0.001, **** = p<0.0001*

3.2.7 Reduced signalling of PDK-1 in the AKT and AGC branch improves intestinal health

Intestinal barrier dysfunction has been associated with age-related mortality in female *Drosophila* (Rera, Clark and Walker, 2012; Clark *et al.*, 2015) and it has been suggested that in females intestinal integrity predicts biological age more accurately than chronologic age, underlining the strong relation between organismal ageing and intestinal health. (Rera, Clark and Walker, 2012; Fan *et al.*, 2015; Regan *et al.*, 2016; Schinaman *et al.*, 2019a; Juricic, Grönke and Partridge, 2020). One of the causes of intestinal barrier dysfunction is age-related over-proliferation of intestinal stem cells (ISC) resulting in an accumulation of undifferentiated entroblasts (EBs) ultimately leading to gut cell crowding and tumour formation (Biteau, Hochmuth and Jasper, 2008; Choi *et al.*, 2008; Biteau *et al.*, 2010; Patel, Dutta and Edgar, 2015). Both rapamycin and IIS mutants like *chico*$^{1/1}$ and *InR*$^{E19/05545}$ attenuate age-related over-proliferation of ISC in the female but not the male gut (Regan et al. 2016, Biteau *et al.*, 2010). Thus, I measured proliferation of ISC with age in PDK-1 mutant females using immunostaining of phosphorylated histone H3 (pH3+), a marker for proliferating cells (Hirata *et al.*, 2004).

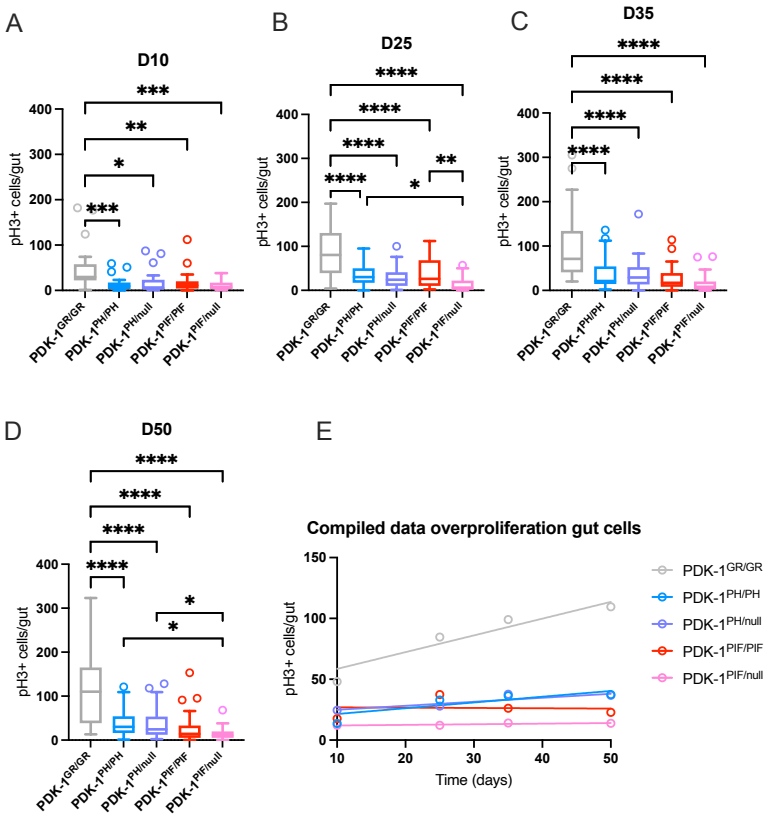

Figure 15: Reduced activity of both the AKT and the AGC branch downstream of PDK-1 attenuate age-related proliferation of intestinal stem cells. *(A-D) Quantification of pH3+ positive cells per gut. n=25-60 guts per condition*
A: d10 PDK-1^{GR} vs PDK-1$^{PH/PH}$ p=0.0008; PDK-1$^{PH/null}$ p=0.0124; PDK-1$^{PIF/PIF}$ p=0.0032; PDK-1$^{PIF/null}$ p=0.0008 and d25, one-way ANOVA.
B: d25 PDK-1^{GR} vs PDK-1$^{PH/PH}$ p<0.0001; PDK-1$^{PH/null}$ p<0.0001; PDK-1$^{PIF/PIF}$ p<0.0001; PDK-1$^{PIF/null}$ p<0.001; d25 PDK-1$^{PIF/null}$ vs PDK-1$^{PH/PH}$ p=0.0113 and PDK-1$^{PIF/null}$ vs PDK-1$^{PIF/PIF}$ p=0.0029 and, one-way ANOVA.
C: d35 PDK-1^{GR} vs PDK-1$^{PH/PH}$ p<0.0001; PDK-1$^{PH/null}$ p<0.0001; PDK-1$^{PIF/PIF}$ p<0.0001; PDK-1$^{PIF/null}$ p<0.001 and one-way ANOVA. D: d50 PDK-1^{GR} vs PDK-1$^{PH/PH}$ p<0.0001; PDK-1$^{PH/null}$ p<0.0001; PDK-1$^{PIF/PIF}$ p<0.0001; PDK-1$^{PIF/null}$ p<0.001, d50 PDK-1$^{PIF/null}$ vs PDK-1$^{PH/PH}$ p=0.0331 and PDK-1$^{PIF/null}$ vs PDK-1$^{PIF/PIF}$ p=0.0360
E: compiled data from all ages. Linear regression model over time. PDK-1^{GR} 0 p=0.0002; PDK-1$^{PH/PH}$ 0 p=0.0009; PDK-1$^{PH/null}$ 0 p=0.0365; PDK-1$^{PIF/PIF}$ 0 p=0.99; PDK-1$^{PIF/null}$ 0 p=0.55. Genotype: PDK-1^{GR} vs PDK-1$^{PH/PH}$ slope p=0.0220; PDK-1^{GR} vs PDK-1$^{PH/null}$ slope p=0.0101; PDK-1^{GR} vs PDK-1$^{PIF/PIF}$ slope p=0.0010; PDK-1^{GR} vs PDK-1$^{PIF/null}$ slope p=0.0001.
** = p<0.05, ** = p<0.01, *** = p<0.001, **** = p<0.0001*

The number of pH3+ cells in the intestine of 10 day old female flies was already significantly reduced in PDK-1$^{PH/PH}$, PDK-1$^{PH/null}$, PDK-1$^{PIF/PIF}$ and PDK-1$^{PIF/null}$ mutants compared to the PDK-1^{GR} control (Figure 15A). Thus, even at young age, loss of PDK-1 function affected proliferation rates of ISCs. The progression from young (day 10) to mid-life (day 25) resulted in a significant increase in the number of pH3+ cells in the intestine of PDK-1^{GR} control flies, and this number further increased at old age (Figure 15E). Compared to PDK-1^{GR} control flies, PDK-1 mutants had significantly lower number of pH3+ cells throughout life (Figure 15B-D). Interestingly, PDK-1$^{PH/PH}$ flies showed an age-related increase in the number of pH3+ cells (Figure 15E), although this increase was lower than in the control flies. In contrast, there was no age-related increase in the number of pH3+ cells observed in PDK-1$^{PIF/PIF}$ mutant guts. PDK-1$^{PH/null}$ mutants showed the same pH3+ cell number as PDK-1$^{PH/PH}$, while ph3+ cell number was further decreased in PDK-1$^{PIF/null}$ flies compared to PDK-1$^{PIF/PIF}$ flies. At old age, PDK-1$^{PIF/null}$ flies contained the lowest number of pH3+ cells in their gut, and this was significantly lower than the number in the intestine of PDK-1$^{PH/PH}$ and PDK-1$^{PH/null}$ mutant flies. Thus, while both the Akt and the AGC branch affected the proliferation of ISCs, only the intervention targeting the AGC branch was sufficient to completely abolish the age-related increase in ISC proliferation, consistent with the important role of the TOR branch of the IIS network in controlling ISC proliferation (Haller *et al.*, 2017).

3.2.8 PDK-1 regulates response to intestinal infection

To assess whether the low mitotic levels observed in the PDK-1 mutants were due to improved homeostasis and not due to impaired mitotic ability, we next measured ISC proliferation upon an acute stimulus. ISC proliferation can be induced by oral infection of flies with the gram-negative bacterium *Erwinia carotovora carotovora* (*Ecc15*). Cell survival, wound healing and DNA repair genes become activated 16h after infection with *Ecc15* and ISC division is induced within 24h post infection (Buchon *et al.*, 2009). Therefore, we quantified the number of pH3+ cells in the gut of PDK-1 mutants after 24h of *Ecc15* infection throughout life.

We did not observe a clear upregulation in pH3 positive cells in the PDK-1^{GR} mutants when stimulated for 24 hours with *Ecc15* at day 10 (Figure 16A). While there was the expected trend and a 1.5 fold increase in pH3 positive cells upon infection, this was not significant, probably due to the relatively high mitotic baseline activity and high variance in the PDK-1^{GR} guts. A clear increase in the number of pH3 positive cells was observed in all mutant genotypes upon infection at young age (day 10) (Figure 16 A-C). Both the PDK-1$^{PIF/PIF}$ and the PDK-1$^{PIF/null}$ mutants showed a 4.0 fold upregulation of the number of pH3 positive cells upon *Ecc15* infection at day 10. Moreover, both the PDK-1$^{PIF/PIF}$ and the PDK-1$^{PIF/null}$ mutants showed an increase of 3.6 and 4.2 fold, respectively. Therefore while PDK-1 mutants showed reduced mitotic activity of ISCs during homeostasis, upon bacterial infection their ISCs were able to proliferate to at least the same extent as those of control animals.

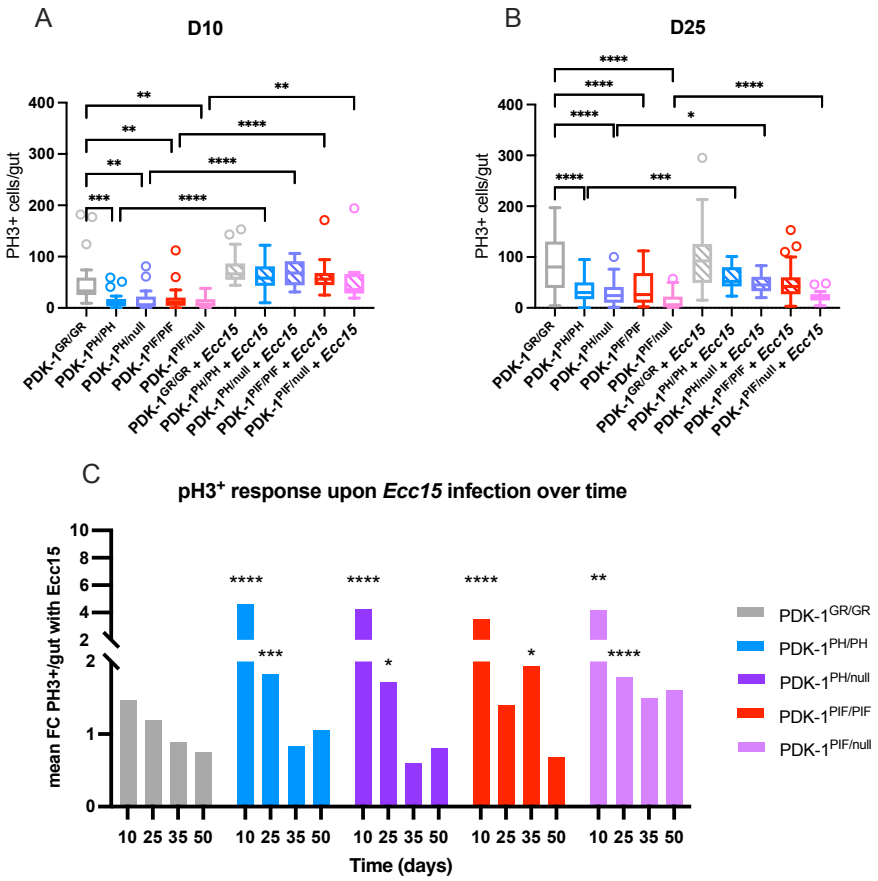

Figure 16: Proliferation capacity of ISCs is not impaired in PDK-1^{PH} and PDK-1$^{PIF/PIF}$
A-C: Quantification of pH3+ positive cells per gut after 24h Ecc15 infection at d10, d25, d35 and d50 (n=25-60 guts per condition).
A: Genotype effect on d10: PDK-1^{GR} vs PDK-1$^{PH/PH}$ p<0.001; PDK-1$^{PH/null}$ p<0.05; PDK-1$^{PIF/PIF}$ p<0.01; PDK-1$^{PIF/null}$ p<0.001. Ecc15 infection effect on d10: PDK-1^{GR} vs +Ecc15 p=n.s.; PDK-1$^{PH/PH}$ vs +Ecc15 p<0.0001; PDK-1$^{PH/null}$ vs +Ecc15 p<0.0001; PDK-1$^{PIF/PIF}$ vs +Ecc15 p<0.0001; PDK-1$^{PIF/null}$ vs +Ecc15 p<0.01. One-way ANOVA
B: Genotype effect on d25: PDK-1^{GR} vs PDK-1$^{PH/PH}$ p<0.0001; PDK-1$^{PH/null}$ p<0.0001; PDK-1$^{PIF/PIF}$ p<0.0001; PDK-1$^{PIF/null}$ p<0.001; d25 PDK-1$^{PIF/null}$ vs PDK-1$^{PH/PH}$ p<0.05 and PDK-1$^{PIF/null}$ vs PDK-1$^{PIF/PIF}$ p<0.01. Ecc15 infection effect on d25: PDK-1^{GR} vs +Ecc15 p=n.s.; PDK-1$^{PH/PH}$ vs +Ecc15 p<0.001; PDK-1$^{PH/null}$ vs +Ecc15 p<0.05; PDK-1$^{PIF/PIF}$ vs +Ecc15 p=n.s.; PDK-1$^{PIF/null}$ vs +Ecc15 p<0.0001 one-way ANOVA.
C: Median fold change of PH3+ cells/gut between normal conditions and Ecc15 infection at day 10, 25, 35 and 50 (n=25-60 guts per condition). Significance is based on individual time comparisons (like graph A+B). two-way ANOVA was performed to investigate the interaction factor. Ecc15 infection effect: PDK-1^{GR} p=n.s.; PDK-1$^{PH/PH}$ p<0.0001; PDK-1$^{PH/null}$ p<0.01; PDK-1$^{PIF/PIF}$ p<0.0001; PKD-1$^{PIF/null}$ p=0.0001.
** = p<0.05, ** = p<0.01, *** = p<0.001, **** = p<0.0001*

When the overall effect of *Ecc15* infection per genotype was investigated, a clear response was observed in all PDK-1 mutants but not in the PDK-1^{GR} flies. Further suggesting that the low baseline levels in the PH domain and PIF pocket mutants are due to a tight regulation rather than defective and unresponsive ISCs. Taken together, we can conclude that both the AKT and the AGC branch are involved in the regulation of mitotic activity in the gut. Meanwhile, downregulation of either branch does not prevent a robust and forceful response to stress. Clearly the proliferation capacity of the ISCs is not negatively affected, if anything the response is stronger than in PDK-1^{GR}.

3.2.9 *Both the PDK-1$^{PH/null}$ and the PDK-1$^{PIF/null}$ mutant withstand intestinal infection better than PDK-1^{GR}*

Upregulation of ISC proliferation in reaction to intestinal infection, is part of a more complex response to maintain the gut barrier during stress. My previous data showed an enhanced upregulation of mitotic activity upon infection in the PDK-1 mutant females, thus the ability to survive and counteract intestinal infection was further investigated. At day 10 flies were switched from normal SYA food to vials containing paper pads with 5% sugar water contaminated *Ecc15* bacteria. The food was refreshed every other day until all flies had died. A control containing only 5% sugar water showed that Ecc15 severely reduced survival. Neither PDK-1$^{PH/PH}$ nor PDK-1$^{PIF/PIF}$ survived longer on food containing *Ecc15* bacteria (Figure 17). However, both PDK-1$^{PH/null}$ and PDK-1$^{PIF/null}$ had a significantly longer median survival compared to PDK-1^{GR}, of +7% and +9% respectively. PDK-1$^{PIF/null}$ mutant were the most long-lived on *Ecc15* bacteria, even when compared to the PDK-1$^{PH/null}$ mutants. In conclusion, both the AKT and the AGC branch improved survival with intestinal *Ecc15* infection, but only when the pathways were strongly down regulated. Furthermore, the increased response of mitotic activity in the gut previously observed, cannot explain the increase in median survival. Both homozygous PDK-1$^{PH/PH}$ and PDK-1$^{PIF/PIF}$ showed equal ability to increase pH3+ cells but showed no improved median survival with an intestinal bacterial stress assay. Other mechanisms affecting the ability to fight intestinal infection, must be involved and regulated via the AKT and the AGC branch.

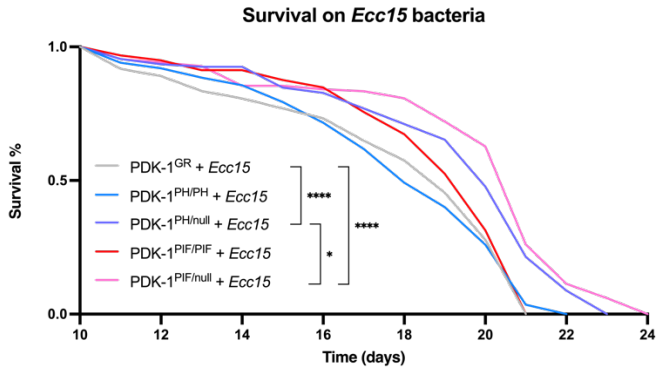

Figure 17: Both PDK-1$^{PH/null}$ and PDK-1$^{PIF/null}$ have improved median survival in intestinal bacterial stress assay. *Survival curve with a diet of 5% sugar H2O with 200 ul Ecc15 medium (Culture grown O.N. at 30°C). Sugar Ecc15 mixture was refreshed every 2 days (N=200 p.g.) PDK-1^{GR} Ecc15 vs PDK-1$^{PH/PH}$ Ecc15 p=n.s.; PDK-1^{GR} Ecc15 vs PDK-1$^{PIF/PIF}$ Ecc15 p=n.s.; PDK-1^{GR} Ecc15 vs PDK-1$^{PH/null}$ Ecc15 p<0.0001; PDK-1^{GR} Ecc15 vs PDK-1$^{PIF/null}$ Ecc15 p<0.0001; PDK-1$^{PH/null}$ Ecc15 vs PDK-1$^{PIF/null}$ Ecc15 p<0.05. log rank test.*
** = p<0.05, **** = p<0.0001*

3.2.10 Reduced signalling of PDK-1 in the AGC branch increases intestinal autophagy

Autophagy is the process of subcellular material degradation via a tightly regulated pathway involving TOR-S6K pathway and various ATG proteins. While autophagy is always active at a basal level, increased levels have been associated with longevity (Bjedov *et al.*, 2010, 2020; Chung and Chung, 2019). CHICO and rapamycin dependent longevity is dependent on autophagy, implicating both the AKT and the AGC branch in autophagy regulation essential for lifespan extension (Bjedov *et al.*, 2010).

Based on the previous results and literature, we hypothesized increased autophagy activity in the PDK-1 mutant guts. Lysotracker is a fluorescent dye that stains acidic organelles like lysosomes and autolysosomes, both indicators of autophagy induction. Interestingly, while the number of

lysotracker-positive punctae was not changed in the mid gut of PDK-1$^{PH/PH}$ and PDK-1$^{PH/null}$ female flies, we observed a significant increase in lysotracker staining in the PDK-1$^{PIF/PIF}$ and the PDK-1$^{PIF/null}$ mutants compared to PDK-1$^{GR/GR}$ (Figure 18). Based on these results we can conclude that the AGC branch but not the AKT branch regulates autophagy downstream of PDK1 in the female gut.

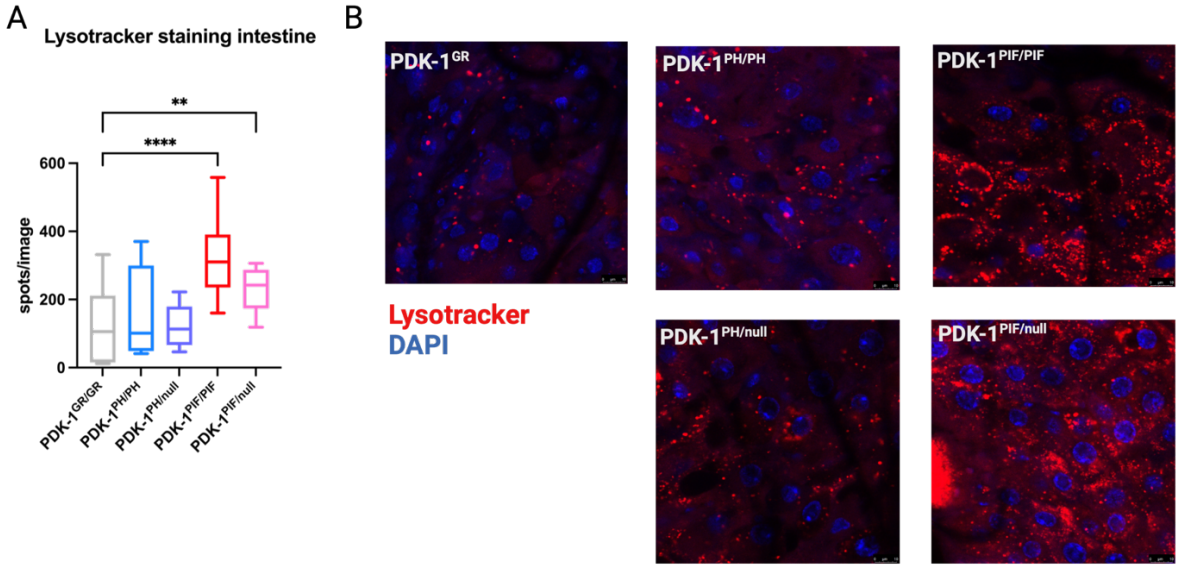

Figure 18: Lysotracker staining of female mid guts at day 10. *(N=6 p.g.) PDK-1^{GR} vs PDK-1$^{PIF/PIF}$ p=0.001' PDK-1^{GR} vs PDK-1$^{PIF/null}$ p=0.001. B: representative images from midgut of PDK-1^{GR,} PDK-1$^{PH/PH}$, PDK-1$^{PH/null}$, PDK-1$^{PIF/PIF}$ and PDK-1$^{PIF/null}$. **p<0.01,* *** = p<0.001

3.3 Investigating the PDK-1 dependent mechanism of longevity

3.3.1 Rapamycin extends lifespan of female PDK-1PH/PH mutants

To understand the underlying mechanisms of PDK-1 dependent lifespan extension, parts of the IIS/TOR network were pharmacologically targeted. This work was done in collaboration with Dr Enric Urena-Sala at the University College London. The epistasis of rapamycin and trametinib with the PDK-1^{PH} and the PDK-1^{PIF} mutants was investigated and will be discussed in relation with the other results.

Rapamycin, an FDA approved TORC1 inhibitor, is a well-known enhancer of lifespan in multiple model organisms (Harrison *et al.*, 2009; Bjedov *et al.*, 2010; Juricic *et al.*, 2022). Furthermore, various genetic early-age onset models have benefitted from rapamycin (Anisimov et al. 2010; Comas et al. 2012; Hasty et al. 2014). Treatment with rapamycin can result in a 10-20% increase in median lifespan in female wild type *Drosophila,* while it does not affect male survival (Bjedov *et al.*, 2010). Rapamycin treatment of heterozygous *chico*^{1} mutants increased longevity, suggesting rapamycin has an additive effect on upstream IIS mutants. However, neither homozygous *chico* or mNSC ablated flies benefitted from rapamycin (Bjedov *et al.*, 2010). Taken together, there seems to be a narrow therapeutic window where simultaneous mTORC1 and IIS downregulation is beneficial.

Treatment with 200 µM rapamycin extended median lifespan of PDK-1^{GR} control females by 11%. Rapamycin treatment also extended lifespan of the already long-lived PDK-1$^{PH/PH}$ mutant females with

an 6% increase in median lifespan compared to non-treated PDK-1$^{PH/PH}$ females (Figure 19A+C+D). Cox proportional hazard analysis showed that the risk of dying was significantly reduced with rapamycin treatment with a hazard ratio of 0.41 (Figure 22B). Thus, combined inhibition of AKT signalling and TORC1 inhibition led to an additional positive effect on lifespan. In contrast to PDK-1$^{PH/PH}$ mutants, there was no additive effect of rapamycin treatment on survival of PDK-1$^{PIF/PIF}$ mutant females, confirmed by the Cox proportional hazard analysis (Figure 22C). The data is consistent with the hypothesis, that the mutation of the PIF pocket specifically affects the TORC1 branch of the IIS/mTOR network.

Rapamycin treatment had no significant effect on lifespan of male PDK-1^{GR} control flies (Figure 19B-D), consistent with previous findings that rapamycin does not increase survival of male wild-type flies (Bjedov *et al.*, 2010). Rapamycin treatment also had no positive effect of the lifespan of PDK-1$^{PH/PH}$ mutant males and even slightly decreased their survival. In PDK-1$^{PIF/PIF}$ mutant males, rapamycin did not affect median survival but it induced increased mortality early in life (Figure 19B). This phenotype has been previously been observed in the male PDK-1$^{PIF/null}$ mutants and reported in IIS mutants with severe downregulation of the pathway. As with the females, the results suggest that rapamycin acts via a shared mechanism as the PIF pocket mutant, as early mortality is only observed in the impaired AGC signalling background.

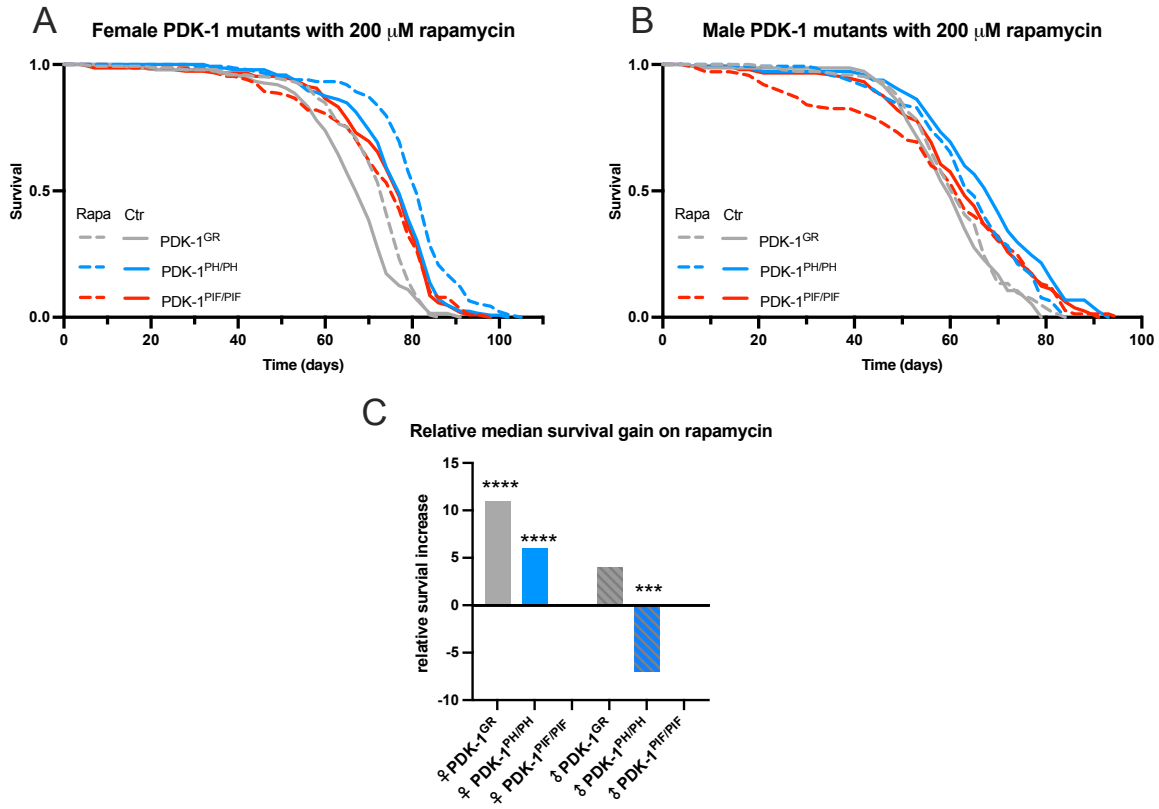

Figure 19: Rapamycin acts via a partly similar mechanism as PDK-1$^{PH/PH}$ and completely shared mechanism with PDK-1$^{PIF/PIF}$. *Lifespan assay on rapamycin treatment in both females and males (N=150 p.g.). Experiments performed by Urena (UCL).*
A + B: females and male lifespan curves, respectively. Median survival compared to PDK-1^{GR} on rapamycin treatment Females: PDK-1^{GR} vs PDK-1^{PH} median 10 % p<0.0001; PDK-1^{GR} vs PDK-1^{PIF} median 3% p<0.001. Males: PDK-1^{GR} vs PDK-1^{PH} median 4% p<0.001; PDK-1^{GR} vs PDK-1^{PIF} p=n.s. log rank test.
C: median survival gain by treatment of rapamycin. Females: PDK-1^{GR} no-rapa vs rapa median 11% p<0.0001; PDK-1$^{PH/PH}$ vs no-rapa vs rapa median 6% p<0.0001; PDK-1$^{PIF/PIF}$ no-rapa vs rapa median p=n.s. Males: PDK-1^{GR} no-rapa vs rapa median 4%

*p=n.s.; PDK-1$^{PH/PH}$ no-rapa vs rapa median -7% p<0.001; PDK-1$^{PIF/PIF}$ no-rapa vs rapa p=n.s. log rank test. * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001*

In summary, rapamycin treatment increased survival in PDK-1$^{PH/PH}$ female, while neither PDK-1$^{PIF/PIF}$ females nor males responded to rapamycin treatment. These findings are consistent with the hypothesis that the PDK-1^{PH} mutation specifically affects the AKT branch, while the PDK-1^{PIF} mutation affects network activity of the mTOR branch.

3.3.2 Trametinib extends lifespan in PDK-1 mutants

The second inhibitor used in the epistasis experiment is trametinib. Trametinib is an FDA approved small molecule inhibitor of MEK kinase, part of the RAS-MEK-ERK signalling cascade (Goitre *et al.*, 2014). Both lifelong and late life treatment with trametinib extend lifespan of wildtype female flies (Slack *et al.*, 2015). Thus, we next addressed, whether trametinib treatment can further extent lifespan of PDK-1 mutant flies. Consistent with previous findings, treatment with Trametinib extended median lifespan of female PDK-1^{GR} control flies by 14% (Figure 20A+C+D). Trametinib treatment also extended lifespan of PDk-1$^{PH/PH}$ mutants with a median lifespan increase of 9% compared to untreated mutant females. In contrast to rapamycin, trametinib also extended the lifespan of PDK-1$^{PIF/PIF}$ females with an increase in median lifespan by 6%. Thus, both PDk-1$^{PH/PH}$ and PDK-1$^{PIF/PIF}$ mutant females showed increased lifespan in response to trametinib treatment. Trametinib resulted in a reduced risk of dying of 0.75 in PDK-1$^{PH/PH}$ females and 0.38 in PDK-1$^{PIF/PIF}$ females. Suggesting that combined inhibition of the AKT and MEK; and mTOR and MEK pathways, respectively, leads to additive positive effects on lifespan, consistent with results from experiments where the drugs were combined (Castillo-Quan *et al.*, 2019). Notably is the difference in the effectiveness in both genotypes, while the PDK-1$^{PH/PH}$ mutants have an equal decrease in risk of dying as the PDK-1^{GR}, the PDK-1$^{PIF/PIF}$ mutants only gain 50% reduction.

Unlike the positive effect of trametinib on survival in females, PDK-1^{GR} control males did not show a significant lifespan extension when fed with trametinib (Figure 20B-D). Similarly, lifespan of male PDK-1$^{PH/PH}$ mutants was not extended by trametinib treatment. In contrast, median lifespan of PDK-1$^{PIF/PIF}$ mutant males was extended by 11% upon trametinib exposure. As we did not observe an increase in the PDK-1^{GR} flies, the effect of MEK inhibition was only apparent in a sensitized background of reduced AGC signalling. It is currently unclear which other pathways in the PDK-1$^{PIF/PIF}$ mutants contribute to increased lifespan from MEK inhibition.

In conclusion, the above results suggest that MEK inhibition plays a minor, if any, role in PDK-1$^{PH/PH}$ longevity as observed by the almost wild type-like response in both females and males. Interestingly, the combination of PDK-1$^{PIF/PIF}$ mutants and trametinib was the only combination of interventions that extended lifespan in both females and males.

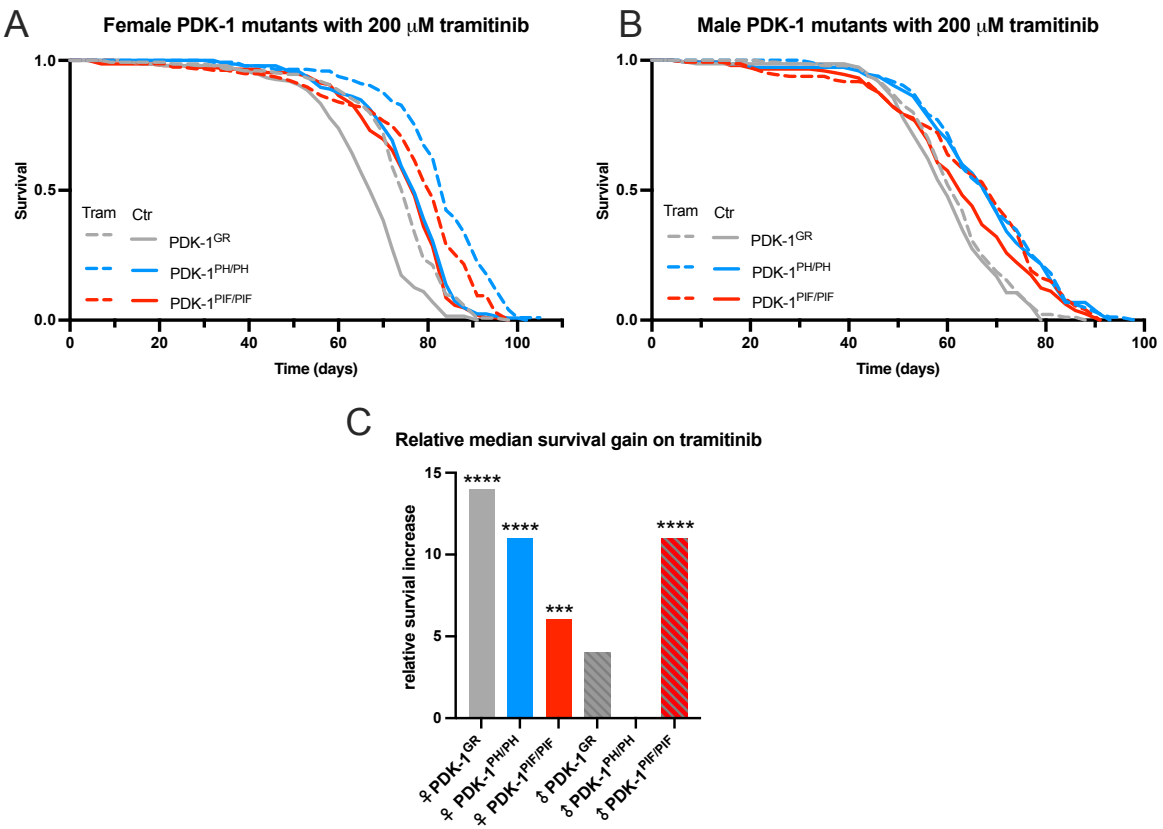

Figure 20: Trametinib acts via a partly similar mechanism as PDK-1$^{PH/PH}$ and PDK-1$^{PIF/PIF}$. *Lifespan assay on tramitinib treatment in both females and males (N=150 p.g.). Experiments performed by Urena (UCL).*
A + B: females and male lifespan curves, respectively. Median survival compared to PDK-1^{GR} on rapamycin treatment. Females: PDK-1^{GR} vs PDK-1^{PH} median 9% p<0.0001; PDK-1^{GR} vs PDK-1^{PIF} median 6% p<0.0001. Males: PDK-1^{GR} vs PDK-1^{PH} median 11% p<0.0001; PDK-1^{GR} vs PDK-1^{PIF} median 11% p<0.0001. log rank test.
D: median survival gain by treatment of rapamycin. Females: PDK-1^{GR} no-rapa vs rapa median 14% p<0.0001; PDK-1$^{PH/PH}$ no-rapa vs rapa median 11% p<0.0001; PDK-1$^{PIF/PIF}$ no-rapa vs rapa median median 6% p<0.001. Males: PDK-1^{GR} no-rapa vs rapa median 4% p=n.s.; PDK-1$^{PH/PH}$ no-rapa vs rapa median -7% p<0.001, PDK-1$^{PIF/PIF}$ no-rapa vs rapa p=n.s. log rank test.
** p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001*

3.3.3 Inhibition of AKT, MEK and mTOR results in the strongest longevity phenotype

Pharmaceutic research in various medical fields has shown that combinations of drugs are often more powerful than single treatments (Keith, Borisy and Stockwell, 2005; Zimmermann, Lehár and Keith, 2007). Not only in the clinic does this principle apply, a combination of trametinib, rapamycin and lithium has been used to extend lifespan in *Drosophila* beyond any singular drug induced improvement (Castillo-Quan *et al.*, 2019). The PDK-1 mutants were treated with a cocktail of rapamycin and trametinib to observe any additive effects.

Combined treatment with rapamycin and trametinib extended median lifespan of PDK-1^{GR} control females by 18% compared to non-treated PDK-1^{GR} females (Figure 21A+C+D). Similarly, cox proportional hazard analysis demonstrated a significantly reduced risk of dying in the PDK-1^{GR} flies when treated with rapamycin or trametinib by 0.34 and 0.74 respectively. The combination of both drugs resulted in an even greater risk reduction of 1.11 (Figure 22A). This extension is stronger than the single drug treatments, demonstrating that for PDK-1^{GR} control flies, rapamycin and trametinib act additively in lifespan extension.

Interestingly, lifespan of PDK-1$^{PH/PH}$ mutants females was also significantly extended by the double drug treatment, resulting in a median lifespan increase of 19%, similar to the extension observed in control flies. Much like the PDK-1^{GR} rapamycin treated reduced risk of death by 0.41, trametinib by 0.75 and the combination of both drugs resulted in 1.11, as calculated by the cox proportional hazard analysis (Figure 22B). This result suggests that combined inhibition of the MEK, AKT and mTOR branch of the network acts additively, consistent with the findings of combined lithium, rapamycin and trametinib treatment (Castillo-Quan *et al.*, 2019). In contrast, combined pharmacological inhibition of TORC1 and MEK did not extend the lifespan of PDK-1$^{PIF/PIF}$ female flies. While indeed the risk of death was significantly reduced by trametinib by 0.36, the combination treatment reduced the gain to 0.05, a non-significant difference in hazard ratio (Figure 22C). So while single treatment of trametinib increased median lifespan of PDK-1$^{PIF/PIF}$ mutant females, the addition of rapamycin abolished this effect, suggesting that a too strong downregulation of the TORC1 branch by the combination of genetic and pharmacological intervention, prevented potential benefits of MEK inhibition. Furthermore, the early mortality observed in the male PDK-1$^{PIF/PIF}$ flies treated with rapamycin was also observed in the females treated with the cocktail. The addition of MEK inhibition clearly diminished survival by reducing the AGC branch signalling too much. This phenotype was previously observed in male PDK-1$^{PIF/null}$ mutants and male PDK-1$^{PIF/PIF}$ mutants treated with rapamycin, although it was not as pronounced.

Treatment with the pharmacological cocktail did not benefit male PDK-1^{GR} lifespan, as did neither treatment alone (Figure 21B-D). Treatment of the PDK-1$^{PH/PH}$ mutants did reduce the lifespan as measured by the log rank testing, but did not affect the likelihood of dying as measured by the cox proportional hazard analysis (Figure 22E). Clearly, stronger downregulation of AGC branch targets in a PDK-1$^{PH/PH}$ sensitized background did not improve male longevity. Similarly, treatment with both rapamycin and trametinib reduced the median lifespan of the PDK-1$^{PIF/PIF}$ mutants significantly. The cox proportional hazard analysis demonstrated a no significantly reduced risk of dying in PDK-1$^{PIF/PIF}$ mutants when treated with a cocktail of rapamycin and trametinib, but show a very strong trend.

In summary, the above results show that combined reduction of the AKT, MEK and mTOR branch of the IIS network results in the strongest positive effect on lifespan in females, while it has detrimental effects in males. Thus, the combination of geroprotective drugs causes sex-specific effects on longevity.

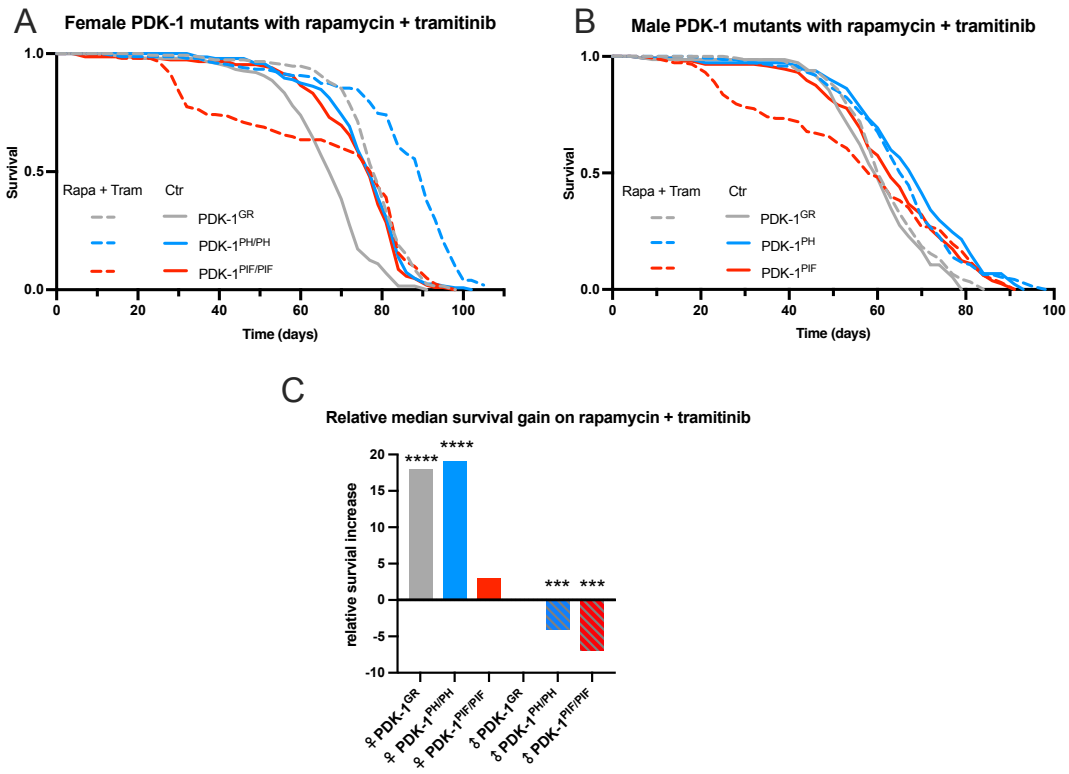

Figure 21: The combination of rapamycin and trametinib extends lifespan of PDK-1$^{PH/PH}$ but not PDK-1$^{PIF/PIF}$ females. *Lifespan assay on rapamycin and trametinib treatment in both females and males (N=150 p.g.). Experiments performed by Urena (UCL).*

A + B: females and male lifespan curves, respectively. Median survival compared to PDK-1^{GR} on rapamycin treatment. Females: PDK-1^{GR} vs PDK-1^{PH} median 15% p<0.0001; PDK-1^{GR} vs PDK-1^{PIF} p=n.s. Males: PDK-1^{GR} vs PDK-1^{PH} median 12% p<0.0001; PDK-1^{GR} vs PDK-1^{PIF} median -3% p=n.s. log rank test.

D: median survival gain by treatment of rapamycin. Females: PDK-1^{GR} no-rapa vs rapa median 18% p<0.0001; PDK-1$^{PH/PH}$ no-rapa vs rapa median 19% p<0.0001; PDK-1$^{PIF/PIF}$ no-rapa vs rapa median 3% p=n.s. Males: PDK-1^{GR} no-rapa vs rapa p=n.s.; PDK-1$^{PH/PH}$ no-rapa vs rapa median -4% p<0.001, PDK-1$^{PIF/PIF}$ no-rapa vs rapa -7% p=0.001. log rank test.

** p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001*

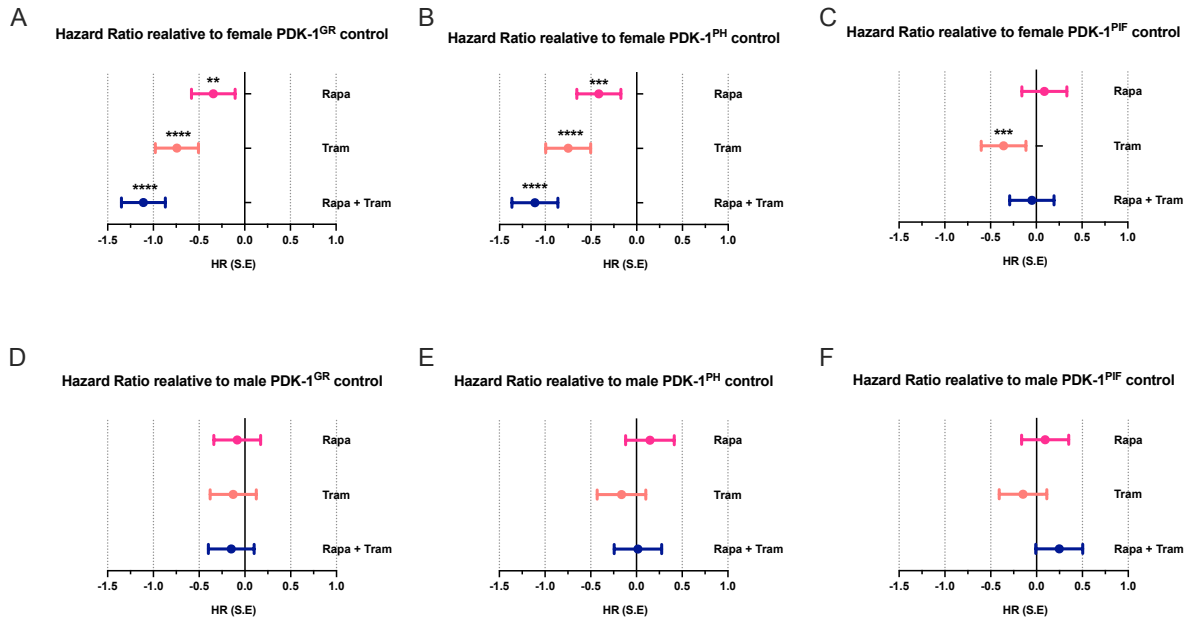

Figure 22: Cox Proportional Hazard analysis of rapamycin, trametinib or combined treatment in PDK-1 mutants. *Hazard ration present as mean ±S.E. of the lifespan experiments shown in Figure 19-22. A: female PDK-1^{GR}: rapa p<0.01; tram p<0.0001, rapa+tram p<0.0001. B: female PDK-1^{PH}: rapa p<0.001; tram p<0.0001, rapa+tram p<0.0001. C: female PDK-1^{PIF}: tram p<0.001. Analysis performed by FLASKI – lifespan analysis. ** p<0.01, *** p<0.001, **** p<0.0001*

3.3.4 *Insulin stimulates IIS in S2 cells*

While our findings were consistent with the hypothesis that the PH mutation mainly affects AKT signalling, while the PIF mutation affects the AGC branch, we currently have no molecular evidence supporting this hypothesis. Thus, it is important to characterize the phosphorylation status of PDK-1 substrates in both the PH domain and PIF pocket mutants, to address how specific the two mutations affect downstream signalling. To establish the measurements of IIS-dependent phosphorylation events, I first used cell culture cells as the system is less complex and pathway induction can be more easily controlled. During homeostasis, potential changes in phosphorylation signalling cascades are hard to detect. Therefore, IIS pathway activity was stimulated before sample collection by treating cells with insulin. To measure IIS-dependent phosphorylation changes, Western Blotting in combination with antibodies against AKT T342 and ERK Y202/204 was used. *Drosophila* Schneider 2 cells were starved for 2 hours in medium without FBS and then stimulated with insulin (5ng/µl) for 30, 60 and 120 sec (Humphrey, Azimifar and Mann, 2015).

No differences in total AKT levels were observed after 30, 60 and 120 sec of insulin stimulation (Figure 23A+E). In contrast, AKT T342 phosphorylation was upregulated and reached significance after 2 minutes of insulin stimulation (Figure 23B). Upon insulin stimulation there was no difference in the levels of total ERK (Figure 23C+F). A significant increase in Y202/204 phosphorylation in Erk was detected at 2 min (Figure 23D). Thus, activation of either the AKT or ERK branch by insulin can be detected with western blotting in cell culture as early as 2 min after stimulation. However, it must be noted that changes must be strong and robust. Both phospho antibodies resulted in higher variance and thus decreased accuracy to detect small biological changes.

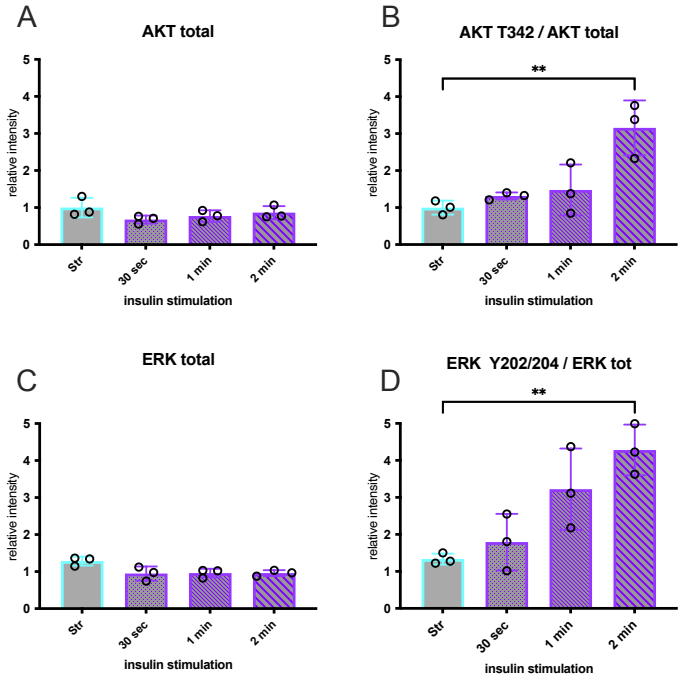

Figure 23: S2 cells stimulated with insulin respond after 2 minutes. *Samples are stimulated with insulin or PBS after 2 hour starvation and then snap lysed and frozen. A+B: immunoblot of AKT and AKT^{T342} of S2 cells (N=3). AKT T342 / AKT total: starvation vs 2 min p<0.01. One-way ANOVA. C+D: immunoblot of AKT and AKT^{T342} of S2 cells (N=3). AKT T342 / AKT total: starvation vs 2 min p<0.01. One-way ANOVA. ** p<0.01*

Unfortunately, antibody availability and specificity are rather limited for *Drosophila* proteins. We only identified one antibody targeting a PDK-1 specific phosphorylation site, namely AKT T342. Despite these limitations, we explored next whether these antibodies also function *in vivo* in flies.

3.3.5 *Ex vivo insulin stimulation of head and thorax did not result in phosphorylation differences*

To measure IIS dependent changes in protein phosphorylation in vivo, we first tried to stimulate the IIS/TOR network before tissue collection by *ex* vivo insulin stimulation of dissected tissues (5ng/µl). Both the heads and the fat body were dissected and stimulated with insulin *ex vivo* and protein extracts were prepared for Western blot analysis. As expected, no difference in total AKT levels was observed in PDK-1^{GR} heads (Figure 24A). However, no significant upregulation of AKT phosphorylation at site T342 was observed in PDK-1^{GR} despite insulin stimulation (Figure 24B). Furthermore, no significant differences were detected in either total AKT or T342 phosphorylation in PDK-1$^{PH/PH}$ nor PDK-1$^{PIF/PIF}$ mutant tissues (Figure 24A-D). There was a slight trend in the fat body with insulin stimulation but it was not significant. Whereas insulin stimulation in cell culture was a robust way of stimulating the IIS/TOR network, this was not easily transferable to the in vivo situation. Additionally, dissection and *ex vivo* insulin stimulation were time consuming and introduced high variance. I therefore decided to stimulate the IIS/TOR network in a more natural way by applying a starvation refeeding regime.

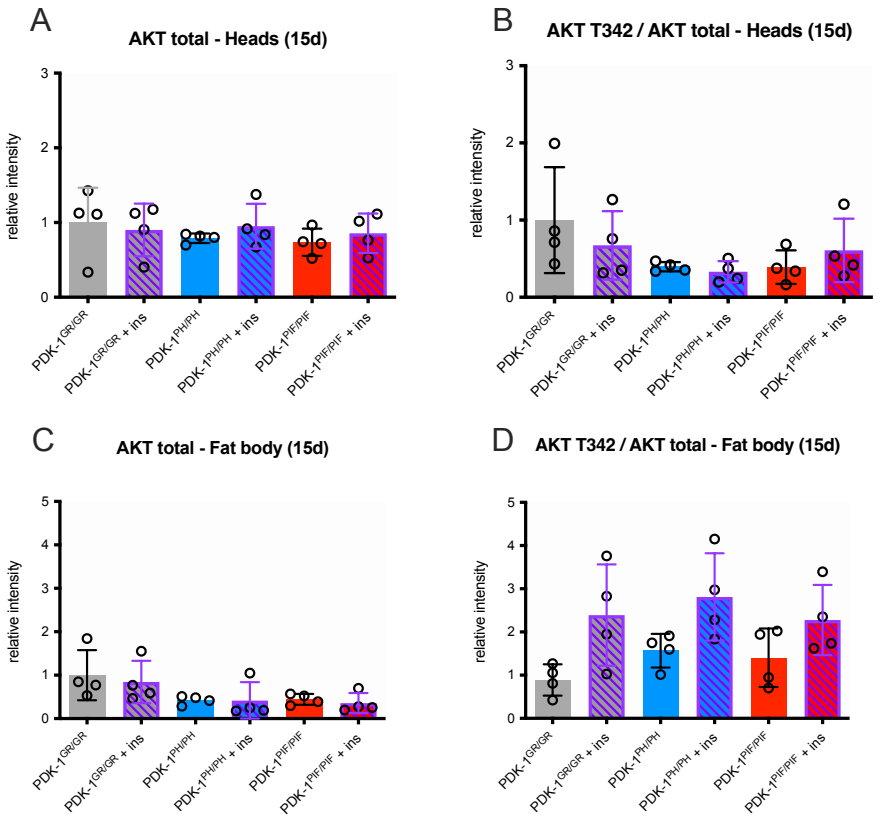

Figure 24: Heads and thorax do not respond to* ex vivo *insulin stimulation. *Flies are dissected in PBS, tissues are broken open using the tweezers and then stimulated with insulin or PBS control* ex vivo *for 5 min. tissues are then snap frozen in liquid nitrogen. A+B: immunoblot of AKT and AKT^{T342} in fly heads at day 15 (N=3 p.g. 25 heads per replicate). One-way ANOVA. C+D: immunoblot of AKT and AKT^{T342} in fly thorax at day 15 (N=3 p.g. 20 thorax per replicate). One-way ANOVA.*

3.3.6 Starvation/refeeding stimulates the IIS in Drosophila

A regime of starvation and refeeding to stimulate the IIS/TOR network *Drosophila* was designed as follows: Continuously fed females acted as control (FED in all graphs), while 24-hour starved females should show reduced IIS signalling, and give an indication of the low baseline (Str in all graphs). Lastly, a refeeding group was established. After an initial 24h starvation period, flies were allowed to feed for a predefined time period, henceforth referred to as "refeeding" (X min in all graphs). This last condition should acutely activate IIS/TOR network activity and give insight into the differences in the signalling cascades between PDK-1 mutants. To determine optimal refeeding time, feeding behaviour on starvation food and during refeeding was investigated. There was a clear reduction after 5h of starvation in feeding. After 24h of starvation, hardly any feeding behaviour was observed (Figure 25A). Thus, flies recognized the absence of food and did not eat. When starved flies were re-introduced to SYA food, this must be taken into account. Additionally, tipping flies from vial-to-vial results in stress and usually no feeding directly after tipping. Indeed, when tipped from starvation food to a vial containing SYA food hardly any feeding was observed during the first 5 minutes in either condition (Figure 25B). We observed a clear peak in feeding of the starved flies at 10 minutes compared to non-starved flies. In both conditions time has a significant effect on feeding behaviour. While the difference was not always prominent, there was a statistically significant effect of history of feeding on the observed feeding behaviour, generally there is heightened feeding in the previously starved flies. Based on these results, we decided to measure IIS/TOR network activity from 10 minutes onwards. Using western blotting to visualize IIS signalling, AKT T342 phosphorylation was significantly upregulated after 30 min refeeding, while total AKT levels remained unchanged in wild type female flies (Figure 25C+D). Taken together, a regime of starvation and refeeding stimulated the IIS/TOR network *in vivo* in a detectable way. Therefore, we continued to test our PDK-1 mutants.

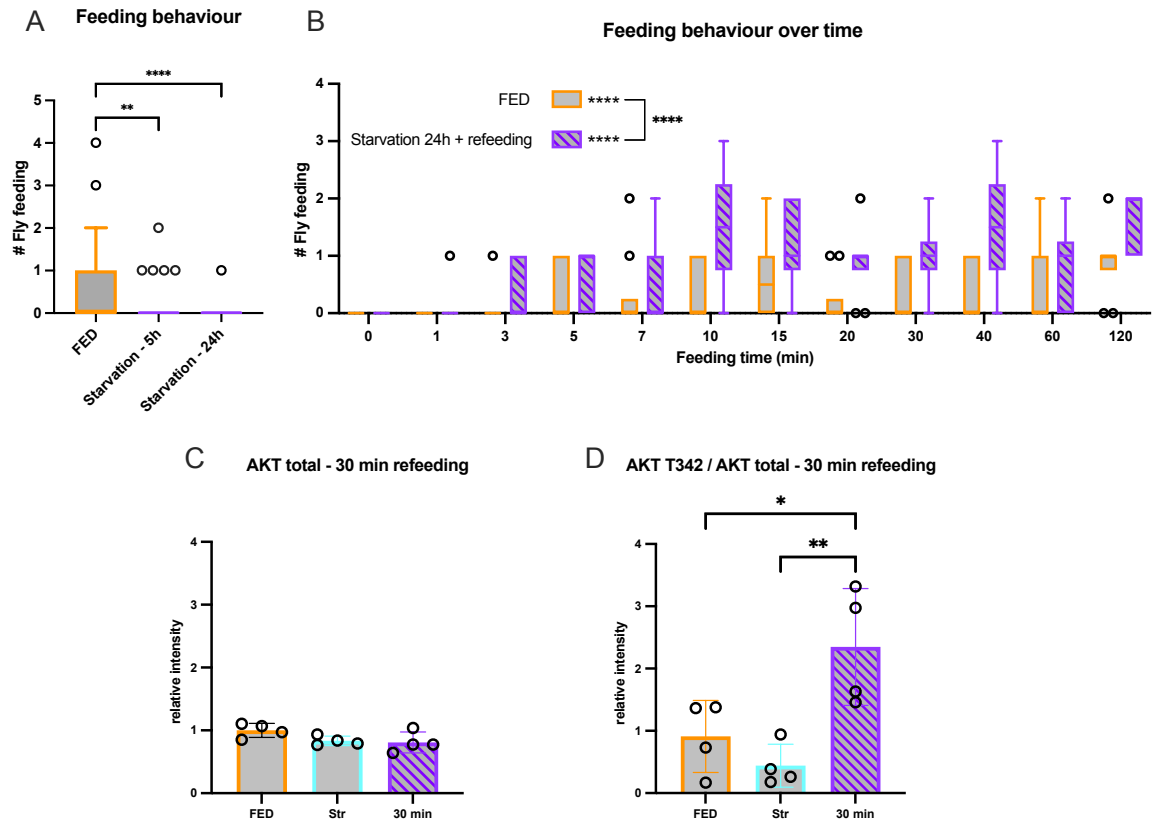

Figure 25: Feeding behaviour of wild type flies depends on the food source and food source history.
A: The number of flies feeding in a time frame of 10 seconds was counted on SYA food (FED) or starvation food (Starvation 5h/Starvation 24h) (N=8 p.g. 5 flies per replicate). Fed vs starvation 5h p<0.01; fed vs starvation 24h p<0.0001. One way ANOVA.
B: Flies are either kept on SYA food (FED) or starved for 24h. After 24h both conditions are tipped to new vials containing SYA food. The number of flies feeding in a time frame of 10 seconds was counted during a time period of 1 minutes till 120 minutes after tipping onto fresh food. (N=8 p.g. 5 flies per replicate). Factor of time in each condition: fed p<0.0001; starvation + refeeding p<0.0001. genotype specific response: p<0.0001. Two-way ANOVA.
*C+D: Flies are kept on SYA food (FED), starved for 24h (Str) or starved for 24h and then refed with SYA food for 30 min (30 min) and then snap frozen in liquid nitrogen. Body parts are separated on dry ice. immunoblot of AKT and AKT^{T342} in fly heads at day 10 (N=4 p.g. 25 heads per replicate). AKT T342 / AKT total: fed vs 30 min p<0.05; starvation vs 30 min p<0.01. One-way ANOVA. * p<0.05, ** p<0.01*

3.3.7 Starvation/refeeding increases AKT^{T342} phosphorylation in both PDK-1^{PH} and PDK-1^{PIF} mutants

I next used the starvation-refeeding regime established above to measure AKT^{T342} phosphorylation in heads and thoraxes of PDK-1 mutants females. Total AKT levels were decreased in PDK-1$^{GR/GR}$ control heads and in the PKD-1$^{PH/PH}$ and the PDK-1$^{PIF/PIF}$ mutants (Figure 26A). Upon refeeding there was a trend for an increase in AKT^{T342}/total AKT levels in PDK-1^{GR} control flies (Figure 26B). Surprisingly, however, AKT T342 phosphorylation relative to total AKT levels was also significantly upregulated in PKD-1$^{PH/PH}$ and the PDK-1$^{PIF/PIF}$ mutants heads upon refeeding (Figure 26B). There was no significant regulation of AKT^{T342} in the thorax as far as we could detect by western blots (Figure 26C+D).

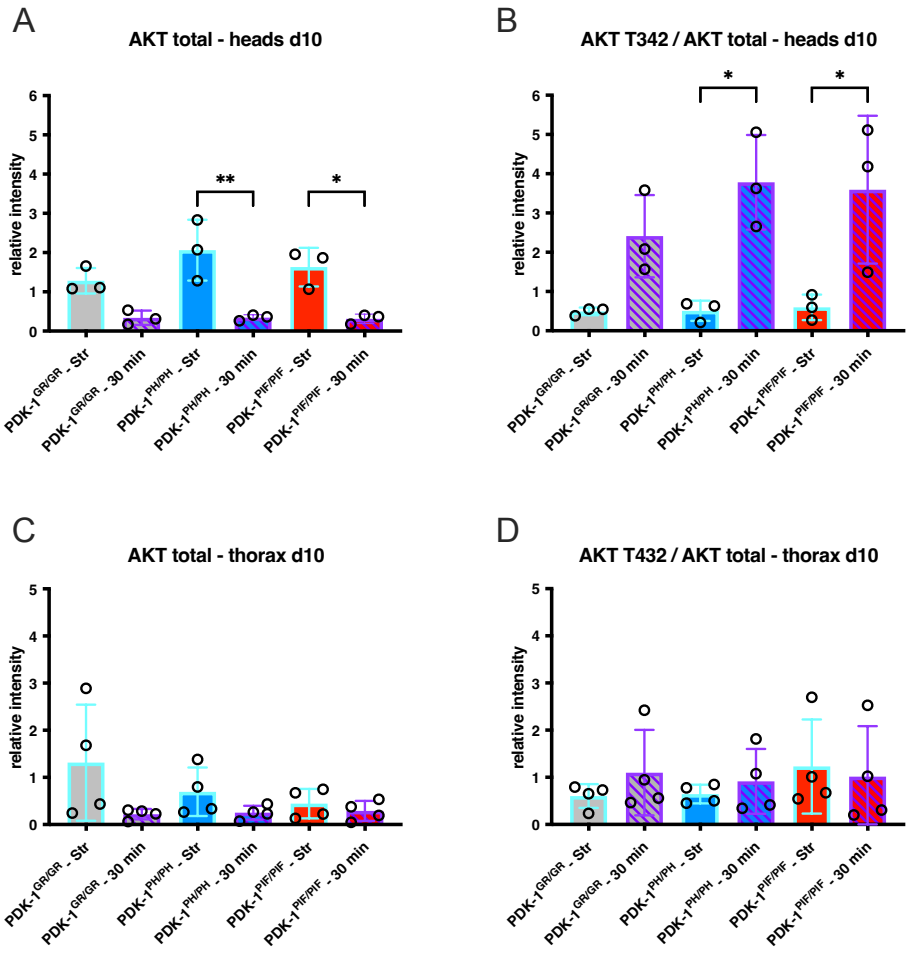

Figure 26: Refeeding resulted in increased AKT^{342} phosphorylation in heads of PDK-1$^{PH/PH}$ and PDK-1$^{PIF/PIF}$. *Flies are kept on SYA food (FED), starved for 24h (Str) or starved for 24h and then refed with SYA food for 30 min (30 min). The flies are then snap frozen in liquid nitrogen. Body parts are separated on dry ice.*
A+B: immunoblot of AKT and AKT^{T342} in fly heads at day 10 (N=3 p.g. 25 heads per replicate). AKT total: PDK-1$^{PH/PH}$ str vs PDK-1$^{PH/PH}$ 30 min p<0.01; PDK-1$^{PIF/PIF}$ str vs PDK-1$^{PIF/PIF}$ 30 min p<0.05. AKT T342 / AKT total: PDK-1$^{PH/PH}$ str vs PDK-1$^{PH/PH}$ 30 min p<0.05; PDK-1$^{PIF/PIF}$ str vs PDK-1$^{PIF/PIF}$ 30 min p<0.05. One-way ANOVA. C+D: immunoblot of AKT and AKT^{T342} in fly thorax at day 10 (N=3-4 p.g. 20 thorax per replicate). One-way ANOVA
** p<0.05, ** p<0.01*

The significant drop in total AKT levels upon 30 minutes refeeding is hard to explain and was not observed in pilot experiments. Additionally, theory predicted a decrease in AKT T342 phosphorylation in PDK-1$^{PH/PH}$ due to the defective PH domain. The current results are therefore hard to interpret. We can however presume that the head is more responsive to refeeding stimulation than the thorax. Unfortunately, we were limited in available antibodies and thus in our ability to understand the phosphorylation changes in our mutants. Consequently, we decided to analyse IIS/TOR network changes upon starvation/refeeding using phosphoproteomics.

3.3.8 Proof of principle: phosphorylation changes with refeeding

Based on the previous refeeding experiments we isolated peptides from whole control flies at day 10 at various different time point after refeeding. The clean peptides mixtures were labelled with TMT labels, phospho-enriched and injected into the mass spectrometer. Like the experiments above, the flies were either continuously fed, starved overnight without refeeding or starved overnight and refed.

As we saw increase in AKT ^{T342} phosphorylation at around 30 min, we focussed our time span around this point. Furthermore, we wanted to see if after 24 hours of refeeding, the starvation effect was gone. Therefore, flies were collected after 5 min, 11 min, 22 min, 30 min, 45 min, 90 min, 2 hours, 4 hours, 6 hours, 8 hours, 11 hours and 24 hours of refeeding. As this was only a pilot study, one sample was used per time point.

While ±5900 phosphorylation sites were detected, we were mostly interested if sites associated with insulin signalling could be detected. Based on the Flybase network of IIS, phosphorylation changes were plotted over time. The base line was the starvation condition and the last time point was fed. While the individual phosphorylation points were hard to interpret, phosphorylation changes were detected. Upon refeeding, phosphorylation of CHICO was clearly altered from 30 minutes onwards (Figure 27A,C+D). However, we could also observe changes as early as 5 minutes after feeding (Figure 27E). Additionally, we saw clear similarities between some phosphorylation sites, phosphorylation of CHICO^{532} and CHICO^{534} were clearly linked. While we observed a strong regulatory phosphorylation change in CHICO, no difference in PDK-1 phosphorylation was observed (Figure 27F+G). This was not unexpected as PDK-1 is mostly dependent on autophosphorylation, as discussed in the introduction.

As we were interested in observing effects of the PDK-1^{PH} and PDK-1^{PIF}mutants it was important to observe components from the AKT and the AGC branch. Indeed, we saw a clear decrease in FOXO^{44} phosphorylation early after refeeding, it returned to the fed phosphorylation level after 90 minutes (Figure 27J). It seemed to be a strong but transitory response to refeeding. Another component of the AKT branch signalling, is SGG, the fly homology of GSK-3. While phosphorylation of site 213 decreased from the moment of refeeding, the phosphorylation of site 9 shot up (Figure 27K+L). Understanding the interplay between phosphorylation sites is hard when limited information is available, but that there was regulation of SGG upon refeeding is undisputable. Furthermore, S6K and TSC-1, both part of the AGC signalling branch, showed increased phosphorylation at site 418 and 449 respectively (Figure 27M+N). None of the changes were significant due to the nature of the experiment, with only one replicate per time point.

Overall, components of the insulin signalling pathway were detected and we observed regulation upon refeeding. We therefore proceeded with the experiment including the PDK1 mutants.

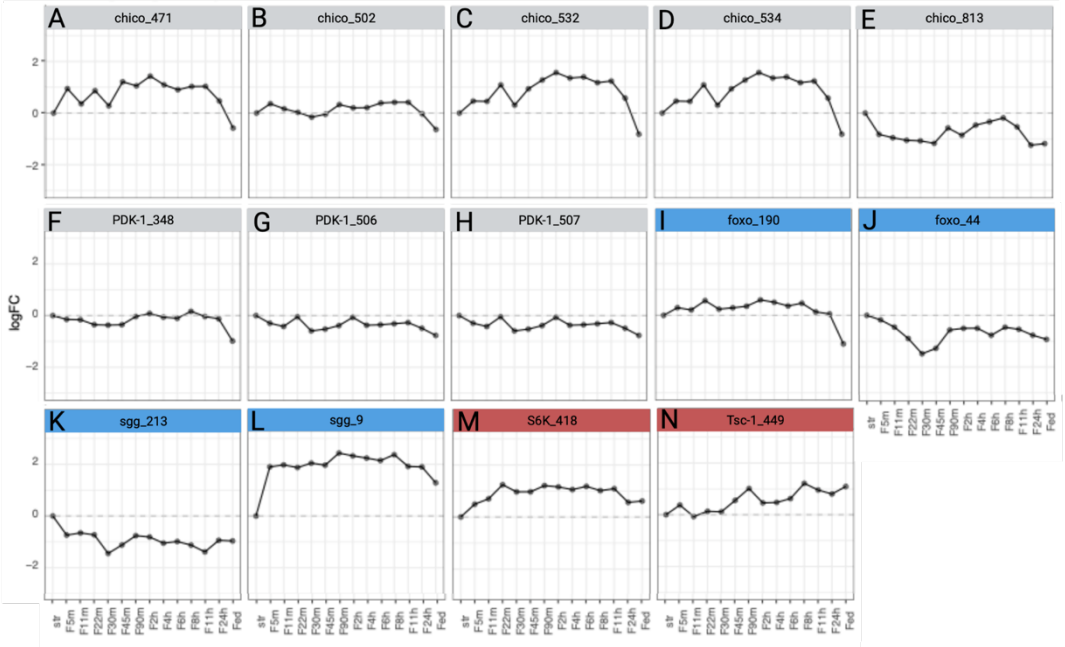

Figure 27: TMT phosphoproteomics can pick up AKT and AGC branch signalling components. *A-N: Flies are kept on SYA food (FED), starved for 24h (Str) or starved for 24h and then refed with SYA food for defined time (5 min, 11 min, 22 min, 30 min, 45 min, 90 min, 2h, 4h, 6h, 8h, 11h and 24h (N=1). The flies are then snap frozen in liquid nitrogen. The Log fold phosphorylation change compared to starvation time point of chico^{471}, chico^{502}, chico^{532}, chico^{534}, chico^{813}, PDK-1^{348}, PDK-1^{506}, PDK-1^{507}, foxo^{190}, foxo^{44}, sgg^{213}, sgg^{9}, S6K^{418} and tsc-1^{449} are depicted. The components predicted to be regulated by the AKT branch are blue and the components regulated by the AGC branch are red.*

3.3.9 Refeeding results in alternative phosphorylation in the PDK-1$^{PH/PH}$ and the PDK-1$^{PIF/PIF}$ mutant

Having proof of principle and indication of the peak time of phosphorylation regulation, we chose 3 time points to investigate in the PDK-1 mutants. Additionally, insulin signalling is tissue specific and we therefore choose to investigate the abdomen. This includes the fat body and gut, a tissue where we saw a clear difference between the PDK-1^{PH} and PDK-1^{PIF} mutants. After starvation the flies were allowed to refeed for 10 min, 20 min or 60 minutes. Additionally, starved and fed flies were included as controls.

We investigated the phosphorylation changes specifically in the IIS/TOR network. While many components were detected, only a few showed regulation. Quite some components were differently phosphorylated in both the PDK-1^{PH} and PDK-1^{PIF} mutants compared to PDK-1^{GR} starvation baseline. We saw a significant change in CHICO^{502}, TSC-1^{449}, RICTOR^{1904} and DSOR-1^{385} in the fed condition (Figure 28A-K). The fold changes were small and often went in a similar direction as the PDK-1^{GR} mutants. The ability to detect significant differences based on such small changes was due to the depth and low variance of the data set. Taken together, we can conclude that both the PDK-1^{PH} and the PDK-1^{PIF}mutant had a slightly elevated phosphorylation upstream in the pathway, around TOR complex regulation and in the Ras-MEK-ERK pathway during fed conditions.

We detected some differences between the PDK-1$^{PH/PH}$ and the PDK-1$^{PIF/PIF}$ mutants. There was a clear and strong upregulation of the phosphorylation of SGG^{9} in PDK-1^{PH} mutants compared to PDK-1^{GR} (Figure 28). Although not significant during the 10, 20 and 60 min of refeeding, the trend seemed to

divert already at 10 minutes from the control. Divertingly, a similar pattern in the SGG^{214} phosphorylation site was not observed (Figure 28F).

Another striking difference between the PDK-$1^{PH/PH}$ and the PDK-$1^{PIF/PIF}$ mutants was the regulation of Lipin (LPIN) in the PDK-$1^{PH/PH}$ mutants (Figure 28G-I). Insulin stimulates the hyperphosphorylation of LPIN (17 sites in mammalian lipin), resulting in inhibitory cytosolic localization (Kok *et al.*, 2012). Two out the three phosphorylation sites in Lpin were increased in PDK-$1^{PH/PH}$ fed conditions, suggesting there was a stronger downregulation in Lpin activity compared to the PDK-$1^{PIF/PIF}$ and PDK-1^{GR} (Figure 28G-I).

Not only the PDK-$1^{PH/PH}$ mutants had specific phosphorylation changes, significant and unique regulation was also observed in the PDK-$1^{PIF/PIF}$ mutants. Firstly, we detected a unique up-regulation of PKC^{522} phosphorylation in the PDK-$1^{PIF/PIF}$ mutants (Figure 28J). Secondly, we saw an absence of regulation in ERK^{198} phosphorylation in the fed conditions in PDK-$1^{PIF/PIF}$ mutants (Figure 28K). in both the wild type like control and in the PDK-1^{PH} mutants we detected a strong down regulation in the fed condition compared to starvation. However, phosphorylation level remained constant in PDK-$1^{PIF/PIF}$ mutants. Clearly, the dephosphorylation process is hampered in the PDK-$1^{PIF/PIF}$ mutants, resulting in a more constantly phosphorylated ERK, even during fed conditions.

Taken all together, we saw differences in phosphorylation between the PH domain and PIF pocket mutants, implying different signalling cascades are regulated. However, most of the phosphorylation changes observed were small and hard to interpret.
During a broader analysis of the phosphoproteomics data set many processes involved in oocyte development were detected. The majority of the regulated phosphoproteome associated with GO term of a developmental or reproductive nature. To draw biological meaningful conclusions, this experiment must be repeated with either the whole fly or with heads only.

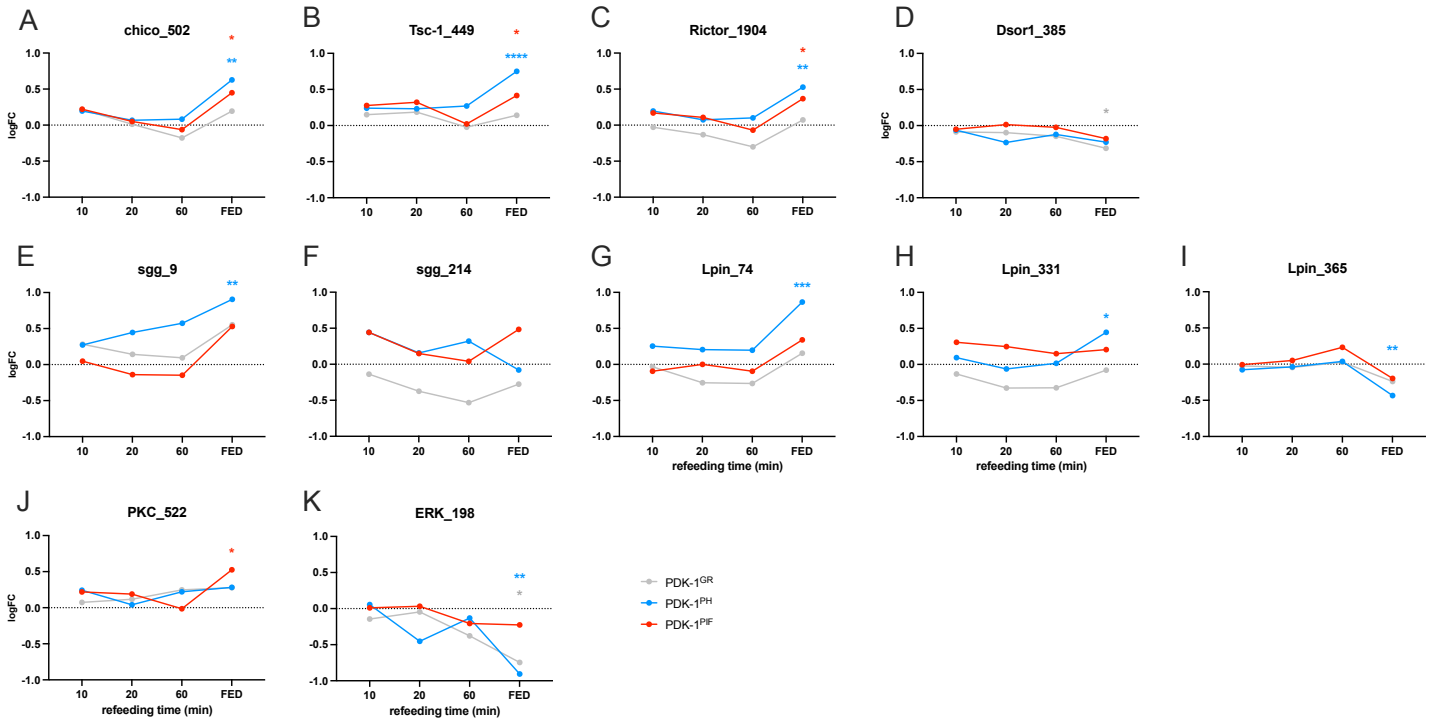

Figure 28: phosphoproteomics show that both the PDK-1$^{PH/PH}$ and the PDK-1$^{PIF/PIF}$ mutants have alternative phosphorylation intensity in various components of the IIS/TOR network. *A-K: Flies are kept on SYA food (FED), starved for 24h or starved for 24h and then refed with SYA food for defined time (10 min, 20 min and 60 min). Flies are snap frozen in liquid nitrogen and body parts are separated on dry ice. (N=4 p.g. 80 abdomen per replica). The Log fold phosphorylation change compared to starvation time point of the corresponding genotype is plotted (adjusted P value below).*
A: chico^{502}: PDK-1$^{PH/PH}$ str vs PDK-1$^{PH/PH}$ fed p<0.01 ; PDK-1$^{PIF/PIF}$ str vs PDK-1$^{PIF/PIF}$ fed p<0.05
B: tsc-1^{449} : PDK-1$^{PH/PH}$ str vs PDK-1$^{PH/PH}$ fed p<0.05 ; PDK-1$^{PIF/PIF}$ str vs PDK-1$^{PIF/PIF}$ fed p<0.05
C: rictor^{1904} : PDK-1$^{PH/PH}$ str vs PDK-1$^{PH/PH}$ fed p<0.01 ; PDK-1$^{PIF/PIF}$ str vs PDK-1$^{PIF/PIF}$ fed p<0.05
D: dsor-1^{385} : PDK-1^{GR} str vs PDK-1^{GR} fed p<0.05
sgg^{9} : PDK-1$^{PH/PH}$ str vs PDK-1$^{PH/PH}$ fed p< 0.01
lpin^{74}: PDK-1$^{PH/PH}$ str vs PDK-1$^{PH/PH}$ fed p<0.001
lpin^{31}: PDK-1$^{PH/PH}$ str vs PDK-1$^{PH/PH}$ fed p< 0.05
lpin^{365}: PDK-1$^{PH/PH}$ str vs PDK-1$^{PH/PH}$ fed p<0.01
PKC^{522} : PDK-1$^{PIF/PIF}$ str vs PDK-1$^{PIF/PIF}$ fed p<0.05
ERK^{198}: PDK-1^{GR} str vs PDK-1^{GR} fed p<0.05; PDK-1$^{PH/PH}$ str vs PDK-1$^{PH/PH}$ fed p<0.01.

3.4 Humanization of PDK-1 in *Drosophila*

3.4.1 *2 types of mammalian PKD-1 inhibitors: GSK233 and MP7*

While genetic engineering is a suitable method to characterize the role of PDK-1 in longevity, it is not transferable to the human population. Thus far, genetic manipulation has been approved for clinical use only 21 times by the FDA (Shahryari *et al.*, 2019; FDA, 2022a). Most of these treatments are for terminal stage cancer or incurable diseases. It is thus more realistic to think about pharmaceutical interventions to treat ageing and age-related phenotypes. As discussed in the introduction, PDK-1 is a highly druggable target. Both academic research and the pharmaceutical industry have studied PDK-

1 inhibitors intensively in relation to cancer. Thus far no pharmaceuticals have reached the clinic, mostly due to side effects or in-effectivity (Falasca *et al.*, 2010; Emmanouilidi and Falasca, 2017).

Significant health- and lifespan improvement in both the PDK-1^{PH} and PDK-1^{PIF} mutants have been observed. As proof of principle, is it possible to use *Drosophila* to investigate the possibility to repurpose mammalian cancer pharmaceutics to treat ageing. Using *Drosophila* to investigate new cytotoxic drug cocktails or personalize treatment has been done before (Sonoshita and Cagan, 2017).

The most promising PDK-1 inhibitors based on their inhibitor type and binding affinity have been selected. GSK2334470 (GSK233) is a specific type I PDK-1 inhibitor, meaning it targets the ATP binding pocket of PDK-1 (Figure 29) (Najafov *et al.*, 2011b). Type 1 inhibitors are targeting one of the most conserved domains of kinome, and specificity is therefore hard to achieve. GSK233 inhibits PDK-1 AKT activation with an IC_{50} value of 15 nM, concentrations a 100 times higher than the IC_{50} did not affect 95 other human kinases. Among those are 13 highly similar AGC-family kinases, indicating that GSK233 has specificity towards PDK-1 (Najafov *et al.*, 2011b). GSK233 inhibits SGK and S6K T-loop phosphorylation even upon IGF1 stimulation at a concentration of 30nM and 0.3µM respectively in mammalian HEK-293 cells. Not only is T-loop phosphorylation inhibited, substrate kinase activity of SGK is abolished at concentrations 0.1 µM, underlining the crucial role of PDK-1 on activation of the AGC branch kinases (Najafov *et al.*, 2011b).

Relatively high concentrations are needed to reduce PDK-1 dependent AKT T-loop phosphorylation on Thr308 (1-3µM) as PH domain association with PIP_3 is very powerful. In the presence of IGF stimulation, GSK233 could not inhibit AKT phosphorylation completely. However, when the PH domain was absent or partially functional, GSK233 could reduce phosphorylation levels successfully. Taken together, the close proximity of PDK-1 and AKT due to their respective PH domains increases the inhibition threshold. Additionally, a clear reduction of AsPC-1 cell survival was observed with concentrations of 2µM, indicating that GSK233 has high potency in cells (Emmanouilidi and Falasca, 2017). Overall, GSK seems to be a potent and specific PDK-1 inhibitor targeting the ATP domain. GSK treatment should mostly downregulate general PDK-1 activity, but allow for AKT branch signalling upon insulin treatment.

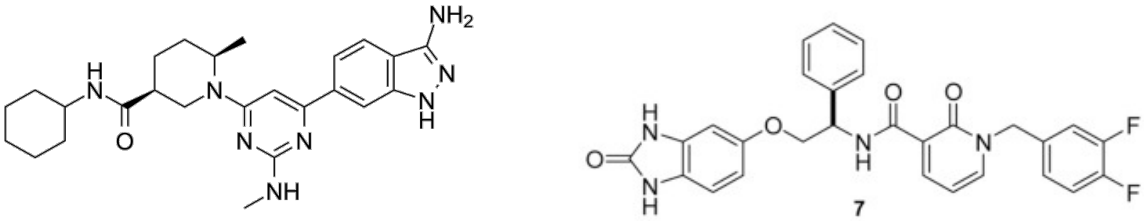

Figure 29: molecular structure of GSK233 (left) and MP7 (right)

In contrast to GSK233, MP7 is a type II inhibitor targeting the DFG-out loop formation (Figure 29) (Erlanson *et al.*, 2011). Unlike the ATP binding site, the DFG-out conformation is highly kinase specific. The DFG-out conformation induces deactivating shift in various key loops, like the T and A loop, both crucial for phosphorylation of a substrate. Additionally, a hydrophobic moiety of MP7 occupies a unique cleft, only present in PDK-1 when in the DFG-out conformation. It is the first Type II inhibitor

targeting an AGC kinase. The IC_{50} value of MP7 is 2nM, concentrations 3000 higher did not result in similar inhibition of kinase activity in the 256 kinases tested (Erlanson *et al.*, 2011; Nagashima *et al.*, 2011). Both numbers show how incredibly specific and potent MP7 is. While this high specificity makes it a very power human drug, it might limit the applicability in *Drosophila.* Not only is MP7 highly PDK-1 specific, it inhibits PDK-1 dependent phosphorylation effectively. MP7 reduces phosphorylation of both AKT and S6K at concentrations of 1 µM in PC-3 cells. Not only does MP7 affect phosphorylation of PKD-1 substrates, it also affects autophosphorylation of Ser241. Autophosphorylation of Ser241 is essential for activation of PDK-1. Based on our current knowledge, the DFG-out conformation will inhibit dimerization via the PIF pocket and thus *trans*-autophosphorylation (Levina *et al.*, 2022). This effect was time dependent, after 48h a 70% reduction in autophosphorylation at Ser-241 was observed (Nagashima *et al.*, 2011). Similarly to GSK, there is a clear reduction of AsPC-1 cell survival with concentrations of 2 µM (Emmanouilidi *et al.*, 2019). Taken all together, MP7 is one of the most powerful PDK-1 inhibitors currently available.

As discussed in the introduction, mammalian and *Drosophila* PDK-1 share many domains and have great similarity. Not surprisingly, the ATP binding site shares 60% percentage of residues. Therefore, it is hypothesized that Type I inhibitors like GSK233 might also inhibit the *Drosophila* PDK-1 protein. Since the type II inhibitor MP7 requires an interaction with a unique pocket present only in the DFG-out conformation, it might not show high affinity towards the *Drosophila* PDK-1 protein.

3.4.2 Mammalian inhibitors do not affect Drosophila PDK-1 in S2+ cells

To determine if the two mammalian PDK-1 inhibitors GSK233 and MP7 can inhibit *Drosophila* PDK-1 an XXT assay was performed. GSK or MP7 at concentrations as low as 2µM reduced viability in mammalian cancer cell lines (Emmanouilidi *et al.*, 2019). As we expected, reduced affinity of these drugs towards the *Drosophila* PDK-1 protein, we treated *Drosophila* S2 cells with 25µM and 500µM of GSK, and 125µM and 500µM of MP7. However, neither treatment with GSK nor MP7 did affect cell viability in the XTT assay (Figure 30A-B). As a positive control we treated cells with lithium, which reduced viability at a dose of 0.1M and was fully lethal at 2.5M (Figure 30A-B). Thus, generally the $S2^{+}$ cells were responsive towards drug treatment, but the two mammalian PDK-1 inhibitors did not cause the expected effect, which indicated that they don´t inhibit the *Drosophila* PDK-1 protein.

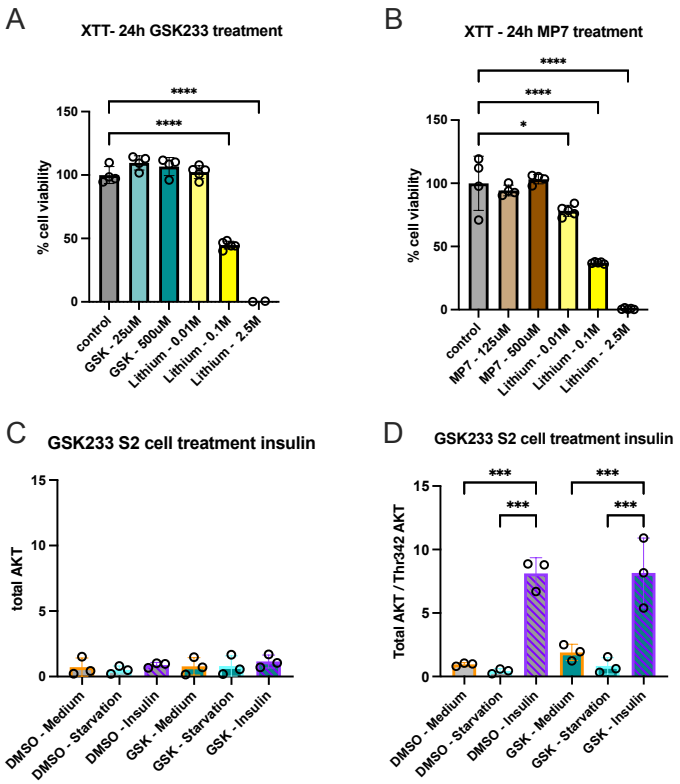

Figure 30: Neither GSK233 nor MP7 affected survival or phosphorylation of AKT in S2⁺ cells. *Comparison of viability of S2⁺ cells with the XTT assay after 24h GSK233 or MP7 treatment. As control lithium treatment was used.*
A: control vs lithium 0.1M p<0.0001; control vs lithium 2.5 M p<0.0001. one-way ANOVA.
B: control vs lithium 0.01M p<0.05; control vs lithium 0.1M p<0.0001; control vs lithium 2.5 M p<0.0001. one-way ANOVA.
S2⁺ cells are treated with DMSO control or with GSK233 250 µM. Samples are either frozen after normal culture conditions (medium), stimulated wih PBS after 2-hour starvation (starvation) or stimulated with insulin for 10 min after 2 hour starvation (insulin). C+D: immunoblot of AKT and AKT^{T342} of S2 cells (N=3). AKT T342 / AKT total: DMSO medium vs DMSO insulin p<0.001; DMSO starvation vs DMSO insulin p<0.001. GSK233 medium vs GSK233 insulin p<0.001; GSK233 starvation vs GSK233 insulin p<0.001. One-way ANOVA.
** p<0.05, *** p<0.001, **** p<0.0001*

In order to test this further, a western blot to measure phosphorylation of the direct PDK-1 downstream target Akt was performed. While GSK233 is binding the relatively conserved ATP binding pocket, MP7 is binding to various outside loops and a pocket uniquely present in the DFG-out conformation of the human PDK-1 protein. Thus, GSK233 is more likely to show sufficient affinity, hence potency, towards the *Drosophila* PDK-1 protein. Therefore S2^{+} cells were treated with 250 µM GSK233 and subsequently stimulated with insulin (5ng/µl) for 10 min. In cells treated with DMSO control a significant elevation of p-AKT^{Thr342} was observed (Figure 30C-D), consistent with the predicted increase in IIS pathway activity. Unexpectantly, cells treated for 24h with 250µM GSK233 showed an equal upregulation of p-AKT^{342} upon insulin stimulation (Figure 30C-D). Thus, despite using an about 800-fold higher concentration than the inhibitory concentration in HEK cells, GSK233 did not block phosphorylation of AKT, suggesting that it is not efficient in inhibiting PDK-1 activity in *Drosophila*. In summary, despite the high similarity between the human and *Drosophila* PDK-1 protein structure neither inhibitor showed affinity or efficacy in S2^{+} cells. Consistently, previous still unpublished work from our lab has shown that a precursor of GSK233 did not affect lifespan in *Drosophila*. Thus, the role of mammalian PDK-1 in organismal lifespan regulation cannot be addressed in flies carrying the *Drosophila* PDK-1 protein.

3.4.3 *Humanization of PDK-1 in Drosophila*

As the Drosophila PDK-1 protein could not be targeted by mammalian PDK-1 inhibitors, we reasoned that if we replace the coding sequence of the Drosophila PDK-1 gene by its human counterpart, the inhibitors might be also functional in flies. The fly would thereby be "humanized" for the PDK-1 protein. In order to achieve this, the PDK-1 founder line (PDK-1$^{\text{Founder line}}$) carrying an attP site in the PDK-1 gene was used and the human sequence was introduced via phi31-mediated recombination. We chose to generate two fly lines: (I) where most of the PDK1 gene was replaced by the human gene, termed fully humanized PDK-1 (PDK-1$^{\text{fully Hum)}}$ and (II) a replacement of only the PH-domain termed PDK-1$^{\text{PH Hum}}$. Due to the location of the attP site and the sequences affected in the PDK-1 founder line (Figure 31), it was not possible to replace the full kinase domain of *Drosophila PDK-1*, but only 80% of it, by the human sequence. However, the most important kinase domains, including but not limited to the APT binding site, the catalytic domain, the PIF pocket and the PH-domain, were all fully humanized. Alignment and kinase domain identification were used to determine the optimal transition sites between the human and the *Drosophila* protein for both the PDK-1$^{\text{Fully Hum}}$ and PDK-1$^{\text{PH Hum}}$. The transition had to be outside any obvious kinase domains, such as ATP site or DFG loop. The human amino acid sequence was codon optimised according to the *Drosophila* codon preference, to optimize translation.

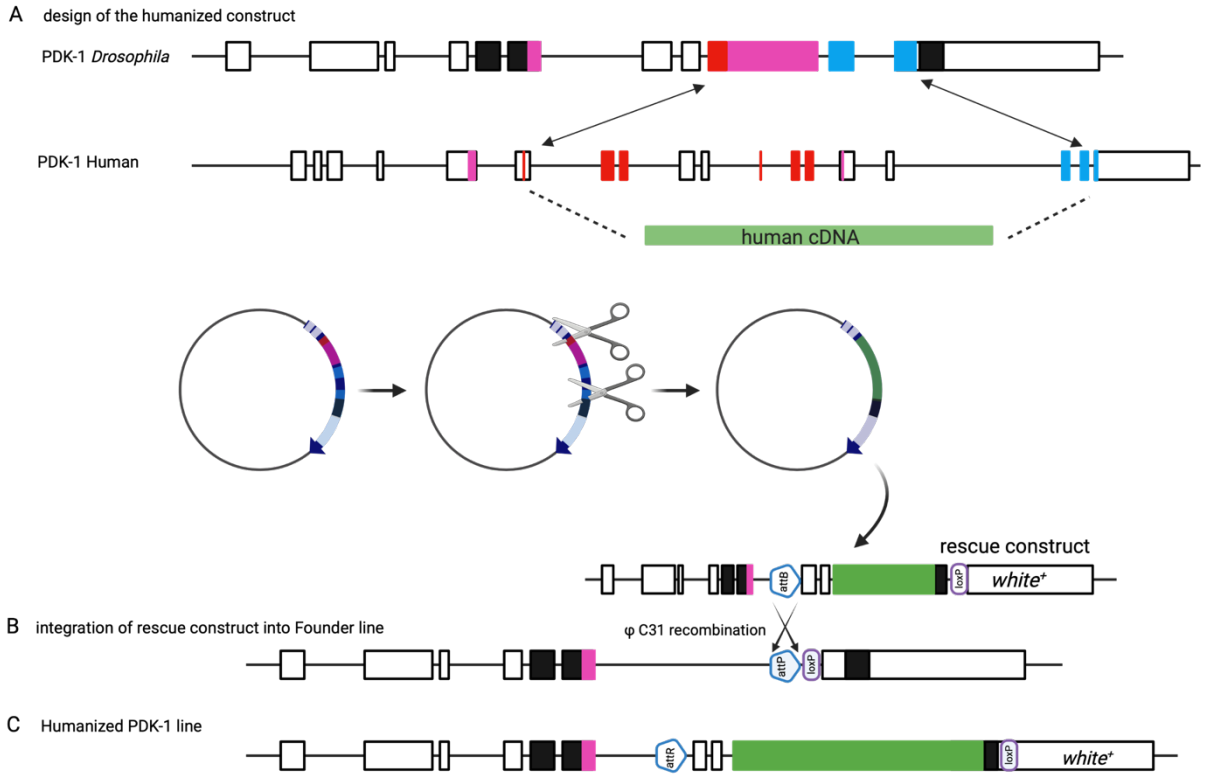

Figure 31: Design strategy of Humanized PDK-1 mutants. *A: Using alignment techniques and knowledge about the key residues in the kinase domains, the Drosophila and human sequence are aligned. B: Human cDNA is cloned into a vector, which is used to create the humanized rescue construct, which is injected into the fly embryo. C: Inserting the rescue construct using φ C31 recombination at the attP site, creating the PDK-1$^{\text{Hum}}$ line.*

Upon injection of the PDK-1$^{PH\ Hum}$ construct, transgenic flies were identified using unique restriction sites and confirmed by sequencing. Unfortunately, despite multiple rounds of injection no transgenic flies were obtained for the PDK-1$^{Fully\ Hum}$ construct. Since both constructs were similar in size and the same integration technique was used, it is unlikely that technical problems explain the inability to receive transgenic flies for the PDK-1$^{Fully\ Hum}$ construct. Thus, these results suggest that integration of the PDK-1$^{Fully\ Hum}$ construct causes dominant lethality in *Drosophila*. Heterozygous flies carrying the PDK-1$^{PH\ Hum}$ transgene were viable and were backcrossed into the wDah background before further phenotyping.

3.4.4 Humanization in PDK-1 in Drosophila is viable

While heterozygous flies carrying the PDK-1$^{PH\ Hum}$ transgene were viable the question remained whether homozygotes for the humanized PDK-1 gene were viable. When PDK-1$^{Hum/Tm3}$ was crossed, offspring without a balancer were observed, suggesting the humanized PDK-1 gene functions in the *Drosophila* cellular landscape and kinome sufficiently for survival. The humanized PDK-1 gene was further investigated by testing the ability of the humanized PDK-1 gene to rescue the lethality observed in the PDK-1$^{null/null}$. Both the wild type and the PDK-1 mutant crossed with a female PDK-1$^{Hum/Hum}$ with male PDK-1$^{null/Tm3}$ resulted in a mean egg-to-adult viability of 95% (Figure 32A). Upon hatching, offspring of the PDK-1^{Hum} cross were inspected and sorted into two groups, those with the PDK-1$^{Hum/null}$ genotype and those with the PDK-1$^{Hum/tm3}$ genotype. Based on the equal hatching of both genotypes, it can be concluded that humanized PDK-1 can rescue the lethal null phenotype completely, hence must function sufficiently to ensure survival. We have therefore created a viable humanized *Drosophila* PDK-1 fly line.

Upon further investigation, while crossing female PDK-1$^{Hum/Hum}$ with male PDK-1$^{Hum/Hum}$ no viable offspring were observed (Figure 32B). To investigate if either sex was sterile, sex specific crosses were set up. When female PDK-1$^{Hum/Hum}$ were mated with male w^{Dah}, 85% egg to adult viability was observed, statistically not different from wild type (Figure 32C). In contrast, when the cross was done using male PDK-1$^{Hum/Hum}$ with female wild type flies, no flies developed. Clearly, humanized male flies were unable to produce viable offspring with either humanized females or wild type females. As discussed in the introduction, PDK-1 signalling is essential for reproduction in mice. In *Drosophila* we observed wild type like egg-to-adult viability in females and a complete loss of reproductive capacity in males. It is currently unknown why males are sterile while females are not.

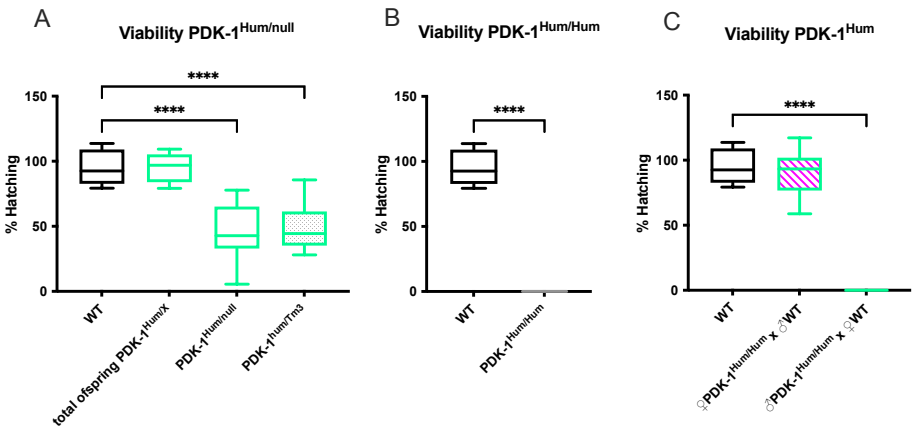

Figure 32: PDK-1$^{Hum/Hum}$ is viable, but reproduction is hampered in both sexes.
A: viability of PDK-1$^{Hum/null}$: w^{Dah} vs PDK-1$^{Hum/null}$ p<0.0001; w^{Dah} vs PDK-1$^{Hum/tm3}$ p<0.0001. One-way ANOVA
B: viability of PDK-1$^{Hum/Hum}$: w^{Dah} vs PDK-1$^{Hum/Hum}$ p<0.0001. student t-test.
C: Viability of PDK-1$^{Hum/Hum}$: w^{Dah} vs Female PDK-1$^{Hum/Hum}$ x male w^{Dah} p=n.s.; w^{Dah} vs male PDK-1$^{Hum/Hum}$ x female w^{Dah} p=0.0001. One-way ANOVA.
***** p<0.0001*

3.4.1 Humanization of the PH domain of Drosophila PDK-1 delays development

Given the effects of PDK-1 mutations on development, we next measured developmental timing. Firstly, we observed a significant delay of ½ day in pupae formation in PDK-1$^{Hum/Hum}$ (Figure 33A). Correspondingly, peak hatching time was delayed by a day (Figure 33B). This is similar to the delay observed in the PDK-1$^{PH/PH}$ and PDK-1$^{PIF/PIF}$ mutants and suggests that humanization of PDK-1 affects signalling during developmental stages, potentially in a similar way to PDK-1^{PH} mutants. Lastly, we see a clear reduction in weight at day 10 between w^{Dah} and PDK-1$^{Hum/Hum}$ flies. Taken together, it implies that the humanization affected developmental timing and organismal size in a significant manner due to unknown limitations in protein function.

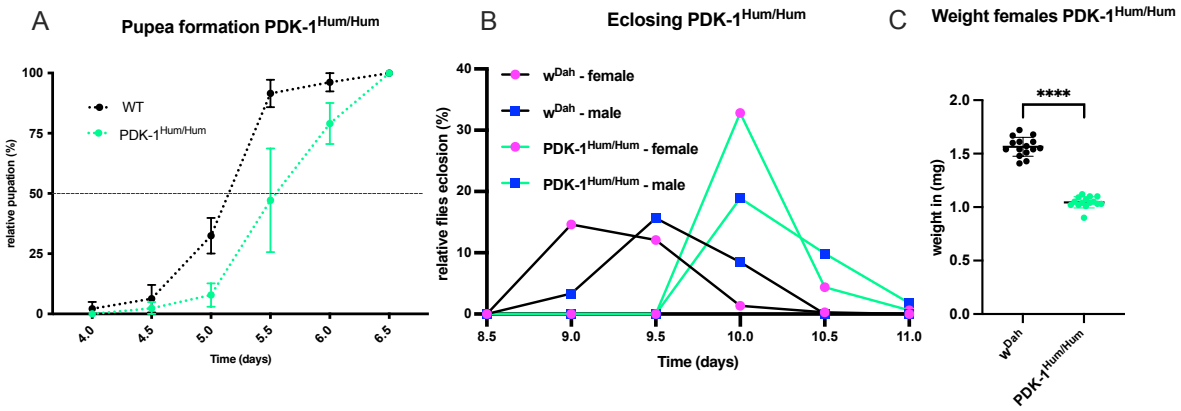

Figure 33: Developmental delay in PDK-1$^{Hum/Hum}$ *A: pupation was scored twice a day. B: eclosure of adult fly was counted twice per day: d9 PDK-1^{WT} vs PDK-1$^{Hum/Hum}$ p<0.001 both sexes, d9.5 PDK-1^{WT} vs PDK-1$^{Hum/Hum}$ p<0.0001 both sexes, d10 PDK-1^{WT} vs PDK-1$^{Hum/Hum}$ p<0.001 both sexes. One-way ANOVA. C: weight at day 10 (N=20 p.g.). p<0.0001. student t-test. * = p<0.05, ** = p<0.01, *** = p<0.001, **** = p<0.0001*

3.4.1 *Humanization of PDK-1 did not affect lifespan*

A mild reduction in functionality in the PH domain in the PDK-1^{PH} flies affected development, lifespan and other health phenotypes. While humanized flies were viable, both viability and development were affected, suggesting the PDK-1 function is affected. Based on the results in both PDK-1^{PH} and PDK-1$^{Hum/Hum}$, lifespan should be characterized before considering treatment with PDK-1 inhibitors. I next measured the lifespan of homozygous humanized PH domain PDK-1 mutant females compared to w^{Dah} control females. There was no significant difference in survival between PDK-1$^{Hum/Hum}$ and w^{Dah} females (Figure 34). Thus, in contrast to PDK-1^{PH} mutant flies, which were long-lived, humanization of the PH domain did not increase lifespan. The absence of a lifespan phenotype in the humanized flies will allow testing the beneficial effects of mammalian PDK-1 inhibitors on longevity independent of an already affected lifespan.

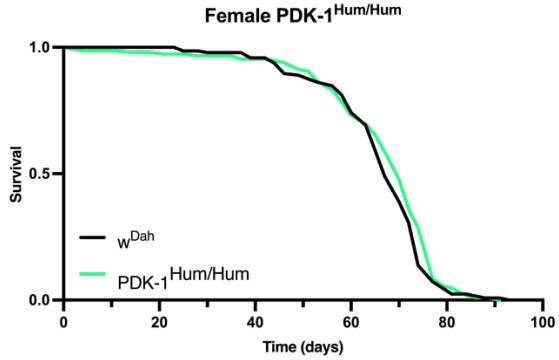

Figure 34: lifespan PDK-1$^{Hum/Hum}$. *W^{Dah} vs PDK-1$^{Hum/Hum}$ p=0.8798*

3.4.1 *Humanization of PDK-1 reduces fertility in both sexes*

Humanization of PDK-1 affected developmental timing but not lifespan as previously observed. Furthermore, female PDK-1$^{Hum/Hum}$ had reduced viability and male PDK-1$^{Hum/Hum}$ were sterile. Lastly, we investigated fecundity. Virgin female flies were crossed with males and after an initial mating period of 24h, scored for egg laying on day 1, 3, 5, 8, 9 and 10. Egg laying of PDK-1$^{Hum/Hum}$ females was severely reduced on all days compared to wild type (Figure 35A). On day 1 a 2.3 fold reduction in egg laying per female was observed. A reduction of 5.5, 5.0, 7.3, 3.7 and 5.5 fold on the consecutive counted days was observed. Thus, humanization of the PDK-1 PH domain in *Drosophila* affects fecundity.

Knowing that PDK-1$^{Hum/Hum}$ are sterile, the effect of mating with wild type males was investigated in female PDK-1$^{Hum/Hum}$. Despite being mated with wild type male flies, a significant reduction in egg laying was observed on all days. (Figure 35B). The reduction was prominent with fold changes between 2.1 and 4 In conclusion, humanization of the PDK-1 gene affects egg laying capacity in female flies, regardless of the genotype of the male. Taken together with previous data it seems that, while the number of produced eggs was severely reduced, the quality of then is similar to w^{Dah}. The data implies that egg maturation might be delayed rather than impaired. Total life fecundity would have to be investigated to draw any definite conclusions.

In contrast to the female, when the male parent was humanized, there was no reduction in egg laying by wild type females. As sperm is not essential for egg laying, there is no significant reduction in

fecundity, despite producing no viable eggs. It is currently unknown if male PKD-1$^{Hum/Hum}$ have severely reduced production of sperm, as with the egg production of females, or if the produced sperm is not viable.

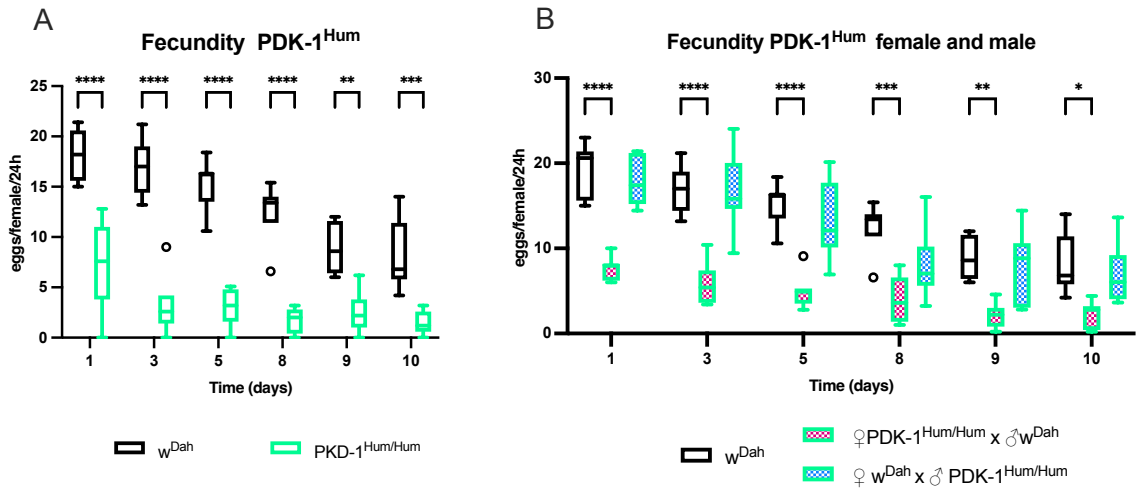

Figure 35: Fecundity of PDK-1$^{Hum/Hum}$ is impaired. *Females are collected as virgins, sorted into vials with 5 females and 3 males and allowed to mate for 24h. Day 1 is counted as the first day after the 24h mating period.*
A: Fecundity w^{Dah} vs PDK-1$^{Hum/Hum}$ x PDK-1$^{Hum/Hum}$: d1 FC 2.3 p<0.0001; d3 FC 5.5 p<0.0001; d5FC 5.0 p<0.0001; d8 FC 7.3 p<0.0001; d9 FC 3.7 p<0.01; d10 FC 5.5 p<0.001, one-way ANOVA.
E: Fecundity w^{Dah} vs female PDK-1$^{Hum/Hum}$ x male w^{Dah} : d1 FC 2.1 p<0.01; d3 FC 2.9 p<0.0001; d5 FC 3.0 p<0.0001; d8 FC 3.2 p<0.0001; d9 FC 4.0 p<0.01; d10 FC 4.0 p<0.05. one-way ANOVA.
** p<0.05, ** p<0.01, **** p<0.0001*

3.4.2 The PH domain specific 2-O-Bn PDK-1 inhibitor

As I only obtained transgenic flies in which the PH domain of PDK-1 was humanized, it was only meaningful to test PDK-1 inhibitors that specifically target the PH domain. As such, I chose 2-*O*-benzyl-*myo*-inositol 1,3,4,5,6-pentakisphosphate (2-O-Bn), which is a Type III PDK-1 specific inhibitor developed by Falasca et al. (2010) that specifically targets the PH domain of human PKD-1. The mechanism of action of 2-O-bn is in direct competition with PIP_3 to bind the PH domain of PDK-1. Occupation of the PH domain with 2-O-Bn prevents recruitment to the plasma membrane, hence conformational changes and close proximity to AKT. Indeed, 2-O-Bn reduces the DPK-1 dependent AKT phosphorylation at Thr308 at concentrations of 20 µM in SKOV-3 and PC3 cells (Falasca *et al.*, 2010). Based upon the proposed model of autoinhibition by the PH domain, allosteric inhibitors targeting the PH domain could prevent any phosphorylation (Levina *et al.*, 2022). Taking everything together, the mechanism of action of 2-O-Bn seems to be two-fold; firstly, there is competitive inhibition of the PDK-1 PH domain. Secondly, there might be a reduction in *trans*-autophosphorylation, affecting the whole PDK-1 signalling pathway.

Due to its beneficial chemical characteristics, high specificity and relative potency, 2-O-Bn is an attractive anti-cancer pharmaceutic. Additionally, treatment with 2-O-Bn reduced AKT signalling in otherwise resistant cell lines. These effects where recapitulated *in vitro*, mice treated with 12,5 mg/kg^{-1} of 2-O-Bn showed reduced tumour growth after 12 days (Falasca *et al.*, 2010). Toxicity studies in mice showed that concentration up to 5 times the therapeutic window can be tolerated. Overall, 2-O-Bn seems to be a promising inhibitor to block the activity of PDK-1 in our humanized *Drosophila* model.

3.4.3 *2-O-Bn does not affect development in PDK-1$^{Hum/Hum}$*

PH domain-specific *Drosophila* PDK-1 mutants showed a delay in development. Thus, I tested whether treating the humanized flies with 2-O-Bn would delay development. Based on previous studies in cell culture (Falasca *et al.*, 2010) and on feasible drug concentrations in fly food, larvae were reared on concentrations of 0.35µM, 3.5µM and 35µM 2-O-Bn. As previously observed, PDK-1$^{Hum/Hum}$ showed a developmental delay of about 12h compared to w^{Dah} control flies. 2-O-Bn treatment did not affect pupation or adult eclosion of w^{Dah} wild type flies (Figure 36A-B), indicating that it has no detrimental off-target effect on *Drosophila* development at the chosen concentrations. However, there was also no effect of 2-O-Bn treatment on the development of PDK-1$^{Hum/Hum}$ mutant flies, neither in pupae formation (Figure 36A) nor on adult eclosion (Figure 36B). Thus, it is currently unclear if the inhibitor did not reach pharmacological concentrations, could bind the humanized PDK-1 protein insufficiently or had low efficiency in *Drosophila* and further test will be necessary to address these points.

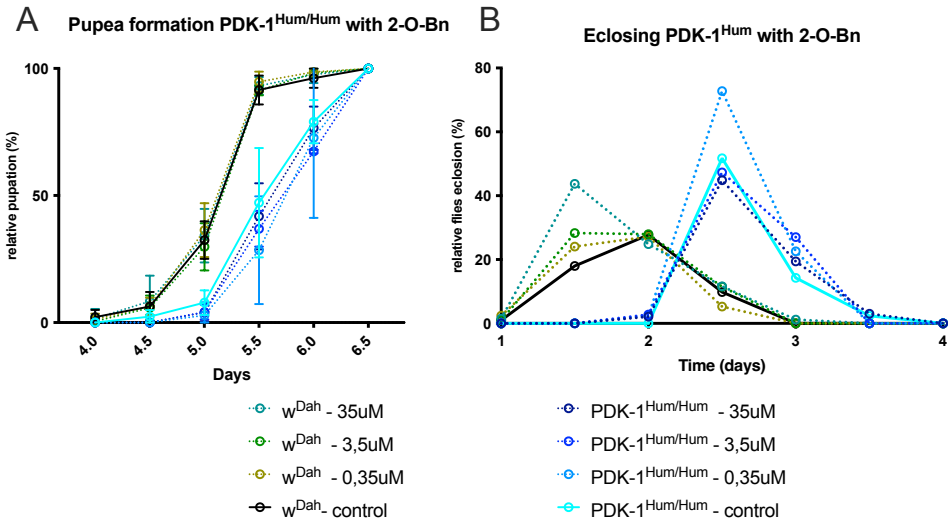

Figure 36: Development of the PDK-1$^{Hum/Hum}$ with 2-O-Bn treatment. *A: pupation was scored twice a day. B: Eclosure of adult fly was counted twice per day. w^{Dah} vs w^{Dah} + 2-O-Bn all concentrations: p= n.s; PDK-1^{Hum} vs PDK-1^{Hum} + 2-O-Bn all concentrations p= n.s. one-way ANOVA.*

4 DISCUSSION

4.1 The burden of regulating ageing

4.1.1 One IIS/TOR network, many different branches affecting longevity

As has been previously discussed, both the insulin and the TOR network are implicated in longevity. Both upstream regulators and downstream effectors can manipulate lifespan in astounding ways. It is remarkable that both reducing functionality of a receptor, an adaptor protein or a downstream effector of that same signalling cascade all extend lifespan (Tatar *et al.*, 2001; Giannakou *et al.*, 2004; Hwangbo *et al.*, 2004; Slack *et al.*, 2010). It implies the presence of either various feedback loops, redundancy or multiple mechanism involved in longevity. The presence of negative feedback loops and redundancy is clearly shown in the IIS/TOR network, explaining partly how downstream effectors have such striking effects on the general activity of the pathway (Puig *et al.*, 2003; Hers and Tavaré, 2005; Taniguchi, Emanuelli and Kahn, 2006). Elucidating the molecular and mechanistic outputs of the various branches of IIS/TOR network that regulated the different aspects of longevity, is one of the main aims of the field. By dissecting the role of a single kinase which two very distinctive mechanisms of activation, we characterized the role of two branches of signalling in health and longevity in *Drosophila* by manipulating two key kinase domains.

4.2 Elucidating phosphorylation pathways

Understanding which pathways are involved in the longevity of both the PH domain and the PIF pocket mutant was one of the main aims of this thesis. Investigating a kinase with two distinctive modes of activating it downstream targets, characterizing phosphorylation changes in the substrates is essential. Designing a new way to stimulate the IIS/TOR network via a natural way, we established a starvation-refeeding protocol. As in the cells, I was able to detect a strong regulation of AKT in *Drosophila* after 30 minutes of refeeding. This new method allowed us to simply and effectively stimulate the pathway, enabling us to perform large scale experiments.

Using the new method to stimulate the pathway, regulation of p-AKT^{342} was investigated using western blots. Unfortunately, either the difference in pathway activation was negligible or the method to detect changes was insufficiently sensitive. As PDK-1^{null} is a lethal mutation, we assume that both the PH domain and the PIF pocket have residual functionality to allow for viable flies. PDK-1^{K465E} mice phosphorylate AKT^{Thr308} upon insulin stimulation, but the response has a 3-5-fold lower maximum and is more transient than in wild type litter mates, showing that we are looking at changes rather and absence of signalling. We therefore investigated elegant and subtle changes in phosphorylation cascades. Additionally, to the sensitivity problem, only one PDK-1 dependent phosphorylation antibody was available for *Drosophila*. We therefore decided to perform phosphoproteomics.

After much optimisation, I was able to create a data set spanning a time line from 5 minutes refeeding till 24 hours. We observed clear regulation of various phospho-sites on known IIS/TOR network components, among others; CHICO; PDK-1; FOXO; SGG; S6K and TSC1. Both PH-domain-dependent as well as PIF-pocket-dependent signalling members were detected. Interestingly, we observed that some sites were regulated as early as 5 minutes, for example SGG^{9}, while others only showed strong persistent regulation after 30 minutes, CHICO^{471}, CHICO^{532} and CHICO^{534}. Using this knowledge

phosphorylation changes after 10 minutes, 30 minutes and 60 minutes of refeeding in the PDK-1 mutants were investigated.

Previous research had shown tissue-specific longevity mechanism upon IIS/TOR network downregulation (Tain *et al.*, 2017). The three *Drosophila* body parts were tested during optimisation. While both heads and abdomen resulted in sufficient depth and reproducibility, peptides isolated from the thorax reduced depth significantly. As muscle contains an abundancy of a few structural proteins which are phosphorylated, it blocked any other IIS/TOR network signal. While reproducibility was good, we could not address our biological questions. With the exception of a few, no peptides associated with the IIS/TOR network were detected.

Based on the depths and diversity of proteins detected, in combination with the role of the gut and fat body in longevity, it was decided to analyse the abdomen. While indeed the data set provided great depth, diversity and reproducibility, upon further analysis the majority of the signal came from the oocytes. Analysis of GO term enrichment resulted in terms only associated with reproduction and development. While this was disappointing, we did detect some IIS/TOR signalling components. Phosphorylation regulation of the IIS/TOR components from the refeeding was almost completely absent and barely detected. The most change was observed between starvation and normal SYA feeding. As this will influence the reproductive machine too, any conclusion must be made hesitantly.

We observed IIS/TOR network dependent regulation which was shared between both mutants. The IIS/TOR network contains many feedback loops, and regulation of CHICO can be expected. $CHICO^{502}$ is predicted to correspond to $IRS2^{591/594}$ based on sequence homology an PRIDE (Ochoa et al., 2019). This phosphorylation has been previously detected in a big phosphoproteomics data set investigating cell cycle dependent phosphorylation (Dephoure et al., 2008; Olsen et al., 2010). It is currently unknown what phosphorylation of $IRS2^{591/59}$, but our data clearly show it is upregulated in nutrient rich environment. Likewise, is the increased phosphorylation of $TSC\text{-}1^{449}$, which is upregulated in both mutants under fed conditions. $TSC\text{-}1^{449}$ is a serine phosphorylation site which is not conserved in humans. However, based on alignment sequences, there is a is a predicted phosphorylation site nearby in the human TSC protein, namely at serine 467. Phosphorylation of S467 and the linked phosphorylation site of S578 results in destabilization of the TSC protein and therefore disassociation of the TSC1/TSC2 complex (Li et al., 2018). Which ultimately results in more TORC1 activation. The phosphorylation of $TSC\text{-}1^{449}$ is up in the FED condition compared to starved, when indeed more TORC1 activation is expected. Surprisingly is however that increased TORC1 signalling is detected in both mutants and not in the wild type like $PDK\text{-}1^{GR}$.

Apart from shared regulation of the IIS/TOR network phosphorylation, we also observed mutant specific phosphorylation, emphasizing that despite many share phenotypes there might be different regulatory signalling cascades underneath. SGG^{9} is an inhibitory phosphorylation, reducing, but not abolishing, the enzyme activity (Frame, Cohen and Biondi, 2001; Beurel, Grieco and Jope, 2015). The phosphorylated serine is conserved based on homology between the protein sequences and corresponds to GSK-$3\alpha^{9}$ and GSK-$3\beta^{21}$. The inhibitory phosphorylation can be performed by AKT or PKA, depending on the signal. Inhibitory phosphorylation by AKT is modulated by growth factors, while the PKA regulation dependents on hormonal stimulation or G protein coupled receptors that are linked to changes in intercellular cAMP levels (Fang et al., 2000; Frame, Cohen and Biondi, 2001).

Reduced SGG activity leads to an upregulation of glycogen synthesis. The observed increase in p-SGG^{9} results in increased glycogen synthesis for storage during nutrient rich conditions. Curiously, our theory would have predicted a reduction in AKT-dependent phosphorylation of SGG, as AKT activity should have been reduced in PDK-1^{PH} mutants. Divertingly, a similar pattern in the SGG^{214} phosphorylation site was not observed (Figure 28F). The phosphorylation site is conserved and corresponds to serine^{278} in humans. Phosphorylation on this site has been reported to be unresponsive to insulin stimulation in HEK293 cells (Frame and Cohen, 2001).

Another striking difference between the PDK-1^{PH} and the PDK-1^{PIF} mutants was the regulation of LPIN in the PDK-1^{PH} mutants. Lipins act as a phosphatidate phosphatases (PAPs) in the glycerolipid biosynthesis pathways. Two sequential acylations of glycerol-3-phosphate produce phosphatidate (PA), which gets converted into diacylglycerol (DG). DG is a key intermediate for the creation of triacylglycerol in the Kennedy pathway of glycerolipid synthesis. Lipins regulate the balance between PA and diacylglycerol. The subcellular location of lipins is regulated by post-translational modifications. Insulin stimulates the hyperphosphorylation of LPIN (17 sites in mammalian lipin), resulting in inhibitory cytosolic localization (Kok et al., 2012). While more kinases are probably responsible for the hyperphosphorylation of Lpin, TORC1 is responsible for at least a few (Peterson et al., 2011). Two out the three phosphorylation sites in Lpin were increased in PDK-1^{PH} fed conditions, suggesting there was a stronger downregulation in Lpin activity than in PDK-1^{GR}. From the 3 phosphorylation sites, only LPIN^{74} is conserved and probably corresponds to phosphorylation serine^{125} in the human lipin protein, based on sequence alignment and PRIDE (Ochoa et al., 2019).

Not only the PDK-1$^{PH/PH}$ mutants had specific phosphorylation changes, significant and unique regulation was also observed in the PDK-1$^{PIF/PIF}$ mutants. PKC^{522} phosphorylation in the PDK-1$^{PIF/PIF}$ mutants was significantly up compared to control (Figure 28J). Unfortunately, there was no predicted phosphorylation site nearby in the human protein, and it is currently unknown if this is an activating or inhibitory phosphorylation.

The Threonine p-ERK^{198}, which he PDK-1$^{PIF/PIF}$ mutants fail to dephosphorylate, is more substantially characterized. During starvation and refeeding both PDK-1 mutants show equal levels of phosphorylation to controls. However, there is a clear dephosphorylation state in both the PDK-1$^{PH/PH}$ and the PDK-1^{GR} flies during abundancy of food. Activation of human ERK1/ERK2 required phosphorylation at both a threonine and tyrosine residue located in a Thr-Glu-Tyr motif in the activation loop (Katz, Amit and Yarden, 2007; Mehdizadeh *et al.*, 2016). Activation by phosphorylation is dependent on the upstream kinase MEK. Inactivation is regulated by two phosphatases, protein tyrosine phosphatase-ERK/Enhancer of Ras1 (PTP-ER) and mitogen-activated protein kinase phosphatase 3 (Mkp3) respectively (Shaul and Seger, 2007). Threonine 198 is part of a Thr-Glu-Tyr motif in *Drosophila* in a highly conserved region, implying this is the 1 of the two activating phosphorylation sites in *Drosophila* ERK. While phosphorylation, hence activation, under starvation is not altered, clearly, the dephosphorylation process is hampered in the PDK-1$^{PIF/PIF}$ mutants, resulting in a more constantly active ERK during fed conditions.

I have shown that indeed *Drosophila* can be used to create a phosphorylation landscape, but only when using whole fly, heads or abdomen without oocytes. As 80 abdomens are required per sample, using either whole fly or 100 heads is more realistic. Investigating the PH domain and PIF pocket

dependent signalling using whole fly would elucidate many open questions. To triage apart which signalling cascades could play a role in the longevity phenotype observed, I will discuss both the PDK-1^{PH} and the PDK-1^{PIF} mutants separately in the coming two chapters.

4.3 Understanding the downstream pathways involved in PH domain PDK-1 longevity

4.3.1 Controlling size and development from a central cockpit

Our understanding of key residues within kinases and their ability to interact with their partners is growing. PDK-1 is a special kinase within the AGC family, having two unique domains influencing its mechanisms of activation. Previously, a PH domain mouse mutant has been created and characterized. We created a Drosophila homologue PDK-1^{K601E}, allowing characterization of the PH domain dependent PDK-1 signalling and the corresponding phenotype. As the PH domain is essential for AKT^{342} phosphorylation, hence activation AKT, it is referred to as the AKT signalling branch.

Lethality was common in the PDK-1 mutants and reduced viability is often observed in IIS mutants, thus a reduced viability was anticipated. Curiously, only the strong hypomorphs of PDK-$1^{PH/null}$ showed a reduced viability, indicating that only severe inhibition of the AKT branch results in morbidity before adulthood. Intensity of AKT branch signalling has previously been correlated with viability, strongly inhibited PI3K signalling severely reduced viability, while moderate inhibition did not in *Drosophila* (Oldham *et al.*, 2002). Not only is viability reduced, but a clear developmental delay proportional to the intensity of the PDK-1 down regulation was observed. Unlike many IIS mutants, the PDK-$1^{PH/PH}$ mutants were not smaller than controls, only stronger downregulation of the AKT branch in the PKD-$1^{PH/null}$ resulted in reduced size. Previously, in mice PH domain specific mutation PKD-1^{K465E} reduction in size due to smaller cells, hence limited cellular mass was observed. This observation is fitting with the knowledge that, CHICO with p60, the regulatory subunit of PI3K, regulates growth but not proliferation rates (Weinkove *et al.*, 1999; Oldham *et al.*, 2002). More recent research showed that FOXO does not regulate cell size in a cell-autonomous way, but via non-autonomous ways (Slack *et al.*, 2011). FOXO regulates the expression and secretion of dILPs by MNCs, hence regulating organismal size. Taken together, we can conclude that the AKT branch does regulate size but only when strongly inhibited. I hypothesize that this effect is regulated in the MNCs, where reduced AKT activation results in reduction in FOXO activated dILP expression. To investigate this hypothesis regarding the mechanisms leading to smaller size in the PDK-1 mutants, one could express wild type PDK-1 in the MNCs cells. If size is solely affected by the reduction in dILPs secreted by the brain and regulated by the AKT branch, PDK-1^{PH} mutants with wild type PDK-1 expression in the brain should develop to normal size.

Reduced IIS/TOR network signalling is often associated with reduced fecundity, however causality has been firmly disproven. Unlike the regulation of growth, dILPs act on reproduction and germ line stem cells in a local niche (Liao, 2020). Accelerated loss of germ line stem cells in *chico* mutants, is counteracted by overexpression of dILP in the ovarian somatic cells (Hsu and Drummond-Barbosa, 2009). As with size, it seems that FOXO affects dILP expression, thereby reducing fecundity. Interestingly, no reduction in fecundity is observed in the PDK-$1^{PH/PH}$ flies, only the stronger

hypermorph PDK-1$^{PH/null}$ shows a severe reduction. While the difference is more striking, it is similar to the size phenotype observed. The moderate reduction in PH domain signalling might not be strong enough to affect FOXO-dependent dILP regulation, resulting in wild type like size and fecundity.

4.3.2 Less is more in PH domain regulated longevity

Manipulation of either IIS or TOR network upstream in their signalling cascades has resulted in longevity (Clancy et al., 2001; Bjedov et al., 2010; Grönke et al., 2010). Equally, when only a single branch of signalling was affected, longevity has been previously observed (Hwangbo et al., 2004; Slack et al., 2015). While some interventions only partly downregulated protein functionality or expression, none of the above mutations investigated a single kinase domain within a protein. Lifespan extension can be achieved by targeting a single kinase domain as shown in both the PDK-1$^{PH/PH}$ and the PDK-1$^{PH/null}$ mutants. The improvement is not as striking as in C. elegans pdk-1^{sa680} but comparable to other IIS Drosophila mutants. By characterizing the phenotype of the PDK-1^{PH} mutants, we hope to triage apart which parts of the nutrient sensing partway are responsible for the PH domain PDK-1 dependent longevity.

By affecting the PH domain in PDK-1 the interaction with PIP_3 is disturbed, supposedly severely reducing or even abolishing of the PDK-1 dependent p-AKT^{Thr342}, but not the p-AKT^{Thr308}. AKT has more than a 100 downstream actors and some of those have been implicated in longevity (Manning and Cantley, 2007). AKT activation diversifies the signalling cascades and touches many downstream effectors, among those the famous longevity gene FOXO. FOXO is essential for the longevity phenotype in both late ablation of the MNCs and adult expression of dominant negative form of the INR (Slack *et al.*, 2011). Additionally, *dfoxo* overexpression in the fat body results in a lifespan extension of 15-20%, underlying that FOXO is not only essential but sufficient for longevity (Giannakou *et al.*, 2004; Hwangbo *et al.*, 2004). The signalling cascade of PIP_3-AKT-FOXO, can explain the longevity phenotype of the PDK-1^{PH} mutants. While I was unable to test FOXO dependency since both *dfoxo^{null};PDK-1$^{PH/PH}$* and *dfoxo^{null};PDK-1$^{PIF/PIF}$* were not viable, it is very likely that the PDK-1^{PH} longevity is partly regulated by FOXO (Unpublished data from UCL Partridge lab). The lifespan extension was clearly dose dependent in females, where a reduction in expression levels is additive to a hampered PH domain functionality. The longest longevity of all mutants is observed in the PDK-1$^{PH/null}$ females, an additional 17% compared to PDK-1$^{PH/PH}$. Notably, males did not benefit from stronger downregulation of the AKT signalling branch. It abolished the lifespan extension observed in the PDK-1$^{PH/PH}$ males, most probably due to early mortality. While the ability to extend lifespan via the AKT signalling cascade is not sex specific, the optimal window of signalling clearly is.

By treating the PDK-1$^{PH/PH}$ mutants with two well-known AGC kinase inhibitors, their role in PH domain PDK-1 dependent longevity was investigated. Targeting TORC1 and MEK with rapamycin and trametinib it was determined if they act synergistically or independently. Rapamycin treatment extended lifespan in the PDK-1$^{PH/PH}$ mutants, although not as profoundly as in wild type like flies. The cox proportional hazard analysis showed that the risk of dying was reduced with rapamycin treatment to a similar extend as in the PDK-1^{GR} + rapamycin flies.

Seemingly contradictory, rapamycin treatment of PDK-1$^{PH/PH}$ males reduced longevity, while not affecting wild type like controls. Analysis with the cox proportional hazard analysis showed no

significant additive hazard in the PDK-1$^{PH/PH}$ mutants. While indeed the effect is opposite, both results indicate that TORC1 and the PDK-1$^{PH/PH}$ work collaboratively. Indeed, regulation of TORC1 substrates has been observed before in PDK-1^{K465E} mice mutants. They showed diminished S6K^{Thr389} phosphorylation, a well-known TORC1 phosphorylation site (Pullen *et al.*, 1998; Fingar *et al.*, 2004; Bayascas *et al.*, 2008).

While in the females we see a clear positively synergistic effect, the males exhibit an antagonistic epistasis phenotype. As mentioned above, the optimal signalling window is sex-specific, and rapamycin clearly disrupts the optimal signalling intensity in males but not in females. This has been observed before, rapamycin treatment in a strongly down regulated IIS signalling environment can be detrimental (Bjedov *et al.*, 2010).

The longest longevity has been observed in the PDK-1$^{PH/null}$, which can be attributed to a potentially more significant downregulation of TORC1, due to more severe reduction in phosphorylation of TSC1/2 by AKT. To test this hypothesis, not only the PDK-1$^{PH/PH}$ mutants but also the PDK-1$^{PH/null}$ mutants should be treated with rapamycin. One would expect a milder gain of median lifespan and reduction in hazard in the PDK-1$^{PH/null}$ mutants, if indeed TORC1 plays a bigger role than the homozygous counter-part.

Not only did TORC1 inhibition increase PH domain PDK-1 mutant longevity, MEK inhibition by trametinib also increased lifespan in females. Once more, the cox proportional hazard analysis shows that the reduction in hazard is roughly equal between female controls and PDK-1$^{PH/PH}$ mutants. Notably, the reduction in hazard is significantly more than with rapamycin treatment in both genotypes. PDK-1$^{PH/PH}$ males do not benefit from either pharmacological intervention, but rather showed no effect like the PDK-1$^{GR/GR}$ controls.

Interestingly, dual treatment with rapamycin and trametinib resulted in a wild type like lifespan extension in the PDK-1$^{PH/PH}$ mutants, a gain of 19%. Indeed, we observe an identical reduction in risk of death in both PDK-1$^{PH/PH}$ and PDK-1^{GR}, which is the sum of either drug individually. Conclusively, inhibition of both TORC1 and MEK works additive, even in the AKT signalling sensitized background. The creation of a double homozygous PDK-1$^{PH/PH;\ PIF/PIF}$ mutant could help us understand the interplay between these two pathways. Intriguing would be to understand if indeed TORC1 and AGC signalling plays a role in PDK-1$^{PH/null}$ longevity. If so, lifespan extension in the PDK-1$^{PH/PH;\ PIF/PIF}$ mutants should increase substantially more than the lifespan of PDK-1$^{PH/null;\ PIF/null}$.

Regarding all data, we have strong evidence that the TORC1 is involved in PDK-1^{PH} longevity. AKT activation is one of the key players in TORC1 activation, hence PDK-1 PH domain mutation could result in a general down regulation of this signalling cascade. While FOXO is an intuitive player in the PDK-1^{PH} longevity, so far no phenotypic evidence is discussed.

4.3.3 Two sides of a coin: of homeostatic metabolism and starvation resistance

To understand which downstream pathways might play a role in the PDK-1^{PH} longevity, additional phenotypes were investigated. Energy expenditure and metabolism are key processes in Drosophila

ageing, both partly under control of PDK-1 signalling (Clancy *et al.*, 2001; Broughton *et al.*, 2005b, 2008; Bjedov *et al.*, 2010; Juricic, Grönke and Partridge, 2020). Previously it was observed that both *chico*$^{-}$ and mNSC-ablated flies have improved starvation resistance (Broughton *et al.*, 2005b, 2008; Bjedov *et al.*, 2010). Not only storage but also the ability to mobilize and utilize TAG play a role in starvation resistance. (Grönke et al., 2005, 2007). During starvation TAG is be utilized, thus the machinery involved in TAG synthesis should be down regulated and TAG is lipolyzed. *Drosophila's* main triglyceride lipase and key player in lipid mobilization, BMM can be activated via PDK-1 signalling of the AKT-FOXO axis (Luong et al., 2006; Wang et al., 2011; Han et al., 2022). Diminished functionality of BMM reduced acute TAG mobilization upon starvation, hence increased TAG preservation thereby improving survival (Grönke et al., 2005). Not only *bmm* is under FOXO control so is *Lip4,* a gene encoding a second enzyme involved in intracellular hydrolysis of TAG. Predictably, FOXO is essential for starvation resistance in *Drosophila* (Kramer, Slade and Staveley, 2008).
Additionally, translation is regulated upon starvation conditions. 4E-BP, which is transcriptionally regulated by FOXO and by phosphorylation by TORC1, is essential for starvation resistance (Tettweiler *et al.*, 2005). 4E-BP is not the only key component in starvation resistance under TORC1 control, both FKH and ATG genes are essential (McPhee and Baehrecke, 2009; Cuesta, Fan and Rand, 2014; Bolukbasi *et al.*, 2017). Apart from all indirect evidence about the role of PDK-1 in starvation, PDK-1 has been directly linked to metabolism of energy reserves in yeast (Pastor-Flores et al., 2011).

Down regulation of PH domain dependent signalling resulted in an increase in starvation resistance in both males and females. Equally to male longevity phenotype, strong down regulation of PH domain signalling resulted in abrogation of the starvation resistance observed with mild downregulation of the pathways. It emphasizes the difference in optimal signalling strength of the AKT branch between the sexes. Intriguingly, both the PDK-1$^{PH/PH}$ and the PDK-1$^{PH/null}$ mutants had increased TAG content in both sexes. Thereby, uncoupling starvation resistance to TAG content, at least in males. As mentioned, the ability to mobilize and utilize previously stored TAG is equally important for starvation resistance. Expression levels of both *bmm* and *lip4* were investigated, but neither was significantly regulated in homeostatic conditions in females. While I did not observe any difference in gene expression of bmm and lip4, we did see regulation on phosphorylation level. LPIN phosphorylation was heightened in the PDK-1$^{PH/PH}$ mutants during nutrient rich conditions. Hyperphosphorylation results in a cytosolic location of LPIN, hence inactivity and stop in the production of TAG. While slightly contradictory with the increase in starvation resistance and accumulated TAG levels, we do observe regulation of starvation pathways at a molecular level in PDK-1$^{PH/PH}$ mutants. A more extensive phosphorylation data set should be obtained to draw definite conclusions. To further investigate the ability to survive starvation one should investigate the ability to mobilize and utilize stored TAG, by looking at pre-, during- and after-starvation TAG content. Additionally, expression levels of *bmm* and *lip4* before and after starvation in both sexes should be investigated.

Starvation resistance has been previously uncoupled from longevity mutants, implying there is no causality for lifespan. Rather, it highlights the altered status of the IIS/TOR network signalling affecting metabolism in the PDK-1 mutants (Brown *et al.*, 2019). As both the AKT-FOXO and the AKT-TORC1 axis are essential for starvation survival, hits are given as too importance of both axis in the PDK-1^{PH} mutants.

4.3.4 Stress resistance and their telling tales about signalling pathways

As every tissue has a distinct role in maintaining organismal homeostasis, it is not surprising that different mechanisms play a role to oppose dysregulated organs (Nie *et al.*, 2022). In *Drosophila* IIS mediated longevity by FOXO is tissue specific; mRNA splicing and nucleosome components in the brain, while in the gut proteasome and ubiquitin-mediated protein catabolism are regulated in a FOXO specific manner (Tain *et al.*, 2017). Ribosomal constituents and proteins are strongly regulated in the fat body. The only mechanism shared between all tissues is regulation of the mitochondrial electron transport chain components. Both the increase in gut proteasome activity and elevating mitochondrial respiration result in improved longevity, suggesting these are organismal-wide pro-longevity mechanisms (Tain *et al.*, 2017).

Considering that regulation of respiration is a pro-longevity mechanisms, and a previously observed ability to withstand oxidative stress in IIS/TOR mutants, the PDK-1^{PH} mutants survival on oxidative stress was investigated. Most Reactive Oxygen Species (ROS) are generated by the mitochondrial electron transport chain during respiration. The very reactive Superoxide (O_2^-) is converted to the slightly more stable H_2O_2 by superoxide dismutase (SOD) and the neutralization is realised by peroxidates. Paraquat reacts with the NADPH oxidases enzyme in the mitochondria converting it into the paraquat radical. This radical interacts with oxygen to form superoxide. Previously, *dilp2-3,5* mutants show improved oxidative stress resistance to H_2O_2 (Grönke et al., 2010). Indeed, both SOD and catalase, a peroxidase, are upregulated in *dilp2-3,5* mutants (unpublished data from J. Paulitz). Another potential signalling cascade to promote oxidative stress resistance is via the AKT-NRF2 axis (Sykiotis and Bohmann, 2008; Wang *et al.*, 2008; Baird and Yamamoto, 2020). NRF2 is associated with longevity and plays a crucial role in dealing with detoxification of oxidative stressors. Rapamycin has also been implicated to improve survival on paraquat (Ravikumar *et al.*, 2006; Feng *et al.*, 2019).

Reduced functionality of the PH domain in PDK-1 resulted in an improved survival on paraquat and H_2O_2 in females. Strikingly, strong down regulation of the AKT branch greatly sensitized male flies to paraquat toxicity, while improving their survival on H_2O_2. We observed a clear sex-specific difference in the ability to combat superoxide radicals produced by paraquat absorption, implying that the males are unable to upregulate SOD in response to oxidative stress, or even have lower general levels. The failure to survive paraquat is not inherent to males, as previously no sex-specific difference in survival on paraquat was observed. Conversely, males seem to be able to adapt to paraquat stress in a superior way than females (Pomatto *et al.*, 2017). We can thus conclude that down regulation of the AKT branch reduces the ability to handle superoxide, meanwhile increase the ability to combat H_2O_2 in males. Generally speaking, both sexes show that the AKT branch affects oxidative homeostasis in *Drosophila*. All pathways discussed above could play a role in PDK-1^{PH} mutants' resistance; FOXO can improve general regulation of respiration or improved ability to combat oxidative stress can be regulated via either the AKT-NRF2 axis or via TORC1 signalling. It would be interesting to investigate the levels of SOD and catalase in both females and males during homeostasis and during oxidative stress. Additionally, investigating if there is a general improvement in mitochondrial respiration and morphology would be elucidating.

I investigated a second stress resistance phenotype, namely the ability to deal with xenobiotic stress. Xenobiotics are substances foreign to *Drosophila* and require neutralization to prevent either

oxidative stress or endoplasmic reticulum (ER) stress (Lafleur, Stevens and Lawrence, 2013; Hofer, 2021). Treatment with DDT affects the nervous system by targeting the voltage-gated sodium channel resulting in uncontrolled nerve firing. Ultimately this results in death, preceded by loss of peripheral muscle control. This is observed in the flies as a “knockdown” with uncontrolled twitching. Generally speaking, detoxification of xenobiotics occurs in three phases. In phase I, cytochrome P450 enzymes (CYPs) and short chain dehydrogenases (SDRs) bioactivate the lipophilic components. A different classes of phase II enzymes then conjugate bulky hydrophilic groups to the molecules to increase their solubility in body fluids and facilitate their excretion. Phase II enzymes include glutathione-S-transferases (GSTs), UDP-glucuronosyl-transferases (UGTs), sulfotransferases, carboxylesterases and others. The third phase is accomplished by the ABC (ATP binding cassette) transporters, which excrete the detoxified molecules (Omiecinski et al., 2011). Many genes implicated in xenobiotic stress resistance are associated with longevity traits, but xenobiotic resistance is not causal for longevity (McElwee *et al.*, 2007b; Sykiotis and Bohmann, 2008; Steinbaugh *et al.*, 2012; Afschar *et al.*, 2016). For instance, overexpression of *Cyp6g1* or *Cyp6w1* gene result in improved DDT resistance in *Drosophila,* but not always longevity (Daborn et al., 2002; Pedra et al., 2004; Schmidt et al., 2017).

Oxidative stress and xenobiotic stress are often correlated, thus shared signalling pathways can be expected. The AKT-NRF2 axis, involved in oxidative stress response is also an important player for xenobiotic metabolism. (Wang *et al.*, 2008; Gill *et al.*, 2010; Ma, 2013; Klotz and Steinbrenner, 2017). Additionally, the AKT-FOXO axis regulates the Nuclear hormone receptors (NHRs) which plays a role in the regulation of detoxification enzymes (Hoffmann and Partridge, 2015; Afschar *et al.*, 2016). NHRs are not only involved in xenobiotic detoxification but also in the regulation of sterol metabolites and fat metabolism, thereby deeply connecting them to the nutrient sensing IIS/TOR network. As detoxification is highly energy consuming, it must tightly linked to the availability of energy (Omiecinski *et al.*, 2011). The ability to regulate NHR involved in xenobiotic metabolism is not *Drosophila* specific and it conserved in mice (Kodama *et al.*, 2004). Resistance to xenobiotic stress is uncoupled from the TORC1-FHK axis in IIS mutants (Slack *et al.*, 2011, 2015; Su *et al.*, 2013; Bolukbasi *et al.*, 2017; Pustylnyak, Gulyaeva and Pustylnyak, 2020). Taken together, it seems that most xenobiotic metabolism signalling cascades go via the signalling node AKT.

We saw a clear increase in DDT resistance in the PDK-1$^{PH/PH}$ and the PDK-1$^{PH/null}$ mutants in females and a significant increase in PDK-1$^{PH/null}$ males. Thus far, no PDK-1$^{PH/PH}$ phenotype indicated the involvement of the AKT-FOXO branch. However, the essential role of FOXO in xenobiotic resistance, strongly implies the downregulation of this axis in the PDK-1^{PH} mutants. Remarkable, only the stronger hypermorph in the males extended lifespan on DDT. Clearly, strong regulation is needed before male allocate more energy to xenobiotic resistance. Opposite of previous phenotypes were strong downregulation of the AKT branch in males was detrimental, it is once more a sex specific optimal window of signalling in the AKT branch.

Contradictory to current literature, we saw a down regulation of *Cyp6w1* in the PDK-1$^{PH/null}$ mutants, while they display the biggest DDT resistance. While upregulation of CYP6g1 enzyme did not improve lifespan, it has been linked with increased resistance (Daborn et al., 2002; Pedra et al., 2004; Schmidt et al., 2017). Further investigation of other genes involved in xenobiotic resistance should be investigated to make definite conclusion about the precise enzymes involved in PDK-1^{PH} dependent resistance.

4.3.5 *Keep on walking*

Flies' innate tendency to climb in their enclosure declines with age and is the most widely used healthspan measure of functional ability of muscles and motoneurons. It must be noted, that improved climbing and longevity are not coupled or predictive of each other (Wilson *et al.*, 2020). While not predictive, loss of movement is a ARD shared between *Drosophila* and humans (Boyd *et al.*, 2005). We use the negative geotaxis, startle-induced vertical movement to investigate general health of PDK-1^{PH} mutants. A striking improvement of climbing was observed from early adulthood till late life. Both mild and strong downregulation of the AKT branch resulted in a delayed onset of this ARD. Additionally, the ARD did not develop till the same severity as in wild type like controls. During life sarcomeric cytoskeleton, the basis unit in muscle, undergoes turnover throughout adult life. Ongoing synthesis and turnover of the key sarcomere structural components such as projectin, myosin and actin, are required to maintain correct sarcomere length and thin filament length (Perkins and Tanentzapf, 2014). Not only healthy muscles are required for locomotion, adaptively coordinating the sequential activation of muscles is equally important. The circuit mechanisms underlying coordinated locomotion are poorly understood. Taken together, improved climbing is an interplay between healthy muscle cells and neurons. Which specific downstream signalling cascades are involved is currently unclear, but one could speculate that the general improvement of energy usage, increased autophagy, reduced oxidative stress and xenobiotic stress, all collide to improve muscle and neuron health.

4.3.6 *A healthy gut feeling*

Equivalently to maintaining oxidative and xenobiotic homeostasis, maintaining the balance between regenerative proliferation and differentiation requires many inter connected signalling cascades. Integrating the nutritional status as a cue to either maintain homeostasis or to upregulate proliferation division has been evolutionary optimised (O'Brien *et al.*, 2011). Intestinal health is a predictor for mortality in female Drosophila and can be labelled a fly ARD (Rera, Clark and Walker, 2012). Barrier epithelia are exposed to frequent environmental challenges and thus under repeated regenerative pressure during a lifespan. Intestinal health can be seen as a combination of preventing cancerous un-controlled cells division and ensuring baseline of healthy cells which make up intestinal barrier.

Tight regulation of cell competition is an important tumour-suppressor mechanism (Baker, 2020). Downregulation of the IIS/TOR network by inhibition of upstream components improved regulation of ISC proliferation rates (Biteau *et al.*, 2010; Lu *et al.*, 2021; Juricic *et al.*, 2022). The role of TSC/TOR signalling in proliferation and maintenance of ISC in response to nutritional conditions is undisputed (McLeod *et al.*, 2010; Kapuria *et al.*, 2012; Haller *et al.*, 2017). Too much downregulation of TORC or IIS signalling results in the accumulation of small clusters of undifferentiated ISCs and detrimental survival effects (Biteau *et al.*, 2010; Kapuria *et al.*, 2012).

Undeniably did down regulation of the AKT branch in the PDK-1^{PH} mutants resulted in diminished uncontrolled proliferation in the intestine. Intensity of the AKT downregulation did not affect the gut proliferation phenotype, hence no difference between PDK-1$^{PH/PH}$ and PDK-1$^{PH/null}$ mutants was observed. Neither was the effect was age dependent, as even at young age there was a significant

reduction compared to wild type like controls. Throughout life levels of pH3+ cells increased in PDK-1^{PH} mutants, confirming that downregulation of AKT did not abolish the intestinal ARD. However, a is significant reduction in uncontrolled intestinal proliferation due to lowered AKT levels, potentially improving quality of life mid-age flies. Much like rapamycin treatment, mitotic levels are most likely controlled via the AKT-TORC1 axis.

Ecc15 infection, a mild enteropathogen, induces a synchronized and transient proliferative response in the epithelium. Upon stimulation by infection, we observe an increase in pH3+ cells in young and middle-aged flies in a PDK-1^{PH} dependent manner. At old age the ability to upregulate proliferation significantly reduced. While it would be logical to assume that the ISCs are depleted and therefore unable to respond to the stimulation, this is most likely not the reason. At young age, repeated regeneration in response to injury or infection, depleted the ISCs percentages. However, while homeostatic ISC percentage is significantly reduced, the proliferation response to infection was equally powerful, showing that number of ISCs are not indicative of proliferation response (Haller *et al.*, 2017). Two signalling cascade play a role in proliferation initiation, the initial phase of proliferation is TOR dependent. Conversely, the late proliferation control is TOR independent, suggesting the role of a currently unknown distinctively different branch of signalling (Haller *et al.*, 2017). As I measured the proliferation 24 hours after infection, both the TOR dependent and TOR independent signalling cascade must have been successfully activated in PDK-1^{PH} flies.

At young age the intestine clearly responded towards infection stress assay with increased proliferation. However, while tight regulation of ISC proliferation and gut barrier function are often associated, they can be uncoupled (Juricic *et al.*, 2022). The ability to survive on *Ecc15* infection is a readout of both intestinal integrity and immune function. Upon infection we did not observe an increased survival in the PDK-$1^{PH/PH}$ mutants but only in the stronger hypermorph. Only strong downregulation of the AKT signalling branch resulted in increases survival, most likely indicating an improved intestinal barrier. One of the mechanism to maintain intestinal health is autophagy and transient upregulation of autophagy in the intestine can extend lifespan in *Drosophila* (Bjedov *et al.*, 2010; Regan *et al.*, 2016; Juricic *et al.*, 2022). Furthermore, autophagy is essential for rapamycin-mediated health improvements in the intestine (Schinaman *et al.*, 2019). Increased autophagy levels are not detected in the PDK-$1^{PH/null}$ mutants, thus cannot explain the improved survival. To confirm the absence of autophagy further investigation of the activation of *Atg* genes should be done both by qPCR and phosphoproteomics data set. Additionally, one could perform the smurf assay, a more direct readout of intestinal barrier integrity.

Taken together, we can conclude TORC1 is most likely a key player in PDK-1^{PH} mutants improved intestinal health, but that another yet unknown signalling cascade enables an improved gut barrier function independent of autophagy.

4.4 Understanding the downstream pathways involved in PIF pocket PDK-1 longevity

4.4.1 Each cell for its own; cell autonomous regulation of size

Despite many attempts to create a viable PDK-1 PIF pocket mutant in mice, identifying an essential non-lethal residue had not been successful so far. I am therefore happy to report that the *Drosophila* PKD-1^{K198A} PIF pocket mutant is viable and develops delayed but into a fertile adult. The corresponding K115 residue in mice is crucial for PIF pocket affinity and functionality in cells, confirming the essential role of this amino acid in the PIF pocket dependent signalling (Biondi et al., 2000, 2001). Of the many PIF pocket dependent PDK-1 substrates, the majority belongs to the AGC kinase family. We therefore refer to this signalling as the AGC branch of PDK-1 dependent signalling.

A severe reduction in AGC kinase signalling resulted in reduced viability. Additionally, we observe a significant sex-independent reduction in size due to either mild or strong reduction of the signalling branch. Furthermore, there is a clear proportional developmental delay. Developmental delays had been previously observed in many of the lethal PIF pocket PDK-1 mice lines (Lawlor *et al.*, 2002; Collins *et al.*, 2003; McManus *et al.*, 2004). While these developmental delays always turned out to be lethal, the developmental delay detected in the *Drosophila* PIF pocket mutant did not prevent hatching into a viable and fertile adult fly. The developmental delay is clearly proportional to the intensity of the PDK-1 down regulation, as the stronger hypermorph showed increased delays.

Overall, the PDK-1^{PIF} mutants behave like classical IIS/TOR mutants regarding size and development. An intriguing observation, as viability and developmental delays are often associated with FOXO. The phosphorylation of MEK by PDK-1 cannot explain the size or developmental delay (Oldham *et al.*, 2002; Slack *et al.*, 2015). Both size and developmental delay in CHICO^{null} mutants can be rescued by expressing a rescue CHICO construct containing a mutant binding site for DRK (Slack *et al.*, 2015). It is the DRK adaptor protein which facilitates the binding with RAS, proving reduced MEK signalling it not crucial in size and developmental timing.

While there are many AGC kinases in the IIS/TOR network, none of those are predicted to control FOXO. The reduction in size can be explained by a reduction in S6K PIF pocket dependent activation, which affects growth in a cell-autonomous way (Montagne *et al.*, 1999; Rintelen *et al.*, 2001; Stocker *et al.*, 2003). S6K$^{-/-}$ mice show reduced total bodyweight and fatmass/body ratio (Selman *et al.*, 2009). Deficient S6K results in small cells, between 15-35%, rather than a reduction in cell number. This effect is not mediated by reduced translation, but rather by increased cell cycle (Montagne *et al.*, 1999; Dowling *et al.*, 2010; Zhang *et al.*, 2022). Similarly, pharmacological inhibition with rapamycin did not reduce translation, implying regulation of translation is a mechanism outside of the TOR network (Garelick *et al.*, 2013).

While many other signalling cascades could be involved, I hypothesize that the reduced activation of S6K results in a reduction in size. This effect should be regulated by a distinctively separate mechanism then translation. A unspecific partial loss of function mutation in PDK-1 resulted in smaller *Drosophila* body parts (Rintelen *et al.*, 2001). So far, these effects were attributed to the AKT branch of PDK-1 signalling. Remarkably, the PDK-1^{PIF} mutants show that the AGC branch of PDK-1 signalling is regulating

these phenotypes equally. As discussed in the section above, it would be interesting to test if the size of PDK-1^{PIF} mutants can be rescued by the non-cell autonomous effects of FOXO expression in the MNCs or by expression of constitutively active S6K.

There was a strong effect on fecundity in both the homozygous and heterozygous stronger hypermorph. Ovarian specific PDK-1^{KO} results in premature ovarian failure (Reddy *et al.*, 2009). This is regulated via a S6K dependent mechanism, as S6K is essential for production in mice in a cell autonomous manner. Additionally, diminished reproductive fitness has been coupled to smaller body-size (Klepsatel *et al.*, 2020). While flies are reared on SYA food, the general down regulation of the AGC branch might mimic poor nutritional environmental signals. Indeed, size and fecundity seem to correlate well in both the PDK-1^{PIF} and the PDK-1^{PH} mutants. As in the PDK-1^{PH} mutants, I predict that reproduction is regulated cell autonomously and it would be interested to investigate size and number of ovarioles in both mutants.

4.4.2 Moderation is the key to PIF pocket mediated longevity

As discussed previously, manipulation of a single kinase domain extended lifespan in *Drosophila.* It resulted in a longevity phenotype which was attributed to the AKT signalling branch, including the AKT-FOXO and the AKT-TORC1. Contradictory, the PIF pocket dependent phosphorylation by PDK-1 does not act via proximity via the PH domain, but on a priming phosphorylation. Substrates must be phosphorylation phosphorylated before the PIF pocket recognizes the hydrophobic motif and forms an interaction. Upon a stabilized interaction, PDK-1 undergoes a conformational change allowing it to add a final phosphorylation to the T loop of the substrate.

Inhibition of PIF pocket phosphorylation results, via either a single signalling cascade branch, but most likely via multiple synergistic or combinatorial signalling cascades, in lifespan extension. The strength of the down regulation, by combining not only a reduction in PIF pocket functionality, but also by reducing the expression levels, did not increase the magnitude of longevity in either sex. Notably, the strong inhibition of the AGG branch of signalling resulted in early mortality in males. This phenotype is not uncommon in very strong IIS/TOR network mutants (Slack *et al.*, 2015). We can therefore conclude that the AGC branch is involved in longevity in both females and males, but that the optimal signalling window is dimorphic. While females benefit from any inhibition, both moderate and strong, males mostly benefit from mild inhibition. This is a distinctly different phenotype than observed in the PDK-1^{PH} mutants, as females benefitted from strong reduction of the AKT branch. It emphasizes the fact that we are dealing with two different branches of the IIS/TOR network activating longevity mechanisms.

Reduced functionality of the PIF pocket increased lifespan with roughly 20% in females and 15% in males. The involvement of various AGC kinases in longevity is not extensively studied, with a few exceptions. It is known that rapamycin extends lifespan in a S6K dependent manner, and down regulation of S6K by itself extends lifespan (Juricic *et al.*, 2022; Zhang *et al.*, 2022). Female females consistently show greater longevity with rapamycin treatment. Furthermore, S6K$^{-/-}$ results in a 19% increase in lifespan in females, but no extension in males, implying a sexual dimorphism (Selman *et al.*, 2009). The exact mechanisms of S6K dependent longevity are unknow but new evidence suggest

that the TORC1-S6K-Syx13 axes regulates endolysosomal morphology, inflammageing and immunosenescence, thereby contributing to longevity.

Additionally, downregulation of MEK, resulting in activation of AOP results in lifespan extension in *Drosophila* (Slack *et al.*, 2015). MEK is not only activated by RAF, but can also be phosphorylated by PDK-1 (Sato, Fujita and Tsuruo, 2004; Slack *et al.*, 2015). The RAS-MEK-ERK pathway activates AOP, and FOXO activation results in enhanced translation of *Aop*. Both FOXO and AOP bind the same genomic loci and affect similar transcriptional programmes. (Alic *et al.*, 2014; Dobson *et al.*, 2019). Their collaboration is not straight forward and depending on the promotors involved, AOP either moderates or promotes FOXO activity. It is clear however, that they oppose the gene-regulatory activity of PNT, which knock down can extend lifespan. Activation of the RAS-MEK-ERK pathway can thus result in FOXO like expression and longevity, potentially in the PDK-1^{PIF} mutants.

Additionally, reduced activation of PKA, a PIF pocket dependent target of PDK-1, increases lifespan and delays onset of ARDs in mice (Enns and Ladiges, 2010). Research has shown reduced PKA signalling can function as caloric restriction, an intervention extending lifespan in many different species (Wei *et al.*, 2008). While evidence in mice is abundant, the one study affecting PKA signalling, did improve neuronal ageing, but did not affect lifespan (Yamazaki *et al.*, 2007). PDK-1^{PIF} longevity could be regulated via this pathway, but more likely are those described above and associated with longevity in *Drosophila.*

Clearly, many investigated, and potentially undiscovered, AGC kinases play a role in longevity. Pharmacological interventions are used to investigate which signalling cascades work synergistically or independently to promote lifespan in the PDK-1^{PIF} mutants. Cox proportional hazard analysis resulted in no significant difference in risk of dying upon rapamycin treatment in either female or male PDK-1$^{PIF/PIF}$ mutants. The inability of rapamycin to positively affect female or male PDK-1$^{PIF/PIF}$ lifespan strongly suggests that PDK-1$^{PIF/PIF}$ longevity is mainly mediated via TORC1. PDK-1$^{PIF/PIF}$ males treated with rapamycin exhibit early life mortality, as observed with the strong hypermorphs. While early mortality can be interpreted as antagonistic synergetic interaction, more likely is that both interventions act on the same signalling node. The strong inhibition pushed TORC1 signalling out of the optimal signalling window. Indeed, this has been observed previously, as potent downregulation of TORC1 in a strongly down regulated IIS signalling environment can be detrimental (Bjedov et al., 2010). Conclusively, TORC1-S6K signalling is reduced in the PDK-1^{PIF} mutants and additional inhibition does not extend and can even diminish longevity.

Trametinib treatment extended lifespan in the PDK-1$^{PIF/PIF}$ mutants of both sexes, a unique observation in all epistasis experiments. In females we see that longevity is expanded by 6% upon inhibition of the MEK-EKR pathway, which is less than half of the gain of longevity in controls. Similarly, cox proportional hazard analysis demonstrated a significantly reduced risk of dying in female PDK-1$^{PIF/PIF}$ flies treated with trametinib. Thus, trametinib acts additively to reduced AGC signalling in females. While longevity was observed in the male PDK-1$^{PIF/PIF}$ flies treated with trametinib, no significant reduction in hazard ratio was observed.

Taken together, we can conclude that MEK-RAS pathway works synergistically with the PIF pocket dependent signalling, at least in females. However, potential mild down regulation of MEK in PDK-1^{PIF}

mutants cannot be ruled out. If regulation is present, inhibition is not strong enough to push MEK-ERK signalling into the most optimal longevity window. The transcription factor AOP, responsible for lifespan effects of trametinib, is therefore predicted to play a minor, if any, role in PDK-1^{PIF} mediated longevity.

The requirement of priming of the background by reducing AGC signalling, could explain the sexual dimorphisms previously observed IIS/TOR network mutants. Interventions need to reduce signalling of multiple branches simultaneously, without hitting detrimental signalling windows, to extend lifespan in males. The optimal activation window of AGC branch of signalling seems to be extremely tightly regulated. Double treatment with rapamycin and trametinib, resulted in a reduction in longevity in males and abolished the longevity effect in females, showing a clear antagonistical effect. This effect was specific to the reduced AGC signalling environment, as the PDK-1$^{PH/PH}$ mutants showed increased survival in females. These results accentuate the difference in signalling between the AKT and the AGC branch of signalling in the IIS/TOR network.

In conclusion, we are the first to show that PIF pocket mutation is not only possible, but extends lifespan in *Drosophila.* Thusfar, the PDK-1$^{PIF/PIF}$ mutants longevity seems to be primarily regulated by down regulation of the rapamycin targets, hence the TORC1-S6K axis, in both sexes. However, the optimal signalling window to promote longevity is sex specific. The RAS-MEK-ERK pathway, affecting the activation of the transcription factor AOP, most likely also contributes to PIF pocket dependent longevity, but in a minor way. To test if AOP is necessary for PDK-1^{PIF} dependency, one should create a PDK-1$^{PIF/PIF}$; PNT^{P1} mutant.

4.4.3 Alternative routes to starvation resistance in Drosophila

By investigating phenotypes typically associated with IIS/TOR mutants, I hoped to triage apart which signalling cascades are affected in the PDK-1^{PIF}mutants. There is much evidence for the essential role of FOXO in starvation resistance, and two key players in lipid mobilization and utilization are activated via FOXO (Luong *et al.*, 2006; Kramer, Slade and Staveley, 2008; Wang *et al.*, 2011; Han *et al.*, 2022). However, FOXO activation is not regulated by PDK-1 PIF pocket signalling cascades, except for the interplay between AOP and FOXO. Yet, this interaction is tissue specific and the RAS-MEK-ERK pathway only seems to be mildly activated in PDK-1^{PIF} mutants.

While regulation of the lipid pathways seems to be FOXO dependent, autophagy and translational responses to starvation are classical responses to rapamycin treatment. 4E-BP, which is transcriptionally regulated by FOXO and phosphoraly regulated by TORC1, is essential for starvation resistance (Tettweiler *et al.*, 2005). 4E-BP is not the only key component in starvation resistance under TORC1 control, both transcription factor FKH and ATG autophagy genes regulated in a TORC1 dependent manner (McPhee and Baehrecke, 2009; Cuesta, Fan and Rand, 2014; Bolukbasi *et al.*, 2017). FKH regulates lipid storage in the gut thereby affecting organismal TAG levels. Unsuitably, none of these mechanisms are regulated by a PDK-1 PIF pocket dependent interaction. Conversely, PIF pocket dependent substrate S6K is not essential for improved starvation resistance in rapamycin fed flies and thus be uncoupled from this phenotype.

Down regulation of PIF pocket dependent signalling resulted in an increase in starvation resistance in both males and females. Both the homozygous and the heterozygous stronger hypermorph improved starvation resistance with 35+% in females. In the males only the homozygous PDK-1$^{PIF/PIF}$ mutants displayed improved survival, showing once again a sex specific optimal window of activation. As discussed above the conventional pathways studied in starvation resistance, cannot account for the AGC branch enhanced starvation survival. Research in mice has shown that PKA regulates the body mass ratio. Loss of the regulatory subunit of PKA expressed in the brain and adipose tissue, resulted in lean mice who remain insulin sensitive during adulthood (Enns *et al.*, 2009). PKA consists of two regulatory subunits, maintaining the two catalytic subunits inactive. The catalytic subunits Cα and Cβ have distinctive expression patterns in mice tissues. Activation by cAMP results in the release of the catalytic subunits, hence the activation of their downstream targets. Especially Cβ is associated with a change in metabolism and susceptibility to obesity (Yu *et al.*, 2004; Enns *et al.*, 2009). Not only mice data links PKA signalling with lipid metabolism. Hormones secreted from the enteroendocrine cells (EEs) regulate systemic lipid homeostasis via a PKA dependent signalling cascade (Song, Veenstra and Perrimon, 2014). Upon starvation EE secreted hormones reduce lipogenesis in ECs and the fatbody. Also under the regulation of PKA is salt-inducible kinase (SIK) (Takemori and Okamoto, 2008). Mutation of SIK increases TAG and glycogen stores and improved starvation resistance (Choi, Kim and Chung, 2011).

Taken together, PDK-1 PIF pocket dependent PKA signalling can explain the starvation resistance phenotype in PDK-1^{PIF} mutants. It would therefore be interesting to investigate if the PKA signalling cascade is involved in the starvation resistance phenotype observed in the PDK-1^{PIF} mutants. Combining the PDK-1^{PIF} mutants with *DCO* mutant, the gene encoding for the major catalytic subunit of PKA, would be highly interesting (Majercak, Kalderon and Edery, 1997).

Both the PDK-1$^{PIF/PIF}$ and the PDK-1$^{PIF/null}$ mutants showed increase in TAG content in a sex independent manner. While the AGC signalling branch clearly regulated TAG storage, it can be uncoupled from starvation resistance and increased translation of either *bmm* or *lip4.* Overall, we see a strong starvation phenotype which is potentially regulated via a PKA dependent mechanisms.

4.4.4 Stress resistance and their telling tales about signalling pathways

Maintaining healthy tissue homeostasis is crucial for longevity (Nie *et al.*, 2022). The ability to neutralize stressors that are encountered throughout life is therefore often increased in long-lived mutants (Grönke et al., 2010). An organism's ability to disarm oxidative radicals, which if left unchecked causes a general decline in homeostasis, is coordinated by a delicate interplay of signalling networks. FOXO mediated longevity results in elevated mitochondrial respiration regulation, implying oxidative stress must be properly controlled in IIS mutants (Tain *et al.*, 2017). Furthermore, rapamycin treatment improved survival on paraquat (Ravikumar *et al.*, 2006; Feng *et al.*, 2019). Paraquat treatment increases superoxide concentration in *Drosophila* with ±30% at concentrations of 20 mM. Mitochondrial SOD was upregulated and required in wild type flies to combat the heightened superoxide levels (Hosamani and Muralidhara, 2013).

A reduction in AGC signalling resulted in improved female survival on paraquat, independent of mild or strong downregulation of the branch. Strikingly, male PDK-1^{PIF}mutants exhibited reduced survival

on paraquat. It strongly suggests that the AGC kinase branch of signalling decreases the expression of SOD in a sex specific manner. The failure to survive paraquat is not inherent to males, as previously no gender specific difference in survival on paraquat was observed (Hosamani and Muralidhara, 2013; Pomatto *et al.*, 2017). Ubiquitous downregulation of the IIS/TOR network enhanced sensitivity specifically in males, as both the PH domain and the PIF pocket mutants demonstrate heightened susceptibility to oxidative stress induced by paraquat. Conversely, the ability to deal with oxidative stress induced by H_2O_2 is equally expanded both sexes. Taken together, the AGC branch of IIS signalling affects oxidative stress resistance in both sexes, but not in equal manners. Understanding the differences between the sexes, one could investigate the ability to upregulate SOD and CAT expression in mitochondria upon paraquat and H_2O_2 stress. Further experiments, investigating the efficiency of the complex I-III enzyme can be investigated.

Known signalling pathways regulating mitochondrial respiration and oxidative resistance are not modulated in a PIF pocket dependent manner. Nor FOXO or TORC1 regulated transcription factor FKH and 4E-BP, both required for oxidative stress resistance, are downstream targets regulated in a PDK-1 PIF pocket dependent manner (Tettweiler *et al.*, 2005; Slack *et al.*, 2015; Bolukbasi *et al.*, 2017). We have to conclude that the AGC kinases play a role in oxidative stress resistance, but currently we are unaware which pathway is involved. Generating a new phosphoproteomics data set, including samples before and during oxidative stress, would help to pinpoint which signalling cascades and mechanisms are involved in PDK-$1^{PIF/PIF}$ mediated resistance.

Like oxidative stress resistance, xenobiotic stress is associated with longevity, sometimes even via similar pathways. The role of CYP genes and other enzymes involved in xenobiotic metabolism is not only linked to longevity in *Drosophila*, but also in humans. It is estimated that genetic variability in the xenobiotic enzymes accounts for 7.7% of the change to survive beyond the age of 89 years (Crocco *et al.*, 2019). Detoxification of xenobiotics is not solely responsible for longevity as overexpression of CYP genes does not improve longevity while improving DDT resistance (Daborn et al., 2002; Pedra et al., 2004; Schmidt et al., 2017).

Resistance to xenobiotic stress is firmly linked with AKT regulated pathways, as discussed in the PDK-1^{PH} section. Nonetheless, the exact mechanisms involved in activation of *cyp* genes, required for phase I over xenobiotic metabolisms, are poorly understood but investigated. The human aryl hydrocarbon receptor (AhR) is directly activated by binding to both harmful endogenous- and xeno-biotics. Upon activation, AhR translocates to the nucleus and binds to the xenobiotic responsive element (XRE), inducing the expression both phase I and II enzymes. Among the genes containing a XRE element are NF-κB and NRF2. The expression of *Nrf2* is associated with increased expression of phase II enzymes, GSTs, UGTs, sulfotransferases, carboxylesterases and others (Xu, Li and Kong, 2005; Kennedy and Tierney, 2012). While gene expression is controlled by AhR, phosphorylation of NRF2 is mediated by AKT (Wang *et al.*, 2008). NF-κB is transcriptionally activated by AhR, but activity is regulated via a phosphorylation signalling cascade via PKC, a PIF dependent PDK-1 substrate. Evidence regarding the role of NF-κB in xenobiotic metabolism is conflicting. While it is clear there is interplay between NF-κB and NRF2, the effect is not one directional (Gao *et al.*, 2022). Induction of immune response, which NF-κB is known for, often leads to a reduction in xenobiotic metabolism (Xie and Tian, 2006). Lastly, MEK-ERK signalling is involved in the regulation of glutathione-S-transferases (GSTs) and cellular detoxification genes (McElwee *et al.*, 2007a; Nässel, Liu and Luo, 2015).

The AGC branch is clearly involved in xenobiotic metabolism, as we see an improved resistance to DDT in females and males. Mild and strong inhibition of the pathway results in enhanced longevity on DDT in females, while strong inhibition of the AGC branch was required to extend lifespan in male *Drosophila.* As described above, the pathways involved in xenobiotic resistance are still poorly understood. None of the previously described pathways are regulated in a PIF pocket PDK-1 dependent manner, with the notable exception of the MEK-ERK axis and the activation of NF-κB. The role of NF-κB is controversial and concluding this pathway is responsible for the resistance phenotype is imprudent. Unfortunately, the significant down regulation of Cyp6w1 in the PDK-1$^{PIF/null}$ mutants, did not help elucidate which mechanism are involved in the improved xenobiotic resistance. As with understanding the signalling cascades involved in the oxidative stress resistance, having a better overview of the phosphorylation signalling cascades affected in the PDK-1^{PIF} mutants would benefit further research into the xenobiotic regulation of the AGC signalling branch.

While the exact downstream pathways in the PDK-1^{PIF} mutants remain elusive, we can conclude they are not only improving lifespan but also generate a healthy and robust homeostasis. Although the many IIS/TOR network interventions had hinted at the potential of PDK-1 as health and longevity modulator, the struggle to create viable PIF pocket mutants in mice indicated how precisely the downregulation had to be targeted. It is therefore remarkable, that we generally see such sticking improvements in stress resistance.

4.4.5 Running never stops

Negative geotaxis performance is a useful metric for quantifying *Drosophila* general health, but not for longevity (Wilson *et al.*, 2020). Regulation and maintenance of cytoskeleton turnover is crucial for healthy muscles (Perkins and Tanentzapf, 2014). Down regulation of PIF-dependent signalling induces an astonishing improvement in climbing from the early adulthood onwards. Both homozygous and heterozygous down regulation provided protection against the age-related decline observed in the controls. At old age the PDK-1^{PIF} mutants retained roughly 50% of their youthful climbing ability. As with the AKT signalling branch, AGC signalling most likely improved the general health of both muscle and neurons involved in locomotion in *Drosophila,* thereby ensuring healthy elderly individuals.

4.4.6 Forever young in the gut

Homeostasis is a interplay between nutrient availability and environmental cues, which ultimately get disbalanced with age (O'Brien *et al.*, 2011). Intestinal health can be seen as a combination of preventing cancerous un-controlled cells division and ensuring baseline of healthy cells which make up intestinal barrier. Intestinal health is a predictor for mortality in *Drosophila* and can be labelled a fly ARD (Rera, Clark and Walker, 2012).

Upon investigating the mitotic activity in the PIF pocket PDK-1 mutants, we saw a clear reduction in the intestine. Proliferation rates were severely reduced during all stages of life in the PDK-1^{PIF} mutants. Both the homozygous and the heterozygous stronger hypermorph effectively reduce mitotic activity in the gut. Even at old age there was no significant increase in pH3+ levels compared to young flies,

suggesting that this ARD is not only delayed in onset but completely abolished by the reduction in AGC signalling.

TOR signalling is a key component of maintaining balance in proliferation, but the PIF pocket dependent downstream target of TOR, S6K is not involved in proliferation rate control in ISC (Kapuria *et al.*, 2012; Quan *et al.*, 2013). The reduced activation of S6K in PDK-1^{PIF} mutants can thus not explain the strong mitotic phenotype observed. Outside of the TOR network, but equally associated with proliferation control is the RAS-MEK-ERK pathway. PDK-1 activation of MEK is often associated with metastasis in human cancers, making it a likely candidate to affect proliferation rates in PDK-1^{PIF} mutants (Du *et al.*, 2015). However, neither trametinib nor adult-onset expression of AOP^{ACT} changed proliferation rates intestine of young or old flies. Neither did it improve gut barrier function, thereby clearly dismissing this pathway as an actor for PDK-1^{PIF} dependent improvement of ISC proliferation. Additionally, we observed a reduction in the dephosphorylation process of ERK^{198} in PDK-1$^{PIF/PIF}$ mutants, resulting in a more constantly active ERK. Increased RAS-MEK-ERK pathway activity would imply an increased mitotic activity rather than a decrease. In conclusion, the MEK-ERK pathway inhibition cannot explain reduced proliferation by diminished AGC branch signalling.

As none of the pathway above explain how reduced TORC1 signalling affects ISC proliferation another signalling cascade must be involved. Indeed, reduced TORC1 signalling positively affects histone levels in *Drosophila* intestine via a non-canonical mechanism (Lu *et al.*, 2021). Expression levels of histone proteins play a key role during ageing (Feser *et al.*, 2010; Benayoun, Pollina and Brunet, 2015; Lu *et al.*, 2021). Inhibition of TORC1 results in an increase in Histone 3 and Histone 4 gene (*H3/H4*) mediated by eukaryotic translation initiation factor 3 (eIF3). Although slightly counter intuitive, as reduced IIS/TOR network signalling usually reduces expression, a complex interplay between inhibition of eIF4 and increased translation via the eIF3-specialised pathway, allows inhibition of TORC1 to increase translation (Lee *et al.*, 2016; Saxton and Sabatini, 2017). Strikingly, rapamycin dependent downregulation of mitotic activity in the ISC is dependent on *H3/H4* expression (Lu *et al.*, 2021). Overexpression of *H3/H4* resulted in a lifespan extension, which could not be further extended by rapamycin. Lastly, elevated *H3/H4* expression resulted in in upregulated transcriptional regulation of autophagy. While PDK-1 does not affect phosphorylation of TORC1 via a PIF pocket dependent mechanism, phosphorylation of the N-terminal tail of H3 is regulated by various PIF pocket dependent PDK-1 substrates, such as PKC and PKA (Y. Wang *et al.*, 2001; Dawson *et al.*, 2009).

While neither the TORC1-S6K, TORC1-FKH nor the MEK-ERK axis are regulated by PDK-1 PIF pocket dependent interaction, H3 phosphorylation is. It might be this pathway which is responsible for the strong downregulation of mitotic activity in the PDK-1^{PIF} mutants. To investigate this hypothesis in the PDK-1^{PIF} mutants, *H3/H4* could be knocked-down in the enterocytes. If indeed PKA and PKC regulation of H3 is mainly responsible for the phenotype, it should be attenuated. If other pathways are involved, some residual protection against elevated mitotic activity should remain.

Reduced signalling in the AGC branch, potentially via H3 regulation, abolished any age-related increase in pH3+ cells. While over-proliferation must be prevented, too much inhibition of mitotic activity by inhibiting the IIS/TOR network is harmful (Biteau *et al.*, 2010; McLeod *et al.*, 2010; Kapuria *et al.*, 2012). By exposing the PDK-1^{PIF} mutants to E*cc15*, a mild enteropathogen, the ability to induce a synchronized and transient proliferative response in the epithelium was investigated. Infection with *Ecc15* resulted

in a robust upregulation of ±5 fold in pH3 positive intestinal cells in young flies. Upregulation is significant during adulthood in the PDK-1$^{PIF/null}$ flies at day 25 and the PDK-1$^{PIF/PIF}$ flies at day 35. While there is some inconsistency, this is probably due to the big variation and repetition might give enough power to prove significance at in both genotypes at both ages. Taken together, we can conclude that while the ISCs are tightly controlled in the mitotic behaviour, when required for repair due to infection, activation is robust and even stronger than wild type like controls.

Whereas control of proliferation rate of ISCs prevents cancerous formation, barrier function is also mediated by healthy enterocytes. Autophagy is essential for rapamycin-mediated health improvements in the intestine (Schinaman *et al.*, 2019b). Not only is autophagy essential, lifelong and short term upregulation is sufficient to maintain intestinal health extend lifespan in *Drosophila* (Bjedov *et al.*, 2010; Regan *et al.*, 2016; Juricic *et al.*, 2022). Upregulation of autophagy in the enterocytes remains even when rapamycin is withdrawn, contradictory to the autophagy status of the ISC which goes down after termination of treatment (Juricic *et al.*, 2022). Specifically, increased upregulation of autophagy in the enterocytes not in the ISC, improves barrier function and longevity (Juricic *et al.*, 2022).

Supplementary to reduced over-proliferation in the gut, we see a strong upregulation of autophagy in PDK-1$^{PIF/PIF}$ and PDK-1$^{PIF/null}$ intestines. This is a distinctive phenotype regulated by the AGC branch, as PH domain inhibition did not affect autophagy. The upregulation of *H3/H4* affects autophagy levels in the intestine, and can explain this phenotype. Additionally, PIF pocket dependent signalling cascade from containing PKC and MEK have been implicated in the regulation of autophagy. Like with the over-proliferation phenotype, the involvement of H3 could be investigated. Additionally, upregulation of the RAS-MEK-ERK pathway, by overexpression of PNT^{CA} can shed light on the role of MEK in the regulation of autophagy in PDK-1^{PIF} mutants. To further investigate improved intestinal health, barrier function can be investigated by analysing tight junctions and performing the smurf assay. If indeed pH3+ levels in the intestine of PDK-1^{PIF} flies is dependent on *H3/H4* expression, both assays should also be investigated in this background.

Lastly, we in investigated survival on a *Ecc15* infection, to have an indication of gut barrier function. Only severe downregulation of the AGC branch resulted in prolonged survival. Survival on *Ecc15* infection is most likely an interplay between maintaining a healthy barrier and a proper immune response. NF-κβ involved in the regulation of immunity, is regulated via a PKC related mechanism. Additionally, the TORC1-S6K-Syx13 axes regulates endolysosomal morphology, inflammageing and immunosenescence, thereby making it a likely candidate for PIF pocket dependent improved *Ecc15* survival. Expression of either NF-κβ or Syn13 could be investigated with qPCR or the creation of a new phosphoproteomics data set. Additionally, immune markers in the gut can be quantified.

4.5 Using *Drosophila* to investigate human pharmaceutics

4.5.1 Being human affects development but not longevity

The idea to use *Drosophila* to investigate human genes is not completely novel. Previously, a human gene has been expressed with fat body specific GAL4/UAS line to investigate the mechanisms of GLUT4

activation (Crivat et al., 2013). While adding expression of a human gene to Drosophila is impressive, the replacement of a Drosophila gene sequence by the human sequence is even more daring (Balasov, Akhmetova and Chesnokov, 2020; Savitsky et al., 2020). This has been accomplished in an epilepsy model investigating the α-subunit of heterotrimer G proteins. The resulting flies had normal weight, locomotion and longevity, indicating that humanization did not negatively affect them (Savitsky et al., 2020). Ultimately, humanized model organisms could be used to develop human medication regimes against ageing.

The ability of the PDK-1^{Hum} to rescue lethality of the PDK-1^{null} mutants, suggested a relatively normal interaction between PDK-1 and its substrates. As discussed before many single residue mutations in PDK-1 are lethal, it can thus be concluded that the PH kinase domain functions at least moderately well in the humanized PDK-1. Additionally, the humanization has most likely not affected the other kinase domains like ATP binding site, catalytic fold or the PIF pocket. The mild developmental delay of ½ day is comparable to the delays observed in the PDK-$1^{PH/PH}$ and PDK-$1^{PIF/PIF}$ mutants. Strong inhibition in the heterozygous hypermorphs of either pathway resulted in a stronger developmental delay, so we can conclude that while the PDK-1^{Hum} are slightly affected in the PDK-1 dependent signalling cascades, the effect must be relatively mild. The PDK-$1^{Hum/Hum}$ female mutants have a reduced size. Conversably, the PDK-$1^{PH/PH}$ had no significant reduction is size, implying maybe not only the PH domain is affected. The viability of the humanized mutants was not affected, as observed by the wild type like eclosion percentage. Taken together, it suggests a delay in egg laying rather than such a severe reduction in fecundity. To test this hypothesis whole life fecundity should be observed.

Humanization of PDK-1 strongly affected reproduction in both sexes. The male humanized mutants were sterile and could not produce any viable offspring, regardless of the genotype of the mother. Female PDK-$1^{Hum/Hum}$ were able to lay viable eggs, but showed a severe reduction in fecundity of roughly 50%. As PDK-1 plays an important role in many signalling cascades crucial for development, it is not surprising that a minor reduction in protein functionality amplified in serious developmental phenotypes (Lawlor *et al.*, 2002). How humanization of the PH domain reduced functionality is currently unclear. It might affect the interaction with PIP_3 due to a lower affinity or the position of the PH domain in regard to the main kinase domain has changed. Since PDK-1 phosphorylation of AKT depends on close proximity, a change in the relative position of the PH domain can affect this interaction. Lastly, the PH domain is suggested to play a n inhibitory role in kinase autophosphorylation (Levina *et al.*, 2022). It is currently unclear which disrupted interaction caused the strong developmental phenotype.

I hypothesize that a the humanized PDK-1 has a general reduction in functionality, thereby slowing down the process of growth and proliferation. Mild reduction in PH domain dependent signalling resulted in normal weight, ½ day delay and no fecundity phenotype. Strong inhibition of the PH domain caused significant smaller flies, delayed development with a day and reduced fecundity with 30%. The PDK-1 humanization flies show significant reduction in weight, ½ day delay in development and a strong fecundity phenotype. Phenotypically there are in between mild and strong inhibition of the PH domain mutants.

In adulthood most of the morphological changes have been completed and signalling via PDK-1 is most likely at lower intensity. The moderate malfunctioning of the humanized PDK-1 is therefore not as

limiting in the signalling cascades in adult flies. Humanization did not affect longevity either positively or negatively. Overall, the humanization of the *Drosophila* PDK-1 was surprisingly smooth.

4.5.2 The inability of 2-O-Bn to affect humanized PDK-1

Due to the nature of the humanized PDK-1 protein, a PH domain specific inhibitor was needed. 2-O-Bn is a Type III PDK-1 specific inhibitor developed by Falasca et al. (2010). Type III inhibitors target allosteric domains rather than of the catalytic domains. 2-O-Bn targets the PH domain of human PKD-1. A wide variety of kinases contain a PH domain, so selectivity towards PDK-1 is complicated. However, the human PDK-1 PH domain has a unique "bulb" structure, allowing for 2-O-Bn specificity towards human PDK-1 (Komander et al., 2004). Typically, type III inhibitors are not as potent as type I (GSK233) or type II (MP7) inhibitors. 2-O-Bn's IC_{50} value is 26.5 nM, which is 2.6 times higher than the IC_{50} value of GSK233 and 13.4 times higher than the IC_{50} value of MP7 (Falasca et al., 2010). Lack of potency is observed when As-PC-1 cells are treated with 2-O-Bn, to reduce cell survival concentrations 15 times higher than either GSK233 or MP7 are needed, emphasizing the lack of potency of 2-O-Bn (Emmanouilidi et al., 2019). While not being extremely potent, 2-O-Bn is highly specific due to targeting the bulb structure in the PDK-1 PH domain. At concentrations 350 times the IC_{50} value, no other kinases are inhibited. Specifically, no AKT isoforms are inhibited (AKT IC_{50} 3000 > PDK-1 IC_{50}).

The mechanism of action of 2-O-Bn is direct competition with PIP_3 to bind the PH domain of PDK-1. Occupation of the PH domain with 2-O-Bn prevents recruitment to the plasma membrane, hence conformational changes and close proximity to AKT. Indeed, 2-O-Bn reduces the PDK-1 dependent AKT phosphorylation at Thr308 at concentrations of 20 µM in SKOV-3 and PC3 cells (Falasca et al., 2010). Based upon the proposed model of autoinhibition by the PH domain, allosteric inhibitors targeting the PH domain could prevent any phosphorylation, hence kinase activity of PDK-1 (Levina et al., 2022). Everything considered, the mechanism of action of 2-O-Bn seems to be two-fold; firstly, there is competitive inhibition of the PDK-1 PH domain. Secondly, there might be a reduction in trans-autophosphorylation, affecting the whole PDK-1 signalling pathway.

Not only cells are affected by 2-O-Bn, it has shown efficiency *in vitro*. Mice treated with 12,5 mg/kg^{-1} of 2-O-Bn showed reduced tumour growth after 12 days by inducing pro-apoptotic pathways (Falasca *et al.*, 2010). Viability in As-PC-1 is reduced when concentrations of 30µM 2-O-Bn are used. It was therefore decided to treat PDK-$1^{Hum/Hum}$ flies with a concentration range of 0.35 to 35 µM. Previous experiments had shown a delayed development in PDK-1^{PH} flies. Additionally, larvae eat more than adult flies, thereby increasing the bioavailability of the drug.

I reared larvae from egg till fly on food containing either 0.35, 3.5 or 35 µM of 2-O-Bn. Unfortunately, the treatment with the PH domain inhibitor did not result in any phenotypical developmental changes. Successful inhibition of the humanized PDK-1 PH domain should either have resulted in lethality, as observed in mice with disrupted PH domain or in developmental delays as observed in the PDK-$1^{PH/PH}$ and PDK-$1^{PH/null}$ mutants (McManus *et al.*, 2004). It must therefore be concluded that neither concentration of 2-O-Bn resulted in sufficient PH domain inhibition to induce a phenotype. There are many potential reasons for lack of response. The concentration used can be outside of the therapeutical window. We are currently unaware of the bioavailability in larvae or strength 2-O-Bn metabolism. Investigating drug and metabolite levels in larvae fed with 2-O-Bn with mass

spectrometry would provide useful insights in the bioavailability. A second reason for the lack of efficiency could be a lack of affinity despite humanization of the PDK-1 protein. 2-O-Bn is excessively optimised to target the human PDK-1 PH domain and not the neighbouring PH domain of AKT. While we have replaced the PH domain of the *Drosophila* protein, we cannot exclude altered folding or post translational modifications. The first stage of protein structure, is the amino acid sequence. By humanizing the *Pdk*-1, we replaced the *Drosophila* amino acids sequence with the human sequence. The secondary stage, the α-helices, β-sheets formation is mostly based on hydrogen bonds between NH and CO groups on the peptides. The tertiary structure refers to the three-dimensional arrangement of all the previously named elements. It is tertiary phase of protein folding which determines where the domains and folds containing crucial motifs are placed (Sun, Foster and Boyington, 2004). After folding, post translational modifications can be added, allowing for regulation of location and activity. By humanizing the *Pdk-1* gene, we have humanized the primary step of protein folding. The formation of α-helices and β-sheets should mostly be similar in humans and *Drosophila*. Unfortunately, controlling the tertiary structure is impossible as this is organism specific. Modelling interaction of the humanized PDK-1 protein with 2-O-Bn could give some insight in affinity, but based on biological experiments, affinity and potency seem to be low. To investigate the potency of 2-O-Bn on Humanized PDK-1, one could perform a competitive ligand binding ELISA.

Unfortunately, humanization of the PH domain in *Drosophila* did not allow for investigating the ability to repurpose PDK-1 inhibitors for ageing. However, using different integration techniques targeting the full kinase or higher concentrations could still allow for successful inhibition of PDK-1^{Hum}. Understanding downstream signalling cascade from kinase domain specific inhibition by pharmaceuticals can be key in treating human ARDs. Many ARDs are still poorly understood and cannot be treated by conventional pharmaceutics. Using model organism with limited lifespan to investigate the potential of these drugs is very attractive. While organ-on-a-chip technology is upcoming and even body-on-a-chip is now possible, this is still a far cry from organismal biology (Ma *et al.*, 2021; Ingber, 2022). Humanization of short-lived model organism might provide an excellent research tool to investigate the potential to repurpose excising pharmaceuticals for treatment of ageing or ARDs.

4.6 Theories on ageing

4.6.1 The evolution of theories of ageing

There have been many theories on ageing throughout the years, while some have been rejected over the years, like the Free radicals, Wear and tear and Disposable soma theory, others have been adjusted and developed (Weismann, 1882; Harman, 1956; Kirkwood, 1977).

The antagonistic pleiotropy theory proposed that evolutionary selecting gene alleles enhancing developmental and reproductive traits will also have deleterious effects (Williams, 1957). Due to the pleiotropic nature of the genes, one allele can positively affect early traits and negatively affect processes involved in late stages of life, making them antagonistic. The trade-off concept of ‘paying’ for benefits received in early life, creates vigorous young and decrepit old organisms. Strikingly, evolution therefore actively, but unknowingly, drives ageing.

A continuation of this theory is the hyperfunction theory. While antagonistic pleiotropy implies that one allele can have both beneficial and negative effects depending on the life stage, it does not provide a more exact mechanism. The hyperfunction theory suggest that alleles are evolutionary optimised to signal at an intensity most beneficial for early traits. However, with age the requirements of the cellular and tissue landscape change. Unfortunately, the signal intensity, evolutionary optimised for development and reproduction over 1000s of years, does not adapt. There is an inappropriate purposeless continued activity of developmental programs causing age-related diseases (Blagosklonny, 2006; Gems, 2022).

Contradictory to this theory is agonistic pleiotropy; evolution selects protective mechanisms to survive until reproductive age and those same mechanisms continue to protect at greater age. An important distinction is that the mechanisms are optimised to function from the first moment of an organism life. During ageing stressors put more pressure on protective mechanisms, which ultimately fail. As the protective mechanisms fail, ARDs arise and mortality increases. However, when these pathways keep functioning properly, they will keep on protecting the organism till late age (Serrano and Blasco, 2007; Alic and Partridge, 2011).

4.6.2 Two kinase domains, two theories

The theories of ageing aim to explain ageing in black and white, trying to fit all aspects of ageing of all living beings on earth into one concept; black or white. However, the hyperfunction theory and the agonistic pleiotropy do not have to be mutually exclusive. This thesis has shown that one kinase can affect various ageing phenotypes via distinctive pathways activated in a kinase domain-specific manner. The picture of developmental and ageing phenotypes in the PDK-1^{PH} and the PDK-1^{PIF} mutants is clearly a clear mixture of both theories.

One the one hand, the long-lived PDK-1$^{PH/PH}$ mutant exhibits no reduction in size or fecundity. Additionally, I have seen improvement in stress resistance and general health in the PDK-1$^{PH/PH}$ mutants. Resistance mechanisms are important to reach the reproductive stage of live and to fight for a mate. Many of the PDK-1$^{PH/PH}$ mutant phenotypes are hypothesized to be regulated by FOXO. Along these lines of reason is FOXO a gene fitting in perfectly with the agonistic pleiotropy theory. FOXO seems to promote protective mechanism which are important from moment one in live, without negatively affecting some developmental or reproductive traits. Not only are these phenotypes observed in the PDK-1$^{PH/PH}$ mutants, they are present in the stronger hypermorph too. A stronger downregulation of the pathways resulted in an even greater longevity. A stronger upregulation of protective mechanisms is clearly beneficial in *Drosophila*.

On the other hand, the PDK-1$^{PIF/PIF}$ mutants showed equal longevity, but severely affected in size and fecundity. Conversely, the PDK-1$^{PIF/PIF}$ mutant show improved stress resistance and general health. It implies that the reduced signalling intensity in the PIF dependent signalling cascades are now tuned for optimal ageing, but wrongly adjusted for development and reproduction. This is also observed by the early life mortality of the males. The notion that indeed reduced signalling intensity is beneficial for ageing, but detrimental for those traits is has been evolutionary optimised for, is perfectly fitting with the hyperfunction theory.

Intestinal over-proliferation is modulated by both the AKT and the AGC branch, most likely via TORC1 and H3 regulation. The active mitotic phenotype, designed to maintain barrier function and fight infection no matter the long-term consequences, can be characterized as a hyperfunction theory signalling cascade. One could say that the signalling cascade is designed to be 'trigger-happy'. At older age this results in accumulation of uncontrolled mitotic intestinal cells. The reduced signalling strength benefits intestinal health, but those same mechanism might result in altered cell cycle progression, hence reduced body size. Being bigger, stronger and faster usually results in more offspring and would have therefore been selected during evolution (Klepsatel *et al.*, 2020). Taken together, the branches regulating intestinal proliferation can be attributed to the hyperfunction theory.

Interestingly, the mortality associated ARD of intestinal dysregulation, is completely abolished in PDK-$1^{PIF/null}$ mutants but not in PDK-$1^{PH/null}$ mutants. Seemingly paradoxically, the PDK-$1^{PH/null}$ mutants show far greater longevity. Longevity can be determined by one ARD reaching lethal levels, while many others are reducing health but not increasing morbidity. Indeed, the reduction in hyperactive genes has resulted in the greatest health improvement in intestinal ARD in PDK-1^{PIF} mutants, however it seems that the activation of pro-protective mechanisms in the PDK-1^{PH} mutants provided a delay in more than one lethal ARD, thereby ensuring the longest lifespan.

In this thesis I have discussed many phenotypes which by themselves did not extend lifespan. Improvement in the ability to deal with oxidative stress or starvation might not lead to longevity, but could greatly enhance healthspan. As mentioned before, one ARD will cause mortality but many will reduce the quality of live. Reducing these ARD simultaneously, either via agonistic pleiotropy mechanisms or via late life induced hyperfunction retuning, will benefit the organism.

conclusively, one kinase acts in ways in concordance with the agonistic pleiotropy and hyperfunction theory. Understanding which pathway is what will greatly help to design anti-ageing treatment. Mechanisms belonging to agonistic pleiotropy will most likely result in beneficial effects from youth to early adulthood. Contradictory, the retuning of signalling cascades associated with the hyperfunction theory should only be done after those pathways played their crucial role during development and reproduction.

4.6.3 The regulation of ageing is by a pallet of grey

The difference between pro-longevity mechanisms and the regulation of mistuned pathways can be a big implication for human treatment of ageing. With the shifting population demographic built-up and the improved lifespan of humans, the proportion of elderly in the population increases (Eurostat, 2021). People who had fulfilling adult lives bearing increasing responsibilities; heading a company, competing at the Olympics or simply the immense task of creating and running a family, become more and more dependent on others. When simply rising from a chair or doing groceries become a hurdle due to frailty, one has to depend on family and the society to help (Searle *et al.*, 2008; Clegg *et al.*, 2013). Loss of self-sufficiency can be a struggle for some and at a dehumanizing experience for others. Studies have shown that frailty can be the main cause of death in elderly living in an elderly home (Gill *et al.*, 2010). Around the age of 60 many ARDs have their onset of and the last 20+ years of live are complex interplay of diseases progression and failing treatments (Kuan *et al.*, 2021; Li *et al.*, 2021).

Understanding which pathways require fine tuning at later age, and which can be promoted to enhance protective mechanisms, is essential to provide the ageing population with the right treatment. There are examples of both theories in humans: osteoporosis is hypothesized to be due to a continued reproductive program to provide calcium-rich milk. Continuation of the reproductive signalling intensity resulting in ARD, identifies it as a classical hyperfunction theory associated signalling cascade (Ezcurra *et al.*, 2018; Gems, 2022). Pro-longevity genes are complicated to identify in the human population(Deelen *et al.*, 2019). Longevity is barely associated with gene alleles, except for the FOXO3A gene, which is a clear example of agonistic pleiotropy gene (Willcox *et al.*, 2008). In conclusion, understanding the role of one kinase that is acting over various different pathways, belong to different theories of ageing, is crucial for the next steps in geroscience.

A complete knock out of the PDK-1 protein it would have never given us the understanding how one kinase acts on ageing via a multitude of mechanism. It is the ability to regulate distinctive pathways via two kinase domains that associates PDK-1 longevity with the two theories of ageing; agonistic pleiotropy and hyperfunction. Conclusively, even within the function of one protein nothing is black and white, like everything in life it is made up by a beautiful diversity of greys.

Table of Figures

Bibliography

Accili, D. and Arden, K. C. (2004) 'FoxOs at the crossroads of cellular metabolism, differentiation, and transformation', *Cell*. Cell, 117(4), pp. 421–426. doi: 10.1016/S0092-8674(04)00452-0.

afar (2022) *TAME - Targeting Aging with Metformin - American Federation for Aging Research*. Available at: https://www.afar.org/tame-trial#tametrialsites (Accessed: 9 November 2022).

Afschar, S. *et al.* (2016) 'Nuclear hormone receptor DHR96 mediates the resistance to xenobiotics but not the increased lifespan of insulin-mutant Drosophila', *Proceedings of the National Academy of Sciences of the United States of America*. Proc Natl Acad Sci U S A, 113(5), pp. 1321–1326. doi: 10.1073/PNAS.1515137113.

Alessi, D. *et al.* (1998) '3-Phosphoinositide-dependent protein kinase 1 (PDK1) phosphorylates and activates the p70 S6 kinase in vivo and in vitro', *Current biology : CB*. Curr Biol, 8(2), pp. 69–81. doi: 10.1016/S0960-9822(98)70037-5.

Alessi, D. R. *et al.* (1994) 'Identification of the sites in MAP kinase kinase-1 phosphorylated by p74raf-1.', *The EMBO Journal*. John Wiley & Sons, Ltd, 13(7), pp. 1610–1619. doi: 10.1002/J.1460-2075.1994.TB06424.X.

Alessi, D. R. *et al.* (1996) 'Mechanism of activation of protein kinase B by insulin and IGF-1.', *The EMBO Journal*. Wiley, 15(23), pp. 6541–6551. doi: 10.1002/j.1460-2075.1996.tb01045.x.

Alessi, D. R. *et al.* (1997) '3-Phosphoinositide-dependent protein kinase-1 (PDK1): Structural and functional homology with the Drosophila DSTPK61 kinase', *Current Biology*. Cell Press, 7(10), pp. 776–789. doi: 10.1016/S0960-9822(06)00336-8.

Alic, N. *et al.* (2014) 'Interplay of dFOXO and Two ETS-Family Transcription Factors Determines Lifespan in Drosophila melanogaster', *PLOS Genetics*. Public Library of Science, 10(9), p. e1004619. doi: 10.1371/JOURNAL.PGEN.1004619.

Alic, N. and Partridge, L. (2011) 'Death and dessert: nutrient signalling pathways and ageing', *Current Opinion in Cell Biology*. Elsevier Current Trends, 23(6), pp. 738–743. doi: 10.1016/J.CEB.2011.07.006.

Anderson, K. E. *et al.* (1998) 'Translocation of PDK-1 to the plasma membrane is important in allowing PDK-1 to activate protein kinase B', *Current Biology*. Cell Press, 8(12), pp. 684–691. doi: 10.1016/S0960-9822(98)70274-X.

Anjum, R. and Blenis, J. (2008) 'The RSK family of kinases: emerging roles in cellular signalling', *Nature Reviews Molecular Cell Biology 2008 9:10*. Nature Publishing Group, 9(10), pp. 747–758. doi: 10.1038/nrm2509.

Arquier, N. *et al.* (2006) 'Analysis of the hypoxia-sensing pathway in Drosophila melanogaster', *The Biochemical journal*. Biochem J, 393(Pt 2), pp. 471–480. doi: 10.1042/BJ20050675.

Atella, V. *et al.* (2019) 'Trends in age-related disease burden and healthcare utilization', *Aging cell*. Aging Cell, 18(1). doi: 10.1111/ACEL.12861.

Baird, L. and Yamamoto, M. (2020) 'The Molecular Mechanisms Regulating the KEAP1-NRF2 Pathway', *Molecular and Cellular Biology*. American Society for Microbiology, 40(13). doi: 10.1128/MCB.00099-20/ASSET/D3421EC7-DA28-468C-A6F1-84B2F174EF31/ASSETS/GRAPHIC/MCB.00099-20-F0006.JPEG.

Baker, N. E. (2020) 'Emerging mechanisms of cell competition', *Nature Reviews Genetics 2020 21:11*. Nature Publishing Group, 21(11), pp. 683–697. doi: 10.1038/s41576-020-0262-8.

Balasov, M., Akhmetova, K. and Chesnokov, I. (2020) 'Humanized Drosophila Model of the Meier-Gorlin Syndrome Reveals Conserved and Divergent Features of the Orc6 Protein', *Genetics*. Oxford Academic, 216(4), pp. 995–1007. doi: 10.1534/GENETICS.120.303698.

Balendran, A. *et al.* (2000) 'A 3-phosphoinositide-dependent protein kinase-1 (PDK1) docking site is required for the phosphorylation of protein kinase Czeta (PKCzeta) and PKC-related kinase 2 by PDK1', *The Journal of biological chemistry*. J Biol Chem, 275(27), pp. 20806–20813. doi: 10.1074/JBC.M000421200.

Bass, T. M. *et al.* (2007) 'Optimization of dietary restriction protocols in Drosophila', *Journals of Gerontology - Series A Biological Sciences and Medical Sciences*. Gerontological Society of America, 62(10), pp. 1071–1081. doi: 10.1093/gerona/62.10.1071.

Bayascas, J. R. (2008) 'Dissecting the role of the 3-phosphoinositide-dependent protein kinase-1 (PDK1) signalling pathways', *Cell Cycle*. Taylor and Francis Inc., pp. 2978–2982. doi: 10.4161/cc.7.19.6810.

Bayascas, J. R. *et al.* (2008) 'Mutation of the PDK1 PH domain inhibits protein kinase B/Akt, leading to small size and insulin resistance', *Molecular and cellular biology*. Mol Cell Biol, 28(10), pp. 3258–3272. doi: 10.1128/MCB.02032-07.

Bayascas, J. R. (2010) 'PDK1: The Major Transducer of PI 3-Kinase Actions', in. Springer, Berlin, Heidelberg, pp. 9–29. doi: 10.1007/82_2010_43.

Benayoun, B. A., Pollina, E. A. and Brunet, A. (2015) 'Epigenetic regulation of ageing: linking environmental inputs to genomic stability', *Nature reviews. Molecular cell biology*. Nat Rev Mol Cell Biol, 16(10), pp. 593–610. doi: 10.1038/NRM4048.

Beramendi, A. *et al.* (2007) 'Neuromuscular junction in abdominal muscles of Drosophila melanogaster during adulthood and aging', *The Journal of comparative neurology*. J Comp Neurol, 501(4), pp. 498–508. doi: 10.1002/CNE.21253.

Beretta, L. *et al.* (1996) 'Rapamycin blocks the phosphorylation of 4E-BP1 and inhibits cap-dependent initiation of translation.', *The EMBO Journal*. European Molecular Biology Organization, 15(3), p. 658. doi: 10.1002/j.1460-2075.1996.tb00398.x.

Beurel, E., Grieco, S. F. and Jope, R. S. (2015) 'Glycogen synthase kinase-3 (GSK3): regulation, actions, and diseases', *Pharmacology & therapeutics*. NIH Public Access, 0, p. 114. doi: 10.1016/J.PHARMTHERA.2014.11.016.

Biondi, R. M. *et al.* (2000) 'Identification of a pocket in the PDK1 kinase domain that interacts with PIF and the C-terminal residues of PKA | The EMBO Journal', *EMBO J*, 19, pp. 979–988. Available at: https://www.embopress.org/doi/full/10.1093/emboj/19.5.979 (Accessed: 30 April 2020).

Biondi, R. M. *et al.* (2001) 'The PIF-binding pocket in PDK1 is essential for activation of S6K and SGK, but not PKB.', *The EMBO journal*, 20(16), pp. 4380–90. doi: 10.1093/emboj/20.16.4380.

Biondi, R. M. *et al.* (2002) 'High resolution crystal structure of the human PDK1 catalytic domain defines the regulatory phosphopeptide docking site', *The EMBO Journal*, 21(16), pp. 4219–4228. doi: Doi: 10.1093/emboj/cdf437.

Biteau, B. *et al.* (2010) 'Lifespan extension by preserving proliferative homeostasis in Drosophila', *PLoS genetics*. PLoS Genet, 6(10), pp. 1–15. doi: 10.1371/JOURNAL.PGEN.1001159.

Biteau, B., Hochmuth, C. E. and Jasper, H. (2008) 'JNK Activity in Somatic Stem Cells Causes Loss of Tissue Homeostasis in the Aging Drosophila Gut', *Cell Stem Cell*. Cell Stem Cell, 3(4), pp. 442–455. doi: 10.1016/j.stem.2008.07.024.

Bjedov, I. *et al.* (2010) 'Mechanisms of Life Span Extension by Rapamycin in the Fruit Fly Drosophila melanogaster', *Cell Metabolism*. Cell Metab, 11(1), pp. 35–46. doi: 10.1016/j.cmet.2009.11.010.

Bjedov, I. *et al.* (2020) 'Fine-tuning autophagy maximises lifespan and is associated with changes in mitochondrial gene expression in Drosophila', *PLoS Genetics*. Public Library of Science, 16(11), p. e1009083. doi: 10.1371/journal.pgen.1009083.

Blagosklonny, M. V. (2006) 'Aging and Immortality: Quasi-Programmed Senescence and Its Pharmacologic Inhibition', *http://dx.doi.org/10.4161/cc.5.18.3288*. Taylor & Francis, 5(18), pp. 2087–2102. doi: 10.4161/CC.5.18.3288.

Blüher, M., Kahn, B. B. and Kahn, C. R. (2003) 'Extended longevity in mice lacking the insulin receptor in adipose tissue', *Science (New York, N.Y.)*. Science, 299(5606), pp. 572–574. doi: 10.1126/SCIENCE.1078223.

Böhni, R. *et al.* (1999) 'Autonomous Control of Cell and Organ Size by CHICO, a Drosophila Homolog of Vertebrate IRS1–4', *Cell*. Cell Press, 97(7), pp. 865–875. doi: 10.1016/S0092-8674(00)80799-0.

Bolster, D. R. *et al.* (2002) 'AMP-activated protein kinase suppresses protein synthesis in rat skeletal muscle through down-regulated mammalian target of rapamycin (mTOR) signaling', *The Journal of biological chemistry*. J Biol Chem, 277(27), pp. 23977–23980. doi: 10.1074/JBC.C200171200.

Bolukbasi, E. *et al.* (2017) 'Intestinal Fork Head Regulates Nutrient Absorption and Promotes Longevity', *Cell Reports*. Elsevier, 21(3), p. 641. doi: 10.1016/J.CELREP.2017.09.042.

Borrás, C. *et al.* (2011) 'RasGrf1 deficiency delays aging in mice', *Aging (Albany NY)*. Impact Journals, LLC, 3(3), p. 262. doi: 10.18632/AGING.100279.

Boyd, C. M. *et al.* (2005) 'Frailty, hospitalization, and progression of disability in a cohort of disabled older women', *The American Journal of Medicine*. Elsevier, 118(11), pp. 1225–1231. doi: 10.1016/J.AMJMED.2005.01.062.

Brogiolo, W. *et al.* (2001) 'An evolutionarily conserved function of the Drosophila insulin receptor and insulin-like peptides in growth control', *Current biology : CB*. Curr Biol, 11(4), pp. 213–221. doi: 10.1016/S0960-9822(01)00068-9.

Broughton, S. *et al.* (2008) 'Reduction of DILP2 in Drosophila triages a metabolic phenotype from lifespan revealing redundancy and compensation among DILPs', *PLoS ONE*. PLoS One, 3(11). doi: 10.1371/journal.pone.0003721.

Broughton, S. J. *et al.* (2005a) 'Longer lifespan, altered metabolism, and stress resistance in Drosophila from ablation of cells making insulin-like ligands', *Proceedings of the National Academy of Sciences of the United States of America*, 102(8), pp. 3105–3110. doi: 10.1073/PNAS.0405775102/SUPPL_FILE/INDEX.HTML.

Broughton, S. J. *et al.* (2005b) 'Longer lifespan, altered metabolism, and stress resistance in Drosophila from ablation of cells making insulin-like ligands', *Proceedings of the National Academy of Sciences of the United States of America*. National Academy of Sciences, 102(8), pp. 3105–3110. doi: 10.1073/pnas.0405775102.

Brown, E. B. *et al.* (2019) 'Starvation resistance is associated with developmentally specified changes in sleep, feeding and metabolic rate', *The Journal of experimental biology*. J Exp Biol, 222(Pt 3). doi: 10.1242/JEB.191049.

Buchon, N. *et al.* (2009) 'Drosophila Intestinal Response to Bacterial Infection: Activation of Host Defense and Stem Cell Proliferation', *Cell Host and Microbe*. Cell Press, 5(2), pp. 200–211. doi: 10.1016/j.chom.2009.01.003.

Campisi, J. *et al.* (2019) 'From discoveries in ageing research to therapeutics for healthy ageing', *Nature 2019 571:7764*. Nature Publishing Group, 571(7764), pp. 183–192. doi: 10.1038/s41586-019-1365-2.

Carvalho, G. B. *et al.* (2017) 'The 4E-BP growth pathway regulates the effect of ambient temperature

on Drosophila metabolism and lifespan', *Proceedings of the National Academy of Sciences of the United States of America*. National Academy of Sciences, 114(36), pp. 9737–9742. doi: 10.1073/PNAS.1618994114/SUPPL_FILE/PNAS.201618994SI.PDF.

Casamayor, A., Morrice, N. A. and Alessi, D. R. (1999) 'Phosphorylation of Ser-241 is essential for the activity of 3-phosphoinositide-dependent protein kinase-1: Identification of five sites of phosphorylation in vivo', *Biochemical Journal*. Portland Press Ltd, 342(2), pp. 287–292. doi: 10.1042/0264-6021:3420287.

Castillo-Quan, J. I. *et al.* (2019) 'A triple drug combination targeting components of the nutrient-sensing network maximizes longevity', *Proceedings of the National Academy of Sciences of the United States of America*. National Academy of Sciences, 116(42), pp. 20817–20819. doi: 10.1073/pnas.1913212116.

Chang, Y. Y. and Neufeld, T. P. (2009) 'An Atg1/Atg13 complex with multiple roles in TOR-mediated autophagy regulation', *Molecular biology of the cell*. Mol Biol Cell, 20(7), pp. 2004–2014. doi: 10.1091/MBC.E08-12-1250.

Chang, Y. Y. and Neufeld, T. P. (2010) 'Autophagy takes flight in Drosophila', *FEBS Letters*. No longer published by Elsevier, 584(7), pp. 1342–1349. doi: 10.1016/J.FEBSLET.2010.01.006.

Chen, J. *et al.* (2021) 'Effect of paraquat on cytotoxicity involved in oxidative stress and inflammatory reaction: A review of mechanisms and ecological implications', *Ecotoxicology and Environmental Safety*. Academic Press, 224, p. 112711. doi: 10.1016/J.ECOENV.2021.112711.

Cheng, X. *et al.* (1998) 'Phosphorylation and activation of cAMP-dependent protein kinase by phosphoinositide-dependent protein kinase', *Proceedings of the National Academy of Sciences of the United States of America*. National Academy of Sciences, 95(17), p. 9849. doi: 10.1073/PNAS.95.17.9849.

Chintapalli, V. R., Wang, J. and Dow, J. A. T. (2007) 'Using FlyAtlas to identify better Drosophila melanogaster models of human disease', *Nature Genetics 2007 39:6*. Nature Publishing Group, 39(6), pp. 715–720. doi: 10.1038/ng2049.

Cho, K. S. *et al.* (2001) 'Drosophila phosphoinositide-dependent kinase-1 regulates apoptosis and growth via the phosphoinositide 3-kinase-dependent signaling pathway', *Proceedings of the National Academy of Sciences of the United States of America*. National Academy of Sciences, 98(11), pp. 6144–6149. doi: 10.1073/pnas.101596998.

Choi, N. H. *et al.* (2008) 'Age-related changes in Drosophila midgut are associated with PVF2, a PDGF/VEGF-like growth factor', *Aging Cell*. Aging Cell, 7(3), pp. 318–334. doi: 10.1111/j.1474-9726.2008.00380.x.

Choi, S., Kim, W. and Chung, J. (2011) 'Drosophila Salt-inducible Kinase (SIK) Regulates Starvation Resistance through cAMP-response Element-binding Protein (CREB)-regulated Transcription Coactivator (CRTC)', *The Journal of Biological Chemistry*. American Society for Biochemistry and Molecular Biology, 286(4), p. 2658. doi: 10.1074/JBC.C110.119222.

Chou, M. M. *et al.* (1998) 'Regulation of protein kinase C zeta by PI 3-kinase and PDK-1', *Current biology : CB*. Curr Biol, 8(19), pp. 1069–1078. doi: 10.1016/S0960-9822(98)70444-0.

Chung, C. L. *et al.* (2019) 'Topical rapamycin reduces markers of senescence and aging in human skin: an exploratory, prospective, randomized trial', *GeroScience*. Geroscience, 41(6), pp. 861–869. doi: 10.1007/S11357-019-00113-Y.

Chung, K. W. and Chung, H. Y. (2019) 'The Effects of Calorie Restriction on Autophagy: Role on Aging Intervention', *Nutrients*. Multidisciplinary Digital Publishing Institute (MDPI), 11(12). doi: 10.3390/NU11122923.

Clancy, D. J. *et al.* (2001) 'Extension of life-span by loss of CHICO, a Drosophila insulin receptor substrate protein', *Science*, 292(5514), pp. 104–106. doi: 10.1126/science.1057991.

Clark, R. I. *et al.* (2015) 'Distinct Shifts in Microbiota Composition during Drosophila Aging Impair Intestinal Function and Drive Mortality', *Cell Reports*. Elsevier B.V., 12(10), pp. 1656–1667. doi: 10.1016/j.celrep.2015.08.004.

Clegg, A. *et al.* (2013) 'Frailty in elderly people', *The Lancet*. Elsevier, 381(9868), pp. 752–762. doi: 10.1016/S0140-6736(12)62167-9.

Cochemé, H. M. *et al.* (2019) 'Enhancing autophagy by redox regulation extends lifespan in Drosophila Authors', *Bioarxiv*.

Cohen, P. and Frame, S. (2001) 'The renaissance of GSK3', *Nature reviews. Molecular cell biology*. Nat Rev Mol Cell Biol, 2(10), pp. 769–776. doi: 10.1038/35096075.

Collins, B. J. *et al.* (2003) 'In vivo role of the PIF-binding docking site of PDK1 defined by knock-in mutation', *The EMBO Journal*. John Wiley & Sons, Ltd, 22(16), pp. 4202–4211. doi: 10.1093/EMBOJ/CDG407.

Cox, J. and Mann, M. (2008) 'MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification', *Nature Biotechnology 2008 26:12*. Nature Publishing Group, 26(12), pp. 1367–1372. doi: 10.1038/nbt.1511.

Crivat, G. *et al.* (2013) 'Insulin Stimulates Translocation of Human GLUT4 to the Membrane in Fat Bodies of Transgenic Drosophila melanogaster', *PLoS ONE*. PLOS, 8(11). doi: 10.1371/JOURNAL.PONE.0077953.

Crocco, P. *et al.* (2019) 'Inter-Individual Variability in Xenobiotic-Metabolizing Enzymes: Implications for Human Aging and Longevity', *Genes*. Multidisciplinary Digital Publishing Institute (MDPI), 10(5). doi: 10.3390/GENES10050403.

Cuesta, E. V.-, Fan, F. and Rand, D. M. (2014) 'Rapamycin reduces Drosophila longevity under low nutrition', *IOSR journal of pharmacy*. NIH Public Access, 4(8), p. 43. doi: 10.9790/3013-0408043051.

Cunningham, J. T. *et al.* (2007) 'mTOR controls mitochondrial oxidative function through a YY1-PGC-1alpha transcriptional complex', *Nature*. Nature, 450(7170), pp. 736–740. doi: 10.1038/NATURE06322.

Currie, R. A. *et al.* (1999) 'Role of phosphatidylinositol 3,4,5-trisphosphate in regulating the activity and localization of 3-phosphoinositide-dependent protein kinase-1', *Biochemical Journal*. Portland Press Ltd, 337(2–3), pp. 575–583. doi: 10.1042/bj3370575.

Daborn, P. J. *et al.* (2002) 'A single p450 allele associated with insecticide resistance in Drosophila', *Science (New York, N.Y.)*. Science, 297(5590), pp. 2253–2256. doi: 10.1126/SCIENCE.1074170.

Dan, H. C. *et al.* (2014) 'Akt-dependent Activation of mTORC1 Complex Involves Phosphorylation of mTOR (Mammalian Target of Rapamycin) by IκB Kinase α (IKKα)', *The Journal of Biological Chemistry*. American Society for Biochemistry and Molecular Biology, 289(36), p. 25227. doi: 10.1074/JBC.M114.554881.

Dawson, M. A. *et al.* (2009) 'JAK2 phosphorylates histone H3Y41 and excludes HP1alpha from chromatin', *Nature*. Nature, 461(7265), pp. 819–822. doi: 10.1038/NATURE08448.

Deelen, J. *et al.* (2019) 'A meta-analysis of genome-wide association studies identifies multiple longevity genes', *Nature Communications 2019 10:1*. Nature Publishing Group, 10(1), pp. 1–14. doi: 10.1038/s41467-019-11558-2.

Dengler, F. (2020) 'Activation of AMPK under Hypoxia: Many Roads Leading to Rome', *International Journal of Molecular Sciences*. Multidisciplinary Digital Publishing Institute (MDPI), 21(7). doi:

10.3390/IJMS21072428.

Dephoure, N. *et al.* (2008) 'A quantitative atlas of mitotic phosphorylation', *Proceedings of the National Academy of Sciences of the United States of America*. Proc Natl Acad Sci U S A, 105(31), pp. 10762–10767. doi: 10.1073/PNAS.0805139105.

Di, R. M. *et al.* (2010) 'PDK1 plays a critical role in regulating cardiac function in mice and human', *Chinese Medical Journal*, 123(17), pp. 2358–2363. doi: 10.3760/CMA.J.ISSN.0366-6999.2010.17.014.

Dobson, A. J. *et al.* (2019) 'Longevity is determined by ETS transcription factors in multiple tissues and diverse species', *PLOS Genetics*. Public Library of Science, 15(7), p. e1008212. doi: 10.1371/JOURNAL.PGEN.1008212.

Dowling, R. J. O. *et al.* (2010) 'mTORC1-mediated cell proliferation, but not cell growth, controlled by the 4E-BPs', *Science (New York, N.Y.)*. Science, 328(5982), pp. 1172–1176. doi: 10.1126/SCIENCE.1187532.

Du, J. *et al.* (2015) 'PDK1 promotes tumor growth and metastasis in a spontaneous breast cancer model', *Oncogene 2016 35:25*. Nature Publishing Group, 35(25), pp. 3314–3323. doi: 10.1038/onc.2015.393.

Dutil, E. M., Toker, A. and Newton, A. C. (1998) 'Regulation of conventional protein kinase C isozymes by phosphoinositide-dependent kinase 1 (PDK-1)', *Current Biology*. Cell Press, 8(25), pp. 1366–1375. doi: 10.1016/S0960-9822(98)00017-7.

Edwards, A. S. and Newton, A. C. (1997) 'Phosphorylation at Conserved Carboxyl-terminal Hydrophobic Motif Regulates the Catalytic and Regulatory Domains of Protein Kinase C *', *Journal of Biological Chemistry*. Elsevier, 272(29), pp. 18382–18390. doi: 10.1074/JBC.272.29.18382.

EMA (2022) *Clinical trials in human medicines | European Medicines Agency*. Available at: https://www.ema.europa.eu/en/human-regulatory/research-development/clinical-trials-human-medicines (Accessed: 9 November 2022).

Emmanouilidi, A. *et al.* (2019) 'Preclinical validation of 3-phosphoinositide-dependent protein kinase 1 inhibition in pancreatic cancer', *Journal of Experimental and Clinical Cancer Research*. BioMed Central Ltd., 38(1), p. 191. doi: 10.1186/s13046-019-1191-2.

Emmanouilidi, A. and Falasca, M. (2017) 'Targeting PDK1 for chemosensitization of cancer cells', *Cancers*. MDPI AG. doi: 10.3390/cancers9100140.

Enns, L. C. *et al.* (2009) 'Disruption of Protein Kinase A in Mice Enhances Healthy Aging', *PLOS ONE*. Public Library of Science, 4(6), p. e5963. doi: 10.1371/JOURNAL.PONE.0005963.

Enns, L. C. and Ladiges, W. (2010) 'Protein kinase A signaling as an anti-aging target', *Ageing research reviews*. Ageing Res Rev, 9(3), pp. 269–272. doi: 10.1016/J.ARR.2010.02.004.

Erlanson, D. A. *et al.* (2011) 'Discovery of a potent and highly selective PDK1 inhibitor via fragment-based drug discovery', *Bioorganic and Medicinal Chemistry Letters*. Bioorg Med Chem Lett, 21(10), pp. 3078–3083. doi: 10.1016/j.bmcl.2011.03.032.

Escher, S. A. and Rasmuson-Lestander, Å. (1999) 'The Drosophila glucose transporter gene: cDNA sequence, phylogenetic comparisons, analysis of functional sites and secondary structures', *Hereditas*. Hereditas, 130(2), pp. 95–103. doi: 10.1111/J.1601-5223.1999.00095.X.

Eurostat (2021) 'Demography of Europe', *Eurostat*.

Evans, D. S. *et al.* (2011) 'TOR signaling never gets old: Aging, longevity and TORC1 activity', *Ageing research reviews*. NIH Public Access, 10(2), p. 225. doi: 10.1016/J.ARR.2010.04.001.

Ezcurra, M. *et al.* (2018) 'C. elegans Eats Its Own Intestine to Make Yolk Leading to Multiple Senescent Pathologies', *Current biology : CB*. Curr Biol, 28(16), pp. 2544-2556.e5. doi:

10.1016/J.CUB.2018.06.035.

Fabbro, D., Cowan-Jacob, S. W. and Moebitz, H. (2015) 'Ten things you should know about protein kinases: IUPHAR Review 14', *British Journal of Pharmacology*. John Wiley and Sons Inc., 172(11), pp. 2675–2700. doi: 10.1111/bph.13096.

Fabrizio, P. *et al.* (2003) 'SOD2 functions downstream of Sch9 to extend longevity in yeast', *Genetics*. Genetics, 163(1), pp. 35–46. doi: 10.1093/GENETICS/163.1.35.

Falasca, M. *et al.* (2010) 'A novel inhibitor of the PI3K/Akt pathway based on the structure of inositol 1,3,4,5,6-pentakisphosphate', *British Journal of Cancer*, 102(1), pp. 104–114. doi: 10.1038/sj.bjc.6605408.

Fan, X. *et al.* (2015) 'Rapamycin preserves gut homeostasis during Drosophila aging', *Oncotarget*. Impact Journals LLC, 6(34), pp. 35274–35283. doi: 10.18632/oncotarget.5895.

Fang, X. *et al.* (2000) 'Phosphorylation and inactivation of glycogen synthase kinase 3 by protein kinase A', *Proceedings of the National Academy of Sciences of the United States of America*. Proc Natl Acad Sci U S A, 97(22), pp. 11960–11965. doi: 10.1073/PNAS.220413597.

FDA (2022a) *Approved Cellular and Gene Therapy Products | FDA*. Available at: https://www.fda.gov/vaccines-blood-biologics/cellular-gene-therapy-products/approved-cellular-and-gene-therapy-products (Accessed: 1 December 2022).

FDA (2022b) *Development & Approval Process | Drugs | FDA*. Available at: https://www.fda.gov/drugs/development-approval-process-drugs (Accessed: 10 November 2022).

FDA (2022c) *Investigational New Drug (IND) Application | FDA*. Available at: https://www.fda.gov/drugs/types-applications/investigational-new-drug-ind-application (Accessed: 9 November 2022).

Feng, N. *et al.* (2019) 'Rapamycin reduces mortality in acute-stage paraquat-induced toxicity in zebrafish', *Singapore Medical Journal*. Wolters Kluwer -- Medknow Publications, 60(5), p. 241. doi: 10.11622/SMEDJ.2018132.

Feser, J. *et al.* (2010) 'Elevated histone expression promotes life span extension', *Molecular cell*. Mol Cell, 39(5), pp. 724–735. doi: 10.1016/J.MOLCEL.2010.08.015.

Fingar, D. C. *et al.* (2004) 'mTOR Controls Cell Cycle Progression through Its Cell Growth Effectors S6K1 and 4E-BP1/Eukaryotic Translation Initiation Factor 4E', *Molecular and Cellular Biology*. Informa UK Limited, 24(1), pp. 200–216. doi: 10.1128/MCB.24.1.200-216.2004.

Fontana, L., Kennedy, B. K. and Longo, V. D. (2014) 'Medical research: Treat ageing', *Nature 2014 511:7510*. Nature Publishing Group, 511(7510), pp. 405–407. doi: 10.1038/511405a.

Fontana, L., Partridge, L. and Longo, V. D. (2010) 'Extending healthy life span-from yeast to humans', *Science*, 328(5976), pp. 321–326. doi: 10.1126/science.1172539.

Frame, S. and Cohen, P. (2001) 'GSK3 takes centre stage more than 20 years after its discovery', *The Biochemical journal*. Biochem J, 359(Pt 1), pp. 1–16. doi: 10.1042/0264-6021:3590001.

Frame, S., Cohen, P. and Biondi, R. M. (2001) 'A common phosphate binding site explains the unique substrate specificity of GSK3 and its inactivation by phosphorylation', *Molecular cell*. Mol Cell, 7(6), pp. 1321–1327. doi: 10.1016/S1097-2765(01)00253-2.

Friedman, D. B. and Johnson, T. E. (1988) 'A mutation in the age-1 gene in Caenorhabditis elegans lengthens life and reduces hermaphrodite fertility', *Genetics*. Genetics, 118(1), pp. 75–86. doi: 10.1093/GENETICS/118.1.75.

Frödin, M. *et al.* (2000) 'A phosphoserine-regulated docking site in the protein kinase RSK2 that recruits and activates PDK1', *The EMBO Journal*. European Molecular Biology Organization, 19(12), p.

2924. doi: 10.1093/EMBOJ/19.12.2924.

Frödin, M. *et al.* (2002) 'A phosphoserine/threonine-binding pocket in AGC kinases and PDK1 mediates activation by hydrophobic motif phosphorylation.', *The EMBO journal*, 21(20), pp. 5396–407. doi: 10.1093/emboj/cdf551.

Gallant, P. *et al.* (1996) 'Myc and Max homologs in Drosophila', *Science (New York, N.Y.)*. Science, 274(5292), pp. 1523–1527. doi: 10.1126/SCIENCE.274.5292.1523.

Gallant, P. (2013) 'Myc Function in Drosophila', *Cold Spring Harbor Perspectives in Medicine*. Cold Spring Harbor Laboratory Press, 3(10). doi: 10.1101/CSHPERSPECT.A014324.

Gao, W. *et al.* (2022) 'Dissecting the Crosstalk Between Nrf2 and NF-κB Response Pathways in Drug-Induced Toxicity', *Frontiers in Cell and Developmental Biology*. Frontiers Media S.A., 9, p. 3940. doi: 10.3389/FCELL.2021.809952/BIBTEX.

Gao, X. *et al.* (2002) 'Tsc tumour suppressor proteins antagonize amino-acid-TOR signalling', *Nature cell biology*. Nat Cell Biol, 4(9), pp. 699–704. doi: 10.1038/NCB847.

Gao, X., Neufeld, T. P. and Pan, D. (2000) 'Drosophila PTEN Regulates Cell Growth and Proliferation through PI3K-Dependent and -Independent Pathways', *Developmental Biology*. Academic Press, 221(2), pp. 404–418. doi: 10.1006/DBIO.2000.9680.

García-Martínez, J. M. and Alessi, D. R. (2008) 'mTOR complex 2 (mTORC2) controls hydrophobic motif phosphorylation and activation of serum- and glucocorticoid-induced protein kinase 1 (SGK1)', *The Biochemical journal*. Biochem J, 416(3), pp. 375–385. doi: 10.1042/BJ20081668.

Garelick, M. G. *et al.* (2013) 'Chronic rapamycin treatment or lack of S6K1 does not reduce ribosome activity in vivo', *Cell cycle (Georgetown, Tex.)*. Cell Cycle, 12(15), pp. 2493–2504. doi: 10.4161/CC.25512.

Gargano, J. W. *et al.* (2005) 'Rapid iterative negative geotaxis (RING): A new method for assessing age-related locomotor decline in Drosophila', *Experimental Gerontology*. Pergamon, 40(5), pp. 386–395. doi: 10.1016/j.exger.2005.02.005.

Gems, D. (2015) 'The aging-disease false dichotomy: Understanding senescence as pathology', *Frontiers in Genetics*. Frontiers Media S.A. doi: 10.3389/fgene.2015.00212.

Gems, D. (2022) 'The hyperfunction theory: An emerging paradigm for the biology of aging', *Ageing Research Reviews*. Elsevier, 74, p. 101557. doi: 10.1016/J.ARR.2021.101557.

Giannakou, M. E. *et al.* (2004) 'Long-Lived Drosophila with Overexpressed dFOXO in Adult Fat Body', *Science*. American Association for the Advancement of Science , 292, p. 12602. doi: 10.1126/SCIENCE.1098219.

Gill, T. M. *et al.* (2010) 'Trajectories of Disability in the Last Year of Life', *New England Journal of Medicine*. Massachusetts Medical Society, 362(13), pp. 1173–1180. doi: 10.1056/NEJMOA0909087/SUPPL_FILE/NEJMOA0909087_DISCLOSURES.PDF.

Gingras, A. C. *et al.* (1998) '4E-BP1, a repressor of mRNA translation, is phosphorylated and inactivated by the Akt(PKB) signaling pathway', *Genes & development*. Genes Dev, 12(4), pp. 502–513. doi: 10.1101/GAD.12.4.502.

Goddeeris, M. M. *et al.* (2003) ' Delayed behavioural aging and altered mortality in Drosophila β integrin mutants ', *Aging Cell*. Wiley, 2(5), pp. 257–264. doi: 10.1046/j.1474-9728.2003.00060.x.

Goitre, L. *et al.* (2014) 'The Ras superfamily of small GTPases: the unlocked secrets', *Methods in molecular biology (Clifton, N.J.)*. Methods Mol Biol, 1120, pp. 1–18. doi: 10.1007/978-1-62703-791-4_1.

Le Good, J. A. *et al.* (1998) 'Protein kinase C isotypes controlled by phosphoinositide 3-kinase through

the protein kinase PDK1', *Science*. American Association for the Advancement of Science, 281(5385), pp. 2042–2045. doi: 10.1126/SCIENCE.281.5385.2042/ASSET/9019403E-1F9D-4B2E-8A48-42E036D67888/ASSETS/GRAPHIC/SE3986855003.JPEG.

Grifoni, D. and Bellosta, P. (2015) 'Drosophila Myc: a master regulator of cellular performance', *Biochimica et biophysica acta*. NIH Public Access, 1849(5), p. 570. doi: 10.1016/J.BBAGRM.2014.06.021.

Grönke, S. *et al.* (2005) 'Brummer lipase is an evolutionary conserved fat storage regulator in Drosophila', *Cell Metabolism*. Cell Press, 1(5), pp. 323–330. doi: 10.1016/J.CMET.2005.04.003.

Grönke, S. *et al.* (2007) 'Dual lipolytic control of body fat storage and mobilization in Drosophila', *PLoS biology*. PLoS Biol, 5(6), pp. 1248–1256. doi: 10.1371/JOURNAL.PBIO.0050137.

Grönke, S. *et al.* (2010) 'Molecular evolution and functional characterization of Drosophila insulin-like peptides', *PLoS Genetics*, 6(2). doi: 10.1371/journal.pgen.1000857.

Haar, E. Vander *et al.* (2007) 'Insulin signalling to mTOR mediated by the Akt/PKB substrate PRAS40', *Nature cell biology*. Nat Cell Biol, 9(3), pp. 316–323. doi: 10.1038/NCB1547.

Haller, S. *et al.* (2017) 'mTORC1 Activation during Repeated Regeneration Impairs Somatic Stem Cell Maintenance', *Stem Cell*, 21, pp. 806-818.e5. doi: 10.1016/j.stem.2017.11.008.

Han, X. *et al.* (2022) 'ATGL deficiency aggravates pressure overload-triggered myocardial hypertrophic remodeling associated with the proteasome-PTEN-mTOR-autophagy pathway', *Cell Biology and Toxicology*. Springer Science and Business Media B.V., 3(3), pp. 1–19. doi: 10.1007/S10565-022-09699-0/FIGURES/7.

Hannah Ritchie, Fiona Spooner and Max Roser (2019) *Causes of death - Our World in Data*. Available at: https://ourworldindata.org/causes-of-death (Accessed: 10 November 2022).

Harman, D. (1956) 'Aging: a theory based on free radical and radiation chemistry', *Journal of gerontology*. J Gerontol, 11(3), pp. 298–300. doi: 10.1093/GERONJ/11.3.298.

Harris, T. E. and Lawrence, J. C. (2003) 'TOR signaling', *Science's STKE : signal transduction knowledge environment*. Sci STKE, 2003(212). doi: 10.1126/STKE.2122003RE15.

Harrison, D. E. *et al.* (2009) 'Rapamycin fed late in life extends lifespan in genetically heterogeneous mice', *Nature*. Nature, 460(7253), pp. 392–395. doi: 10.1038/NATURE08221.

Haruta, T. *et al.* (2000) 'A Rapamycin-Sensitive Pathway Down-Regulates Insulin Signaling via Phosphorylation and Proteasomal Degradation of Insulin Receptor Substrate-1', *Molecular Endocrinology*. Oxford Academic, 14(6), pp. 783–794. doi: 10.1210/MEND.14.6.0446.

Hers, I. and Tavaré, J. M. (2005) 'Mechanism of feedback regulation of insulin receptor substrate-1 phosphorylation in primary adipocytes', *Biochemical Journal*. Portland Press Ltd, 388(Pt 2), p. 713. doi: 10.1042/BJ20041531.

Hers, I., Vincent, E. E. and Tavaré, J. M. (2011) 'Akt signalling in health and disease', *Cellular Signalling*. Pergamon, 23(10), pp. 1515–1527. doi: 10.1016/J.CELLSIG.2011.05.004.

Hirata, A. *et al.* (2004) 'Characterization of a monoclonal antibody, HTA28, recognizing a histone H3 phosphorylation site as a useful marker of M-phase cells', *The journal of histochemistry and cytochemistry : official journal of the Histochemistry Society*. J Histochem Cytochem, 52(11), pp. 1503–1509. doi: 10.1369/JHC.4A6285.2004.

Hofer, T. (2021) 'Oxidative Stress in Human Toxicology', *Antioxidants 2021, Vol. 10, Page 1159*. Multidisciplinary Digital Publishing Institute, 10(8), p. 1159. doi: 10.3390/ANTIOX10081159.

Hoffmann, J. M. and Partridge, L. (2015) 'Nuclear hormone receptors: Roles of xenobiotic detoxification and sterol homeostasis in healthy aging',

https://doi.org/10.3109/10409238.2015.1067186. Taylor & Francis, 50(5), pp. 380–392. doi: 10.3109/10409238.2015.1067186.

Holz, M. K. *et al.* (2005) 'mTOR and S6K1 Mediate Assembly of the Translation Preinitiation Complex through Dynamic Protein Interchange and Ordered Phosphorylation Events', *Cell*. Cell Press, 123(4), pp. 569–580. doi: 10.1016/J.CELL.2005.10.024.

Hosamani, R. and Muralidhara (2013) 'Acute exposure of drosophila melanogaster to paraquat causes oxidative stress and mitochondrial dysfunction', *Archives of Insect Biochemistry and Physiology*, 83(1), pp. 25–40. doi: 10.1002/ARCH.21094.

Hsu, H. J. and Drummond-Barbosa, D. (2009) 'Insulin levels control female germline stem cell maintenance via the niche in Drosophila', *Proceedings of the National Academy of Sciences of the United States of America*. National Academy of Sciences, 106(4), p. 1117. doi: 10.1073/PNAS.0809144106.

Huang, J. *et al.* (2009) 'Directed, efficient, and versatile modifications of the Drosophila genome by genomic engineering', *Proceedings of the National Academy of Sciences of the United States of America*. National Academy of Sciences, 106(20), pp. 8284–8289. doi: 10.1073/pnas.0900641106.

Huang, S. and Czech, M. P. (2007) 'The GLUT4 glucose transporter', *Cell metabolism*. Cell Metab, 5(4), pp. 237–252. doi: 10.1016/J.CMET.2007.03.006.

Humphrey, S. J., Azimifar, S. B. and Mann, M. (2015) 'High-throughput phosphoproteomics reveals in vivo insulin signaling dynamics', *Nature Biotechnology*. Nature Publishing Group, 33(9), pp. 990–995. doi: 10.1038/nbt.3327.

Hwangbo, D. S. *et al.* (2004) 'Drosophila dFOXO controls lifespan and regulates insulin signalling in brain and fat body', *Nature 2004 429:6991*. Nature Publishing Group, 429(6991), pp. 562–566. doi: 10.1038/nature02549.

Ingber, D. E. (2022) 'Human organs-on-chips for disease modelling, drug development and personalized medicine', *Nature Reviews Genetics 2022 23:8*. Nature Publishing Group, 23(8), pp. 467–491. doi: 10.1038/s41576-022-00466-9.

Inoki, K. *et al.* (2005) 'Signaling by Target of Rapamycin Proteins in Cell Growth Control', *Microbiology and Molecular Biology Reviews*. American Society for Microbiology, 69(1), pp. 79–100. doi: 10.1128/MMBR.69.1.79-100.2005/ASSET/1CEC7E36-1B76-4673-AC69-14C25C110734/ASSETS/GRAPHIC/ZMR0010520790006.JPEG.

Inoki, K. *et al.* (2006) 'TSC2 Integrates Wnt and Energy Signals via a Coordinated Phosphorylation by AMPK and GSK3 to Regulate Cell Growth', *Cell*. Cell Press, 126(5), pp. 955–968. doi: 10.1016/J.CELL.2006.06.055.

Jang, Y. C. and Van Remmen, H. (2011) 'Age-associated alterations of the neuromuscular junction', *Experimental Gerontology*. Pergamon, pp. 193–198. doi: 10.1016/j.exger.2010.08.029.

Jensen, C. J. *et al.* (1999) '90-kDa Ribosomal S6 Kinase Is Phosphorylated and Activated by 3-Phosphoinositide-dependent Protein Kinase-1 *', *Journal of Biological Chemistry*. Elsevier, 274(38), pp. 27168–27176. doi: 10.1074/JBC.274.38.27168.

Juricic, P. *et al.* (2022) 'Long-lasting geroprotection from brief rapamycin treatment in early adulthood by persistently increased intestinal autophagy', *Nature Aging 2022 2:9*. Nature Publishing Group, 2(9), pp. 824–836. doi: 10.1038/s43587-022-00278-w.

Juricic, P., Grönke, S. and Partridge, L. (2020) 'Branched-Chain Amino Acids Have Equivalent Effects to Other Essential Amino Acids on Lifespan and Aging-Related Traits in Drosophila', *The Journals of Gerontology: Series A*. Edited by A. Salmon. Oxford University Press, 75(1), pp. 24–31. doi: 10.1093/gerona/glz080.

Kahn, B. B. *et al.* (2005) 'AMP-activated protein kinase: Ancient energy gauge provides clues to modern understanding of metabolism', *Cell Metabolism*. Cell Press, 1(1), pp. 15–25. doi: 10.1016/J.CMET.2004.12.003.

Kamada, Y. *et al.* (2000) 'Tor-mediated induction of autophagy via an Apg1 protein kinase complex', *The Journal of cell biology*. J Cell Biol, 150(6), pp. 1507–1513. doi: 10.1083/JCB.150.6.1507.

Kapahi, P. *et al.* (2004) 'Regulation of Lifespan in Drosophila by Modulation of Genes in the TOR Signaling Pathway', *Current Biology*. Cell Press, 14(10), pp. 885–890. doi: 10.1016/J.CUB.2004.03.059.

Kapuria, S. *et al.* (2012) 'Notch-mediated suppression of TSC2 expression regulates cell differentiation in the Drosophila intestinal stem cell lineage', *PLoS genetics*. PLoS Genet, 8(11). doi: 10.1371/JOURNAL.PGEN.1003045.

Katz, M., Amit, I. and Yarden, Y. (2007) 'Regulation of MAPKs by growth factors and receptor tyrosine kinases', *Biochimica et Biophysica Acta - Molecular Cell Research*, 1773(8), pp. 1161–1176. doi: 10.1016/J.BBAMCR.2007.01.002.

Keith, C. T., Borisy, A. A. and Stockwell, B. R. (2005) 'Multicomponent therapeutics for networked systems', *Nature reviews. Drug discovery*. Nat Rev Drug Discov, 4(1), pp. 71–78. doi: 10.1038/NRD1609.

Kennedy, C. J. and Tierney, K. B. (2012) 'Xenobiotic Protection protection /Resistance Mechanisms in Organisms', *Encyclopedia of Sustainability Science and Technology*. Springer, New York, NY, pp. 12293–12314. doi: 10.1007/978-1-4419-0851-3_51.

Kenyon, C. *et al.* (1993) 'A C. elegans mutant that lives twice as long as wild type', *Nature*, 366(6454), pp. 461–464. doi: 10.1038/366461a0.

Kiehart, D. P., Crawford, J. M. and Montague, R. A. (2000) 'No Title', in *Drosophila Protocols;* New York: Cold Spring Harbor Laboratory Press, Cold Spring Harbor, pp. 345–359.

Kim, E. *et al.* (2008) 'Regulation of TORC1 by Rag GTPases in nutrient response', *Nature Cell Biology 2008 10:8*. Nature Publishing Group, 10(8), pp. 935–945. doi: 10.1038/ncb1753.

Kirkwood, T. B. L. (1977) 'Evolution of ageing', *Nature 1977 270:5635*. Nature Publishing Group, 270(5635), pp. 301–304. doi: 10.1038/270301a0.

Klepsatel, P. *et al.* (2020) 'The influence of developmental diet on reproduction and metabolism in Drosophila', *BMC Evolutionary Biology*. BioMed Central, 20(1). doi: 10.1186/S12862-020-01663-Y.

Klotz, L. O. and Steinbrenner, H. (2017) 'Cellular adaptation to xenobiotics: Interplay between xenosensors, reactive oxygen species and FOXO transcription factors', *Redox biology*. Redox Biol, 13, pp. 646–654. doi: 10.1016/J.REDOX.2017.07.015.

Kobayashi, T. and Cohen, P. (1999) 'Activation of serum- and glucocorticoid-regulated protein kinase by agonists that activate phosphatidylinositide 3-kinase is mediated by 3-phosphoinositide-dependent protein kinase-1 (PDK1) and PDK2.', *Biochemical Journal*. Portland Press Ltd, 339(Pt 2), p. 319. doi: 10.1042/0264-6021:3390319.

Kodama, S. *et al.* (2004) 'Nuclear receptors CAR and PXR cross talk with FOXO1 to regulate genes that encode drug-metabolizing and gluconeogenic enzymes', *Molecular and cellular biology*. Mol Cell Biol, 24(18), pp. 7931–7940. doi: 10.1128/MCB.24.18.7931-7940.2004.

Kok, B. P. C. *et al.* (2012) 'Unlike two peas in a pod: Lipid phosphate phosphatases and phosphatidate phosphatases', *Chemical Reviews*, 112(10), pp. 5121–5146. doi: 10.1021/CR200433M.

Komander, D. *et al.* (2004) 'Structural insights into the regulation of PDK1 by phosphoinositides and inositol phosphates', *EMBO Journal*. EMBO J, 23(20), pp. 3918–3928. doi: 10.1038/sj.emboj.7600379.

Komander, D. *et al.* (2005) 'Role of T-loop phosphorylation in PDK1 activation, stability, and substrate

binding', *The Journal of biological chemistry*. J Biol Chem, 280(19), pp. 18797–18802. doi: 10.1074/JBC.M500977200.

Kramer, J. M., Slade, J. D. and Staveley, B. E. (2008) 'foxo is required for resistance to amino acid starvation in Drosophila', *Genome*. Genome, 51(8), pp. 668–672. doi: 10.1139/G08-047.

Kuan, V. *et al.* (2021) 'Data-driven identification of ageing-related diseases from electronic health records', *Scientific Reports*. Nature Research, 11(1). doi: 10.1038/S41598-021-82459-Y.

Kulkarni, A. S. *et al.* (2022) 'Geroscience-guided repurposing of FDA-approved drugs to target aging: A proposed process and prioritization', *Aging Cell*. Wiley-Blackwell, 21(4). doi: 10.1111/ACEL.13596.

Kuningas, M. *et al.* (2007) 'Haplotypes in the human Foxo1a and Foxo3a genes; impact on disease and mortality at old age', *European journal of human genetics : EJHG*. Eur J Hum Genet, 15(3), pp. 294–301. doi: 10.1038/SJ.EJHG.5201766.

Lafleur, M. A., Stevens, J. L. and Lawrence, J. W. (2013) 'Xenobiotic perturbation of ER stress and the unfolded protein response', *Toxicologic pathology*. Toxicol Pathol, 41(2), pp. 235–262. doi: 10.1177/0192623312470764.

Lawlor, M. A. *et al.* (2002) 'Essential role of PDK1 in regulating cell size and development in mice', *EMBO Journal*, 21(14), pp. 3728–3738. doi: 10.1093/EMBOJ/CDF387.

Lee, A. S. Y. *et al.* (2016) 'eIF3d is an mRNA cap-binding protein that is required for specialized translation initiation', *Nature*. Nature, 536(7614), pp. 96–99. doi: 10.1038/NATURE18954.

Leevers, S. J., Vanhaesebroeck, B. and Waterfield, M. D. (1999) 'Signalling through phosphoinositide 3-kinases: the lipids take centre stage', *Current opinion in cell biology*. Curr Opin Cell Biol, 11(2), pp. 219–225. doi: 10.1016/S0955-0674(99)80029-5.

Leonard, T. A. *et al.* (2011) 'Crystal structure and allosteric activation of protein kinase C βII', *Cell*. Cell, 144(1), pp. 55–66. doi: 10.1016/J.CELL.2010.12.013.

Levina, A. *et al.* (2022) 'Activation of the essential kinase PDK1 by phosphoinositide-driven trans-autophosphorylation', *Nature Communications 2022 13:1*. Nature Publishing Group, 13(1), pp. 1–16. doi: 10.1038/s41467-022-29368-4.

Li, Z. *et al.* (2018) 'Plk1-Mediated Phosphorylation of TSC1 Enhances the Efficacy of Rapamycin', *Cancer research*. Cancer Res, 78(11), pp. 2864–2875. doi: 10.1158/0008-5472.CAN-17-3046.

Li, Z. *et al.* (2021) 'Aging and age-related diseases: from mechanisms to therapeutic strategies', *Biogerontology 2021 22:2*. Springer, 22(2), pp. 165–187. doi: 10.1007/S10522-021-09910-5.

Liao, S. (2020) 'Drosophila Insulin-Like Peptide 8 (DILP8) in Ovarian Follicle Cells Regulates Ovulation and Metabolism', *Frontiers in Endocrinology*. Frontiers Media S.A., 11, p. 461. doi: 10.3389/FENDO.2020.00461/XML/NLM.

Lin, K. *et al.* (1997) 'daf-16: An HNF-3/forkhead family member that can function to double the life-span of Caenorhabditis elegans', *Science (New York, N.Y.)*. Science, 278(5341), pp. 1319–1322. doi: 10.1126/SCIENCE.278.5341.1319.

Loewith, R. *et al.* (2002) 'Two TOR Complexes, Only One of which Is Rapamycin Sensitive, Have Distinct Roles in Cell Growth Control', *Molecular Cell*. Cell Press, 10(3), pp. 457–468. doi: 10.1016/S1097-2765(02)00636-6.

López-Otín, C. *et al.* (2013) 'The hallmarks of aging', *Cell*. Cell Press, p. 1194. doi: 10.1016/j.cell.2013.05.039.

Lu, Y. X. *et al.* (2021) 'A torc1-histone axis regulates chromatin organisation and non-canonical induction of autophagy to ameliorate ageing', *eLife*. eLife Sciences Publications Ltd, 10. doi: 10.7554/ELIFE.62233.

Luong, N. *et al.* (2006) 'Activated FOXO-mediated insulin resistance is blocked by reduction of TOR activity', *Cell Metabolism*. Cell Press, 4(2), pp. 133–142. doi: 10.1016/J.CMET.2006.05.013.

Ma, C. *et al.* (2021) 'Organ-on-a-Chip: A new paradigm for drug development', *Trends in pharmacological sciences*. NIH Public Access, 42(2), p. 119. doi: 10.1016/J.TIPS.2020.11.009.

Ma, Q. (2013) 'Role of Nrf2 in Oxidative Stress and Toxicity', *Annual review of pharmacology and toxicology*. NIH Public Access, 53, p. 401. doi: 10.1146/ANNUREV-PHARMTOX-011112-140320.

Maehama, T. and Dixon, J. E. (1999) 'PTEN: a tumour suppressor that functions as a phospholipid phosphatase', *Trends in cell biology*. Trends Cell Biol, 9(4), pp. 125–128. doi: 10.1016/S0962-8924(99)01519-6.

de Magalhães, J. P., Stevens, M. and Thornton, D. (2017) 'The Business of Anti-Aging Science', *Trends in Biotechnology*. Elsevier Current Trends, 35(11), pp. 1062–1073. doi: 10.1016/J.TIBTECH.2017.07.004.

Majercak, J., Kalderon, D. and Edery, I. (1997) 'Drosophila melanogaster Deficient in Protein Kinase A Manifests Behavior-Specific Arrhythmia but Normal Clock Function', *MOLECULAR AND CELLULAR BIOLOGY*, 17(10), pp. 5915–5922.

Mannick, J. B. *et al.* (2018) 'TORC1 inhibition enhances immune function and reduces infections in the elderly', *Science translational medicine*. Sci Transl Med, 10(449). doi: 10.1126/SCITRANSLMED.AAQ1564.

Manning, B. D. *et al.* (2002) 'Identification of the tuberous sclerosis complex-2 tumor suppressor gene product tuberin as a target of the phosphoinositide 3-kinase/akt pathway', *Molecular cell*. Mol Cell, 10(1), pp. 151–162. doi: 10.1016/S1097-2765(02)00568-3.

Manning, B. D. and Cantley, L. C. (2007) 'AKT/PKB Signaling: Navigating Downstream', *Cell*, 129(7), pp. 1261–1274. doi: 10.1016/J.CELL.2007.06.009.

Masters, T. A. *et al.* (2010) 'Regulation of 3-phosphoinositide-dependent protein kinase 1 activity by homodimerization in live cells', *Science Signaling*. American Association for the Advancement of Science, 3(145). doi: 10.1126/SCISIGNAL.2000738/SUPPL_FILE/3_RA78_SM.PDF.

McElwee, J. J. *et al.* (2007a) 'Evolutionary conservation of regulated longevity assurance mechanisms', *Genome Biology*. BioMed Central, 8(7), pp. 1–16. doi: 10.1186/GB-2007-8-7-R132/FIGURES/5.

McElwee, J. J. *et al.* (2007b) 'Evolutionary conservation of regulated longevity assurance mechanisms', *Genome biology*. Genome Biol, 8(7). doi: 10.1186/GB-2007-8-7-R132.

McKay, M. M. and Morrison, D. K. (2007) 'Integrating signals from RTKs to ERK/MAPK', *Oncogene 2007 26:22*. Nature Publishing Group, 26(22), pp. 3113–3121. doi: 10.1038/sj.onc.1210394.

McLeod, C. J. *et al.* (2010) 'Stem cell dynamics in response to nutrient availability', *Current biology : CB*. Curr Biol, 20(23), pp. 2100–2105. doi: 10.1016/J.CUB.2010.10.038.

McManus, E. J. *et al.* (2004) 'The in vivo role of PtdIns(3,4,5)P3 binding to PDK1 PH domain defined by knockin mutation', *The EMBO journal*. EMBO J, 23(10), pp. 2071–2082. doi: 10.1038/SJ.EMBOJ.7600218.

McPhee, C. K. and Baehrecke, E. H. (2009) 'Autophagy in Drosophila melanogaster', *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research*. Elsevier, 1793(9), pp. 1452–1460. doi: 10.1016/J.BBAMCR.2009.02.009.

Mehdizadeh, A. *et al.* (2016) 'Extracellular signal-regulated kinase 1 and 2 in cancer therapy: a focus on hepatocellular carcinoma', *Molecular Biology Reports*. Springer Netherlands, 43(2), pp. 107–116. doi: 10.1007/S11033-016-3943-9.

Mendoza, M. C., Er, E. E. and Blenis, J. (2011) 'The Ras-ERK and PI3K-mTOR Pathways: Cross-talk and

Compensation', *Trends in biochemical sciences*. NIH Public Access, 36(6), p. 320. doi: 10.1016/J.TIBS.2011.03.006.

Meyts, P. De (2016) 'The Insulin Receptor and Its Signal Transduction Network', *Endotext*. MDText.com, Inc. Available at: https://www.ncbi.nlm.nih.gov/books/NBK378978/ (Accessed: 12 November 2022).

Meyuhas, O. (2000) 'Synthesis of the translational apparatus is regulated at the translational level', *European journal of biochemistry*. Eur J Biochem, 267(21), pp. 6321–6330. doi: 10.1046/J.1432-1327.2000.01719.X.

Milburn, C. C. *et al.* (2003) 'Binding of phosphatidylinositol 3,4,5-trisphosphate to the pleckstrin homology domain of protein kinase B induces a conformational change', *The Biochemical journal*. Biochem J, 375(Pt 3), pp. 531–538. doi: 10.1042/BJ20031229.

Milne, D. M. *et al.* (1994) 'Phosphorylation of the tumor suppressor protein p53 by mitogen-activated protein kinases.', *Journal of Biological Chemistry*. Elsevier, 269(12), pp. 9253–9260. doi: 10.1016/S0021-9258(17)37101-6.

Minois, N., Khazaeli, A. A. and Curtsinger, J. W. (2001) 'Locomotor activity as a function of age and life span in Drosophila melanogaster overexpressing hsp70', *Experimental Gerontology*. Pergamon, 36(7), pp. 1137–1153. doi: 10.1016/S0531-5565(00)00263-1.

Mizushima, N. and Levine, B. (2010) 'Autophagy in mammalian development and differentiation', *Nature cell biology*. Nat Cell Biol, 12(9), pp. 823–830. doi: 10.1038/NCB0910-823.

Montagne, J. *et al.* (1999) 'Drosophila S6 kinase: a regulator of cell size', *Science (New York, N.Y.)*. Science, 285(5436), pp. 2126–2129. doi: 10.1126/SCIENCE.285.5436.2126.

Mora, A. *et al.* (2005) 'Deficiency of PDK1 in liver results in glucose intolerance, impairment of insulin-regulated gene expression and liver failure', *The Biochemical journal*. Biochem J, 385(Pt 3), pp. 639–648. doi: 10.1042/BJ20041782.

Morton, S. *et al.* (2003) 'A reinvestigation of the multisite phosphorylation of the transcription factor c-Jun', *The EMBO Journal*. John Wiley & Sons, Ltd, 22(15), pp. 3876–3886. doi: 10.1093/EMBOJ/CDG388.

Nagashima, K. *et al.* (2011) 'Genetic and pharmacological inhibition of PDK1 in cancer cells: Characterization of a selective allosteric kinase inhibitor', *Journal of Biological Chemistry*. American Society for Biochemistry and Molecular Biology, 286(8), pp. 6433–6448. doi: 10.1074/jbc.M110.156463.

Najafov, A. *et al.* (2011a) 'Characterization of GSK2334470, a novel and highly specific inhibitor of PDK1', *Biochemical Journal*. Biochem J, 433(2), pp. 357–369. doi: 10.1042/BJ20101732.

Najafov, A. *et al.* (2011b) 'Characterization of GSK2334470, a novel and highly specific inhibitor of PDK1', *Biochemical Journal*. Portland Press, 433(2), pp. 357–369. doi: 10.1042/BJ20101732.

Najafov, A., Shpiro, N. and Alessi, D. R. (2012) 'Akt is efficiently activated by PIF-pocket- and PtdIns(3,4,5)P3-dependent mechanisms leading to resistance to PDK1 inhibitors', *The Biochemical journal*. Biochem J, 448(2), pp. 285–295. doi: 10.1042/BJ20121287.

Nässel, D. R., Liu, Y. and Luo, J. (2015) 'Insulin/IGF signaling and its regulation in Drosophila', *General and Comparative Endocrinology*. Academic Press, 221, pp. 255–266. doi: 10.1016/J.YGCEN.2014.11.021.

Nie, C. *et al.* (2022) 'Distinct biological ages of organs and systems identified from a multi-omics study', *Cell Reports*. Cell Press, 38(10), p. 110459. doi: 10.1016/J.CELREP.2022.110459.

Noda, T. and Ohsumi, Y. (1998) 'Tor, a phosphatidylinositol kinase homologue, controls autophagy in

yeast', *The Journal of biological chemistry*. J Biol Chem, 273(7), pp. 3963–3966. doi: 10.1074/JBC.273.7.3963.

O'Brien, L. E. *et al.* (2011) 'Altered modes of stem cell division drive adaptive intestinal growth', *Cell*. Cell, 147(3), pp. 603–614. doi: 10.1016/J.CELL.2011.08.048.

Ochoa, D. *et al.* (2019) 'The functional landscape of the human phosphoproteome.', *Nature Biotechnology*. Nature Research, 38(3), pp. 365–373. doi: 10.1038/S41587-019-0344-3.

Oldham, S. *et al.* (2002) 'The Drosophila insulin/IGF receptor controls growth and size by modulating PtdInsP3 levels', *Development*. The Company of Biologists, 129(17), pp. 4103–4109. doi: 10.1242/DEV.129.17.4103.

Olsen, J. V. *et al.* (2010) 'Quantitative phosphoproteomics reveals widespread full phosphorylation site occupancy during mitosis', *Science signaling*. Sci Signal, 3(104). doi: 10.1126/SCISIGNAL.2000475.

Omiecinski, C. J. *et al.* (2011) 'Xenobiotic metabolism, disposition, and regulation by receptors: from biochemical phenomenon to predictors of major toxicities', *Toxicological sciences : an official journal of the Society of Toxicology*. Toxicol Sci, 120 Suppl 1(Suppl 1). doi: 10.1093/TOXSCI/KFQ338.

Paradis, S. *et al.* (1999) 'A PDK1 homolog is necessary and sufficient to transduce AGE-1 PI3 kinase signals that regulate diapause in Caenorhabditis elegans', *Genes & Development*. Cold Spring Harbor Laboratory Press, 13(11), p. 1438. doi: 10.1101/GAD.13.11.1438.

Partridge, L. (2010) 'The new biology of ageing', *Philosophical Transactions of the Royal Society B: Biological Sciences*. The Royal Society, 365(1537), p. 147. doi: 10.1098/RSTB.2009.0222.

Pastor-Flores, D. *et al.* (2011) 'Depletion of yeast PDK1 orthologs triggers a stress-like transcriptional response'. doi: 10.1186/s12864-015-1903-8.

Patel, P. H., Dutta, D. and Edgar, B. A. (2015) 'Niche appropriation by Drosophila intestinal stem cell tumours', *Nature Cell Biology*. Nature Publishing Group, 17(9), pp. 1182–1192. doi: 10.1038/ncb3214.

Pawlikowska, L. *et al.* (2009) 'Association of common genetic variation in the insulin/IGF1 signaling pathway with human longevity', *Aging cell*. Aging Cell, 8(4), pp. 460–472. doi: 10.1111/J.1474-9726.2009.00493.X.

Pearce, L. R., Komander, D. and Alessi, D. R. (2010) 'The nuts and bolts of AGC protein kinases', *Nature Reviews Molecular Cell Biology*. Nature Publishing Group, pp. 9–22. doi: 10.1038/nrm2822.

Pedra, J. H. F. *et al.* (2004) 'Genome-wide transcription profile of field- and laboratory-selected dichlorodiphenyltrichloroethane (DDT)-resistant Drosophila', *Proceedings of the National Academy of Sciences of the United States of America*, 101(18), pp. 7034–7039. doi: 10.1073/PNAS.0400580101/SUPPL_FILE/00580TABLE5.XLS.

Perkins, A. D. and Tanentzapf, G. (2014) 'An ongoing role for structural sarcomeric components in maintaining Drosophila melanogaster muscle function and structure', *PloS one*. PLoS One, 9(6). doi: 10.1371/JOURNAL.PONE.0099362.

Peterson, T. R. *et al.* (2011) 'mTOR complex 1 regulates lipin 1 localization to control the SREBP pathway', *Cell*. Cell, 146(3), pp. 408–420. doi: 10.1016/J.CELL.2011.06.034.

Pokorny, D. *et al.* (2021) 'In vitro reconstitution of Sgk3 activation by phosphatidylinositol 3-phosphate', *The Journal of biological chemistry*. J Biol Chem, 297(2). doi: 10.1016/J.JBC.2021.100919.

Pomatto, L. C. D. *et al.* (2017) 'The Mitochondrial Lon Protease Is Required for Age-Specific and Sex-Specific Adaptation to Oxidative Stress', *Current biology : CB*. Curr Biol, 27(1), pp. 1–15. doi: 10.1016/J.CUB.2016.10.044.

Post, S. *et al.* (2018) 'Drosophila insulin-like peptides DILP2 and DILP5 differentially stimulate cell signaling and glycogen phosphorylase to regulate longevity', *Frontiers in Endocrinology*. Frontiers

Media S.A., 9(MAY), p. 245. doi: 10.3389/FENDO.2018.00245/BIBTEX.

Puig, O. *et al.* (2003) 'Control of cell number by Drosophila FOXO: downstream and feedback regulation of the insulin receptor pathway', *Genes & development*. Genes Dev, 17(16), pp. 2006–2020. doi: 10.1101/GAD.1098703.

Pullen, N. *et al.* (1998) 'Phosphorylation and activation of p70s6k by PDK1', *Science (New York, N.Y.)*. Science, 279(5351), pp. 707–710. doi: 10.1126/SCIENCE.279.5351.707.

Pustylnyak, Y. A., Gulyaeva, L. F. and Pustylnyak, V. O. (2020) 'Noncanonical Constitutive Androstane Receptor Signaling in Gene Regulation', *International Journal of Molecular Sciences 2020, Vol. 21, Page 6735*. Multidisciplinary Digital Publishing Institute, 21(18), p. 6735. doi: 10.3390/IJMS21186735.

Qin, X., Jiang, B. and Zhang, Y. (2016) '4E-BP1, a multifactor regulated multifunctional protein', *Cell Cycle*. Taylor & Francis, 15(6), p. 781. doi: 10.1080/15384101.2016.1151581.

Quan, Z. *et al.* (2013) 'TSC1/2 regulates intestinal stem cell maintenance and lineage differentiation through Rheb-TORC1-S6K but independently of nutritional status or Notch regulation', *Journal of cell science*. J Cell Sci, 126(Pt 17), pp. 3884–3892. doi: 10.1242/JCS.125294.

R Core Team (2017) R A Language and Environment for Statistical Computing. - References - Scientific Research Publishing (no date). Available at: https://www.scirp.org/(S(351jmbntvnsjt1aadkposzje))/reference/ReferencesPapers.aspx?ReferenceID=2144573 (Accessed: 13 February 2023).

Radimerski, T. *et al.* (2002) 'dS6K-regulated cell growth is dPKB/dPI(3)K-independent, but requires dPDK1', *Nature Cell Biology 2002 4:3*. Nature Publishing Group, 4(3), pp. 251–255. doi: 10.1038/ncb763.

Ransy, C. *et al.* (2020) 'Use of H2O2 to Cause Oxidative Stress, the Catalase Issue', *International Journal of Molecular Sciences*. Multidisciplinary Digital Publishing Institute (MDPI), 21(23), pp. 1–14. doi: 10.3390/IJMS21239149.

Ratliff, E. P. *et al.* (2015) 'Aging and autophagic function influences the progressive decline of adult Drosophila behaviors', *PLoS ONE*. Public Library of Science, 10(7). doi: 10.1371/journal.pone.0132768.

Ravikumar, B. *et al.* (2006) 'Rapamycin pre-treatment protects against apoptosis', *Human Molecular Genetics*, 15(7), pp. 1209–1216. doi: 10.1093/HMG/DDL036.

Reddy, P. *et al.* (2009) 'PDK1 signaling in oocytes controls reproductive aging and lifespan by manipulating the survival of primordial follicles', *Human molecular genetics*. Hum Mol Genet, 18(15), pp. 2813–2824. doi: 10.1093/HMG/DDP217.

Regan, J. C. *et al.* (2016) 'Sex difference in pathology of the ageing gut mediates the greater response of female lifespan to dietary restriction', *eLife*. eLife Sciences Publications Ltd, 5(FEBRUARY2016). doi: 10.7554/eLife.10956.

Reiling, J. H. and Hafen, E. (2004) 'The hypoxia-induced paralogs Scylla and Charybdis inhibit growth by down-regulating S6K activity upstream of TSC in Drosophila', *Genes & Development*. Cold Spring Harbor Laboratory Press, 18(23), p. 2879. doi: 10.1101/GAD.322704.

Rera, M., Clark, R. I. and Walker, D. W. (2012) 'Intestinal barrier dysfunction links metabolic and inflammatory markers of aging to death in Drosophila', *Proceedings of the National Academy of Sciences of the United States of America*. National Academy of Sciences, 109(52), pp. 21528–21533. doi: 10.1073/pnas.1215849110.

Rintelen, F. *et al.* (2001) 'PDK1 regulates growth through Akt and S6K in Drosophila', *Proceedings of the National Academy of Sciences of the United States of America*. National Academy of Sciences, 98(26), pp. 15020–15025. doi: 10.1073/pnas.011318098.

Ritchie, M. E. *et al.* (2015) 'limma powers differential expression analyses for RNA-sequencing and microarray studies', *Nucleic acids research*. Nucleic Acids Res, 43(7), p. e47. doi: 10.1093/NAR/GKV007.

Roser, M., Ortiz-Ospina, E. and Ritchie, H. (2019) *Life Expectancy - Our World in Data*. Available at: https://ourworldindata.org/life-expectancy (Accessed: 9 November 2022).

Rulifson, E. J., Kim, S. K. and Nusse, R. (2002) 'Ablation of insulin-producing neurons in flies: growth and diabetic phenotypes', *Science (New York, N.Y.)*. Science, 296(5570), pp. 1118–1120. doi: 10.1126/SCIENCE.1070058.

Sancak, Y. *et al.* (2008) 'The Rag GTPases bind raptor and mediate amino acid signaling to mTORC1', *Science (New York, N.Y.)*. Science, 320(5882), pp. 1496–1501. doi: 10.1126/SCIENCE.1157535.

Sano, H. *et al.* (2003) 'Insulin-stimulated phosphorylation of a Rab GTPase-activating protein regulates GLUT4 translocation', *The Journal of biological chemistry*. J Biol Chem, 278(17), pp. 14599–14602. doi: 10.1074/JBC.C300063200.

Sarbassov, D. D. *et al.* (2005) 'Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex', *Science*. American Association for the Advancement of Science , 307(5712), pp. 1098–1101. doi: 10.1126/SCIENCE.1106148/SUPPL_FILE/SARBASSOV.SOM.PDF.

Sasaki, A. *et al.* (2021) 'white regulates proliferative homeostasis of intestinal stem cells during ageing in Drosophila', *Nature Metabolism 2021 3:4*. Nature Publishing Group, 3(4), pp. 546–557. doi: 10.1038/s42255-021-00375-x.

Sato, S., Fujita, N. and Tsuruo, T. (2004) 'Involvement of 3-phosphoinositide-dependent protein kinase-1 in the MEK/MAPK signal transduction pathway', *Journal of Biological Chemistry*. J Biol Chem, 279(32), pp. 33759–33767. doi: 10.1074/jbc.M402055200.

Savitsky, M. *et al.* (2020) 'Humanization of Drosophila Gαo to Model GNAO1 Paediatric Encephalopathies', *Biomedicines*. Multidisciplinary Digital Publishing Institute (MDPI), 8(10), pp. 1–16. doi: 10.3390/BIOMEDICINES8100395.

Saxton, R. A. and Sabatini, D. M. (2017) 'mTOR Signaling in Growth, Metabolism, and Disease', *Cell*. Cell, 168(6), pp. 960–976. doi: 10.1016/J.CELL.2017.02.004.

Schinaman, J. M. *et al.* (2019a) 'Rapamycin modulates tissue aging and lifespan independently of the gut microbiota in Drosophila', *Scientific Reports*. Nature Publishing Group, 9(1), pp. 1–10. doi: 10.1038/s41598-019-44106-5.

Schinaman, J. M. *et al.* (2019b) 'Rapamycin modulates tissue aging and lifespan independently of the gut microbiota in Drosophila', *Scientific Reports*. Nature Publishing Group, 9(1), pp. 1–10. doi: 10.1038/s41598-019-44106-5.

Schmidt, J. M. *et al.* (2017) 'Insights into DDT resistance from the drosophila melanogaster genetic reference panel', *Genetics*. Genetics Society of America, 207(3), pp. 1181–1193. doi: 10.1534/genetics.117.300310.

Schulze, J. O. *et al.* (2016) 'Bidirectional Allosteric Communication between the ATP-Binding Site and the Regulatory PIF Pocket in PDK1 Protein Kinase', *Cell chemical biology*. Cell Chem Biol, 23(10), pp. 1193–1205. doi: 10.1016/J.CHEMBIOL.2016.06.017.

Searle, S. D. *et al.* (2008) 'A standard procedure for creating a frailty index', *BMC Geriatrics*. BioMed Central, 8(1), pp. 1–10. doi: 10.1186/1471-2318-8-24/TABLES/3.

Selman, C. *et al.* (2008) 'Evidence for lifespan extension and delayed age–related biomarkers in insulin receptor substrate 1 null mice', *The FASEB Journal*. Wiley, 22(3), pp. 807–818. doi: 10.1096/fj.07-9261com.

Selman, C. *et al.* (2009) 'Ribosomal protein S6 kinase 1 signaling regulates mammalian life span', *Science*. Sheffield Academic Press, 326(5949), pp. 140–144. doi: 10.1126/science.1177221.

Semaniuk, U. *et al.* (2021) 'Drosophila insulin-like peptides: from expression to functions – a review', *Entomologia Experimentalis et Applicata*. John Wiley & Sons, Ltd, 169(2), pp. 195–208. doi: 10.1111/EEA.12981.

Semaniuk, U. V. *et al.* (2018) 'Insulin-like peptides regulate feeding preference and metabolism in Drosophila', *Frontiers in Physiology*. Frontiers Media S.A., 9(AUG), p. 1083. doi: 10.3389/FPHYS.2018.01083/BIBTEX.

Sengupta, S., Peterson, T. R. and Sabatini, D. M. (2010) 'Regulation of the mTOR Complex 1 Pathway by Nutrients, Growth Factors, and Stress', *Molecular Cell*. Cell Press, 40(2), pp. 310–322. doi: 10.1016/J.MOLCEL.2010.09.026.

Serrano, M. and Blasco, M. A. (2007) 'Cancer and ageing: convergent and divergent mechanisms', *Nature Reviews Molecular Cell Biology 2007 8:9*. Nature Publishing Group, 8(9), pp. 715–722. doi: 10.1038/nrm2242.

Shah, O. J. and Hunter, T. (2006) 'Turnover of the active fraction of IRS1 involves raptor-mTOR- and S6K1-dependent serine phosphorylation in cell culture models of tuberous sclerosis', *Molecular and cellular biology*. Mol Cell Biol, 26(17), pp. 6425–6434. doi: 10.1128/MCB.01254-05.

Shahryari, A. *et al.* (2019) 'Development and Clinical Translation of Approved Gene Therapy Products for Genetic Disorders', *Frontiers in Genetics*. Frontiers, 10, p. 868. doi: 10.3389/FGENE.2019.00868.

Shamji, A. F., Nghiem, P. and Schreiber, S. L. (2003) 'Integration of Growth Factor and Nutrient Signaling: Implications for Cancer Biology', *Molecular Cell*. Cell Press, 12(2), pp. 271–280. doi: 10.1016/J.MOLCEL.2003.08.016.

Shaul, Y. D. and Seger, R. (2007) 'The MEK/ERK cascade: From signaling specificity to diverse functions', *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research*. Elsevier, 1773(8), pp. 1213–1226. doi: 10.1016/J.BBAMCR.2006.10.005.

Sheridan, P. E., Mair, C. A. and Quinõnes, A. R. (2019) 'Associations between prevalent multimorbidity combinations and prospective disability and self-rated health among older adults in Europe', *BMC geriatrics*. BMC Geriatr, 19(1). doi: 10.1186/S12877-019-1214-Z.

Slack, C. *et al.* (2010) 'Regulation of lifespan, metabolism, and stress responses by the Drosophila SH2B protein, Lnk', *PLoS Genetics*. PLoS Genet, 6(3). doi: 10.1371/journal.pgen.1000881.

Slack, C. *et al.* (2011) 'dFOXO-independent effects of reduced insulin-like signaling in Drosophila', *Aging Cell*. Aging Cell, 10(5), pp. 735–748. doi: 10.1111/j.1474-9726.2011.00707.x.

Slack, C. *et al.* (2015) 'The Ras-Erk-ETS-Signaling Pathway Is a Drug Target for Longevity', *Cell*. Cell Press, 162(1), pp. 72–83. doi: 10.1016/j.cell.2015.06.023.

Song, W., Veenstra, J. A. and Perrimon, N. (2014) 'Control of lipid metabolism by tachykinin in Drosophila', *Cell reports*. Cell Rep, 9(1), pp. 40–47. doi: 10.1016/J.CELREP.2014.08.060.

Sonoshita, M. and Cagan, R. L. (2017) 'Modeling Human Cancers in Drosophila', *Current topics in developmental biology*. Curr Top Dev Biol, 121, pp. 287–309. doi: 10.1016/BS.CTDB.2016.07.008.

Steinbaugh, M. J. *et al.* (2012) 'Activation of genes involved in xenobiotic metabolism is a shared signature of mouse models with extended lifespan', *American journal of physiology. Endocrinology and metabolism*. Am J Physiol Endocrinol Metab, 303(4). doi: 10.1152/AJPENDO.00110.2012.

Stocker, H. *et al.* (2003) 'Rheb is an essential regulator of S6K in controlling cell growth in Drosophila', *Nature Cell Biology 2003 5:6*. Nature Publishing Group, 5(6), pp. 559–566. doi: 10.1038/ncb995.

Stokoe, D. *et al.* (1997) 'Dual role of phosphatidylinositol-3,4,5-trisphosphate in the activation of

protein kinase B', *Science (New York, N.Y.)*. Science, 277(5325), pp. 567–570. doi: 10.1126/SCIENCE.277.5325.567.

Su, Z.-Y. *et al.* (2013) 'A perspective on dietary phytochemicals and cancer chemoprevention: oxidative stress, nrf2, and epigenomics', *Topics in current chemistry*. Top Curr Chem, 329, pp. 133–162. doi: 10.1007/128_2012_340.

Sun, P. D., Foster, C. E. and Boyington, J. C. (2004) 'Overview of Protein Structural and Functional Folds', *Current Protocols in Protein Science*. Wiley-Blackwell, 35(1), p. 1711. doi: 10.1002/0471140864.PS1701S35.

Svendsen, A. M. *et al.* (2014) 'Down-regulation of cyclin G2 by insulin, IGF-I (insulin-like growth factor 1) and X10 (AspB10 insulin): role in mitogenesis', *The Biochemical journal*. Biochem J, 457(1), pp. 69–77. doi: 10.1042/BJ20130490.

Sykiotis, G. P. and Bohmann, D. (2008) 'Keap1/Nrf2 Signaling Regulates Oxidative Stress Tolerance and Lifespan in Drosophila', *Developmental Cell*. Cell Press, 14(1), pp. 76–85. doi: 10.1016/J.DEVCEL.2007.12.002.

Tacutu, R. *et al.* (2018) 'Human Ageing Genomic Resources: new and updated databases', *Nucleic acids research*. Nucleic Acids Res, 46(D1), pp. D1083–D1090. doi: 10.1093/NAR/GKX1042.

Tain, L. S. *et al.* (2017) 'A proteomic atlas of insulin signalling reveals tissue-specific mechanisms of longevity assurance', *Molecular Systems Biology*. EMBO, 13(9), p. 939. doi: 10.15252/msb.20177663.

Takemori, H. and Okamoto, M. (2008) 'Regulation of CREB-mediated gene expression by salt inducible kinase', *The Journal of steroid biochemistry and molecular biology*. J Steroid Biochem Mol Biol, 108(3–5), pp. 287–291. doi: 10.1016/J.JSBMB.2007.09.006.

Taniguchi, C. M., Emanuelli, B. and Kahn, C. R. (2006) 'Critical nodes in signalling pathways: Insights into insulin action', *Nature Reviews Molecular Cell Biology*. Nature Publishing Group, pp. 85–96. doi: 10.1038/nrm1837.

Tatar, M. *et al.* (2001) 'A mutant Drosophila insulin receptor homolog that extends life-span and impairs neuroendocrine function', *Science*. American Association for the Advancement of Science, 292(5514), pp. 107–110. doi: 10.1126/SCIENCE.1057987/ASSET/5887CD9A-46A6-48C2-8A3A-D3B240B5B6B8/ASSETS/GRAPHIC/SE1319318005.JPEG.

Teixeira, L. *et al.* (2003) 'Drosophila Perilipin/ADRP homologue Lsd2 regulates lipid metabolism', *Mechanisms of development*. Mech Dev, 120(9), pp. 1071–1081. doi: 10.1016/S0925-4773(03)00158-8.

Teleman, A. A. *et al.* (2008) 'Nutritional Control of Protein Biosynthetic Capacity by Insulin via Myc in Drosophila', *Cell Metabolism*. Cell Press, 7(1), pp. 21–32. doi: 10.1016/J.CMET.2007.11.010.

Terada, N. *et al.* (1994) 'Rapamycin selectively inhibits translation of mRNAs encoding elongation factors and ribosomal proteins.', *Proceedings of the National Academy of Sciences of the United States of America*. National Academy of Sciences, 91(24), p. 11477. doi: 10.1073/PNAS.91.24.11477.

Tettweiler, G. *et al.* (2005) 'Starvation and oxidative stress resistance in Drosophila are mediated through the eIF4E-binding protein, d4E-BP', *Genes and Development*. Cold Spring Harbor Laboratory Press, 19(16), pp. 1840–1843. doi: 10.1101/gad.1311805.

Truebestein, L. *et al.* (2021) 'Structure of autoinhibited Akt1 reveals mechanism of PIP3-mediated activation', *Proceedings of the National Academy of Sciences of the United States of America*. Proc Natl Acad Sci U S A, 118(33). doi: 10.1073/PNAS.2101496118.

Ugrankar, R. *et al.* (2015) 'Drosophila glucome screening identifies Ck1alpha as a regulator of mammalian glucose metabolism', *Nature Communications 2015 6:1*. Nature Publishing Group, 6(1), pp. 1–10. doi: 10.1038/ncomms8102.

United Nations (2021) *Knowledge for healthy ageing*. Available at: https://www.decadeofhealthyageing.org/ (Accessed: 9 November 2022).

Vellai, T. *et al.* (2003) 'Influence of TOR kinase on lifespan in C. elegans', *Nature 2003 426:6967*. Nature Publishing Group, 426(6967), pp. 620–620. doi: 10.1038/426620a.

Vihervaara, T. and Puig, O. (2008) 'dFOXO regulates transcription of a Drosophila acid lipase', *Journal of molecular biology*. J Mol Biol, 376(5), pp. 1215–1223. doi: 10.1016/J.JMB.2007.12.042.

Walkiewicz, M. A. and Stern, M. (2009) 'Increased Insulin/Insulin Growth Factor Signaling Advances the Onset of Metamorphosis in Drosophila', *PLOS ONE*. Public Library of Science, 4(4), p. e5072. doi: 10.1371/JOURNAL.PONE.0005072.

Wang, B. *et al.* (2011) 'A Hormone-Dependent Module Regulating Energy Balance', *Cell*. Cell Press, 145(4), pp. 596–606. doi: 10.1016/J.CELL.2011.04.013.

Wang, L. *et al.* (2007) 'PRAS40 regulates mTORC1 kinase activity by functioning as a direct inhibitor of substrate binding', *The Journal of biological chemistry*. J Biol Chem, 282(27), pp. 20036–20044. doi: 10.1074/JBC.M702376200.

Wang, L. *et al.* (2008) 'Essential Roles of the PI3 Kinase/Akt Pathway in Regulating Nrf2-Dependent Antioxidant Functions in the RPE', *Investigative ophthalmology & visual science*. NIH Public Access, 49(4), p. 1671. doi: 10.1167/IOVS.07-1099.

Wang, X. *et al.* (2001) 'Regulation of elongation factor 2 kinase by p90(RSK1) and p70 S6 kinase', *The EMBO journal*. EMBO J, 20(16), pp. 4370–4379. doi: 10.1093/EMBOJ/20.16.4370.

Wang, Y. *et al.* (2001) 'The JIL-1 tandem kinase mediates histone H3 phosphorylation and is required for maintenance of chromatin structure in Drosophila', *Cell*. Cell, 105(4), pp. 433–443. doi: 10.1016/S0092-8674(01)00325-7.

Wang, Y. *et al.* (2018) 'Superoxide dismutases: Dual roles in controlling ROS damage and regulating ROS signaling', *Journal of Cell Biology*. The Rockefeller University Press, 217(6), pp. 1915–1928. doi: 10.1083/JCB.201708007.

Watson, J. and Zalzala, S. (2020) *Participatory Evaluation (of) Aging (With) Rapamycin (for) Longevity Study - Full Text View - ClinicalTrials.gov*. Available at: https://clinicaltrials.gov/ct2/show/NCT04488601 (Accessed: 11 November 2022).

Wei, M. *et al.* (2008) 'Life span extension by calorie restriction depends on Rim15 and transcription factors downstream of Ras/PKA, Tor, and Sch9', *PLoS genetics*. PLoS Genet, 4(1), pp. 0139–0149. doi: 10.1371/JOURNAL.PGEN.0040013.

Weinkove, D. *et al.* (1999) 'Regulation of imaginal disc cell size, cell number and organ size by Drosophila class I A phosphoinositide 3-kinase and its adaptor'. Available at: http://biomednet.com/elecref/0960982200901019 (Accessed: 31 January 2023).

Weinreich, J. *et al.* (2011) 'Rapamycin-induced impaired wound healing is associated with compromised tissue lactate accumulation and extracellular matrix remodeling', *European surgical research. Europaische chirurgische Forschung. Recherches chirurgicales europeennes*. Eur Surg Res, 47(1), pp. 39–44. doi: 10.1159/000327972.

Weismann, A. (1882) 'Theory of ageing: Wear and Tear'.

Werz, C. *et al.* (2009) 'The Drosophila SH2B family adaptor Lnk acts in parallel to chico in the insulin signaling pathway', *PLoS Genetics*, 5(8). doi: 10.1371/JOURNAL.PGEN.1000596.

Wheeler, D. L. and Yarden, Y. (2015) 'Receptor tyrosine kinases: Structure, functions and role in human disease', *Receptor Tyrosine Kinases: Structure, Functions and Role in Human Disease*. Springer New York, pp. 1–440. doi: 10.1007/978-1-4939-2053-2.

WHO (2022) *Ageing and health*, *1 october*. Available at: https://www.who.int/news-room/fact-sheets/detail/ageing-and-health (Accessed: 9 November 2022).

Wick, M. J. *et al.* (2003) 'Mouse 3-Phosphoinositide-dependent Protein Kinase-1 Undergoes Dimerization and trans-Phosphorylation in the Activation Loop', *Journal of Biological Chemistry*. J Biol Chem, 278(44), pp. 42913–42919. doi: 10.1074/jbc.M304172200.

Willcox, B. J. *et al.* (2008) 'FOXO3A genotype is strongly associated with human longevity', *Proceedings of the National Academy of Sciences of the United States of America*. National Academy of Sciences, 105(37), p. 13987. doi: 10.1073/PNAS.0801030105.

Williams, G. C. (1957) 'Pleiotropy, Natural Selection, and the Evolution of Senescence', *Evolution*. JSTOR, 11(4), p. 398. doi: 10.2307/2406060.

Williams, M. R. *et al.* (2000) 'The role of 3-phosphoinositide-dependent protein kinase 1 in activating AGC kinases defined in embryonic stem cells', *Current Biology*. Cell Press, 10(8), pp. 439–448. doi: 10.1016/S0960-9822(00)00441-3.

Wilson, K. A. *et al.* (2020) 'GWAS for Lifespan and Decline in Climbing Ability in Flies upon Dietary Restriction Reveal decima as a Mediator of Insulin-like Peptide Production', *Current Biology*. Cell Press, 30(14), pp. 2749-2760.e3. doi: 10.1016/J.CUB.2020.05.020.

Wullschleger, S., Loewith, R. and Hall, M. N. (2006) 'TOR signaling in growth and metabolism', *Cell*. Elsevier, pp. 471–484. doi: 10.1016/j.cell.2006.01.016.

Xie, W. and Tian, Y. (2006) 'Xenobiotic receptor meets NF-κB, a collision in the small bowel', *Cell Metabolism*. Cell Press, 4(3), pp. 177–178. doi: 10.1016/J.CMET.2006.08.004.

Xu, C., Kim, N. G. and Gumbiner, B. M. (2009) 'Regulation of protein stability by GSK3 mediated phosphorylation', *http://dx.doi.org/10.4161/cc.8.24.10111*. Taylor & Francis, 8(24), pp. 4032–4039. doi: 10.4161/CC.8.24.10111.

Xu, C., Li, C. Y. T. and Kong, A. N. T. (2005) 'Induction of phase I, II and III drug metabolism/transport by xenobiotics', *Archives of Pharmacal Research 2005 28:3*. Springer, 28(3), pp. 249–268. doi: 10.1007/BF02977789.

Yamazaki, D. *et al.* (2007) 'The Drosophila DCO mutation suppresses age-related memory impairment without affecting lifespan', *Nature Neuroscience 2007 10:4*. Nature Publishing Group, 10(4), pp. 478–484. doi: 10.1038/nn1863.

Yang, B.-S. *et al.* (1996) 'Ras-mediated phosphorylation of a conserved threonine residue enhances the transactivation activities of c-Ets1 and c-Ets2', *Molecular and Cellular Biology*. American Society for Microbiology, 16(2), pp. 538–547. doi: 10.1128/MCB.16.2.538.

Yu, S. H. *et al.* (2004) 'Stimulation of c-Rel transcriptional activity by PKA catalytic subunit beta', *Journal of molecular medicine (Berlin, Germany)*. J Mol Med (Berl), 82(9), pp. 621–628. doi: 10.1007/S00109-004-0559-7.

Zhang, H. *et al.* (2000) 'Regulation of cellular growth by the Drosophila target of rapamycin dTOR', *Genes & development*. Genes Dev, 14(21), pp. 2712–2724. doi: 10.1101/GAD.835000.

Zhang, H. H. *et al.* (2009) 'Insulin stimulates adipogenesis through the Akt-TSC2-mTORC1 pathway', *PloS one*. PLoS One, 4(7). doi: 10.1371/JOURNAL.PONE.0006189.

Zhang, P. *et al.* (2022) 'Inhibition of S6K lowers age-related inflammation and immunosenescence and increases lifespan through the endolysosomal system', *bioRxiv*. Cold Spring Harbor Laboratory, p. 2022.08.25.505264. doi: 10.1101/2022.08.25.505264.

Zhang, Y. *et al.* (2003) 'Rheb is a direct target of the tuberous sclerosis tumour suppressor proteins', *Nature cell biology*. Nat Cell Biol, 5(6), pp. 578–581. doi: 10.1038/NCB999.

Zhang, Z. Y. (2002) ‘Protein tyrosine phosphatases: structure and function, substrate specificity, and inhibitor development’, *Annual review of pharmacology and toxicology*. Annu Rev Pharmacol Toxicol, 42, pp. 209–234. doi: 10.1146/ANNUREV.PHARMTOX.42.083001.144616.

Zid, B. M. *et al.* (2009) ‘4E-BP Extends Lifespan upon Dietary Restriction by Enhancing Mitochondrial Activity in Drosophila’, *Cell*. Cell Press, 139(1), pp. 149–160. doi: 10.1016/J.CELL.2009.07.034.

Zimmermann, G. R., Lehár, J. and Keith, C. T. (2007) ‘Multi-target therapeutics: when the whole is greater than the sum of the parts’, *Drug discovery today*. Drug Discov Today, 12(1–2), pp. 34–42. doi: 10.1016/J.DRUDIS.2006.11.008.

Zoncu, R., Efeyan, A. and Sabatini, D. M. (2011) ‘mTOR: from growth signal integration to cancer, diabetes and ageing’, *Nature reviews. Molecular cell biology*. Nat Rev Mol Cell Biol, 12(1), pp. 21–35. doi: 10.1038/NRM3025.

Erklärung zur Dissertation
gemäß der Promotionsordnung vom 12. März 2020

Diese Erklärung muss in der Dissertation enthalten sein.
(*This version must be included in the doctoral thesis*)

„Hiermit versichere ich an Eides statt, dass ich die vorliegende Dissertation selbstständig und ohne die Benutzung anderer als der angegebenen Hilfsmittel und Literatur angefertigt habe. Alle Stellen, die wörtlich oder sinngemäß aus veröffentlichten und nicht veröffentlichten Werken dem Wortlaut oder dem Sinn nach entnommen wurden, sind als solche kenntlich gemacht. Ich versichere an Eides statt, dass diese Dissertation noch keiner anderen Fakultät oder Universität zur Prüfung vorgelegen hat; dass sie - abgesehen von unten angegebenen Teilpublikationen und eingebundenen Artikeln und Manuskripten - noch nicht veröffentlicht worden ist sowie, dass ich eine Veröffentlichung der Dissertation vor Abschluss der Promotion nicht ohne Genehmigung des Promotionsausschusses vornehmen werde. Die Bestimmungen dieser Ordnung sind mir bekannt. Darüber hinaus erkläre ich hiermit, dass ich die Ordnung zur Sicherung guter wissenschaftlicher Praxis und zum Umgang mit wissenschaftlichem Fehlverhalten der Universität zu Köln gelesen und sie bei der Durchführung der Dissertation zugrundeliegenden Arbeiten und der schriftlich verfassten Dissertation beachtet habe und verpflichte mich hiermit, die dort genannten Vorgaben bei allen wissenschaftlichen Tätigkeiten zu beachten und umzusetzen. Ich versichere, dass die eingereichte elektronische Fassung der eingereichten Druckfassung vollständig entspricht."

Teilpublikationen:

Datum, Name und Unterschrift

05.12.2022, Köln, Sophie Louise van Schelt

Declaration for the doctoral thesis (dissertation)

according to the doctoral regulations published 12th March 2020

Non-official English translation of the "Erklärung zur Dissertation"
(*The German version must be included in the doctoral thesis*)

"I hereby declare that I have completed the present dissertation independently and without the use of any aids or literature other than those referred to. All passages that have been taken, either literally or in sense, from published and unpublished works, are marked as such. I declare that this dissertation has not been submitted to any other faculty or university; that - apart from the partial publications and included articles and manuscripts listed below - it has not yet been published, and that I will not publish the dissertation before completing my doctorate without the permission of the PhD Committee. I am aware of the terms of the doctoral regulations. In addition, I hereby declare that I am aware of the "Regulations for Safeguarding Good Scientific Practice and Dealing with Scientific Misconduct" of the University of Cologne, and that I have observed them during the work on the thesis project and the written doctoral thesis. I hereby commit myself to observe and implement the guidelines mentioned there in all scientific activities. I assure that the submitted electronic version is identical to the submitted printed version".

Partial publications of the thesis:

Date, name, and signature

05.12.2022, Köln, Sophie Louise van Schelt

Erklärung zum Gesuch um Zulassung zur Promotion

gemäß der Promotionsordnung vom 12. März 2020

1. Zugänglichkeit von Daten und Materialien

Die Dissertation beinhaltet die Gewinnung von Primärdaten oder die Analyse solcher Daten oder die Reproduzierbarkeit der in der Dissertation dargestellten Ergebnisse setzt die Verfügbarkeit von Datenanalysen, Versuchsprotokollen oder Probenmaterial voraus.

☐ Trifft nicht zu

☒ Trifft zu.
In der Dissertation ist dargelegt wie diese Daten und Materialien gesichert und zugänglich sind (entsprechend den Vorgaben des Fachgebiets beziehungsweise der Betreuerin oder des Betreuers).

2. Frühere Promotionsverfahren

Ich habe bereits einen Doktortitel erworben oder ehrenhalber verliehen bekommen.

Oder: Für mich ist an einer anderen Fakultät oder Universität ein Promotionsverfahren eröffnet worden, aber noch nicht abgeschlossen.

Oder: Ich bin in einem Promotionsverfahren gescheitert.

☒ Trifft nicht zu

☐ Zutreffend
Erläuterung:

3. Straftat

Ich bin nicht zu einer vorsätzlichen Straftat verurteilt worden, bei deren Vorbereitung oder Begehung der Status einer Doktorandin oder eines Doktoranden missbraucht wurde.

Ich versichere, alle Angaben wahrheitsgemäß gemacht zu haben.

Datum	Name	Unterschrift
05.12.2022, Köln	Sophie Louise van Schelt	

Declaration on the application for admission to the doctoral examinations
according to the doctoral regulations published 12th March 2020

1. Accessibility of data and materials

The dissertation involves the acquisition of primary data or the analysis of such data or the reproducibility of the results presented in the dissertation requires the availability of data analyses, experimental protocols or sample material.

☐ not applicable

☒ applicable
I have described in the dissertation how these data and materials are secured and accessible (according to the specifications of the subject area or supervisor).

2. Previous doctoral examinations

I have already obtained a doctorate or been awarded an honorary doctoral degree.
Or: A doctoral examination has been opened at another faculty or university, but not yet completed.
Or: I have failed in a doctoral examination.

☒ not applicable

☐ applicable
explanatory note:

3. Criminal offense

I have not been convicted of a deliberate criminal offence in the preparation or commission of which the status as a doctoral candidate was abused.

I declare that all information provided is truthful.

Date	Name	Signature
05.12.2022, Köln	Sophie Louise van Schelt	