The MAPK/ERK signalling pathway: functional characterisation of rare human genetic variants associated with longevity

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III. Abbreviations

AMPK	Adenosine monophosphate-activated protein kinase
AKT	Protein kinase B
ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
ATP	Adenosine triphosphate
BCA	Bicinchoninic acid
CADD	Combined annotation-dependent depletion
C. elegans	Caenorhabditis elegans
D.melanogaster	Drosophila melanogaster
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
CO2	Carbon dioxide
CVD	Cardiovascular disease
DAPI	4′,6-Diamidin-2-phenylindol
DEP	Differentially expressed proteins
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EMA	European Medicine Agency
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinase
EtOH	Ethanol
FA	Formic acid
FACS	Fluorescence activated cell sorting
FBS	Fetal bovine serum
FDA	Food and Drug Administration
FDR	False discovery rate
FELASA	Federation of the European Laboratory Animal Science Association
FoxO	Forkhead box protein O
GAP	GTPase-activating proteins
GEF	Guanine nucleotide exchange factors

GWAS	Genome-wide association studies
HCL	Hydrochloride
HRP	Horseradish peroxidase
H2O2	Hydrogen peroxide
ISC	Intestinal stem cell
IIS	Insulin/insulin-like growth factor signalling
KEGG	Kyoto Encyclopedia of Genes and Genomes
KOH	Potassium hydroxide
LB	Luria-Bertani
LC-MS/MS	Liquid Chromatography with tandem mass spectrometry
LLS	Leiden Longevity Study
LOF	Loss-of-function
LUMC	Leiden University Medical Centre
MAF	Minor allele frequency
MAPK	Mitogen-activated protein kinase
MEF	Mouse embryonic fibroblast
mESC	Mouse embryonic stem cell
MPI Age	Max Planck Institute for Biology of Ageing
MS	Mass spectrometry
MTBE	Methyl tertiary-butyl ether
mTOR	Mechanistic target of rapamycin
NaCL	Sodium chloride
ORA	Overrepresentation analysis
OXPHOS	Oxidative phosphorylation
PAM	Protospacer adjacent motif
PBS	Phosphate buffered saline
PCA	Principal component analysis
PDGF	Platelet-derived growth factor
PFA	Paraformaldehyde
pH3	Phospho-histone 3
PIP3	Phospha-tidylinositol (3,4,5) triphosphates
PI3K	Phosphatidylinositol 3-kinase
RGS	Rigosertib
SAH	S-adenosyl-L-homocysteine

SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SYA	Sugar/yeast/agar
TBST	Tris-buffered saline with Tween
TCA cycle	Tricarboxylic acid cycle
TMT	Tandem mass tag
wDah	White Dahomey
WGS	Whole-genome sequencing
WT	Wildtype

IV. Summary

Observational and experimental studies have revealed that ageing is malleable. Research in different organisms has shown that dietary, pharmacological and genetic interventions can extend health- and lifespan. Similarly in humans, diet and other lifestyle factors such as exercise, have been shown to slow the ageing process, however, the role of genetics is less clear. For human lifespan, the heritability is somewhere in the range of 12-25%, but for longevity, i.e. belonging to the top 10% longest-lived, the heritability remains elusive. Investigations in this field have, thus far, identified a limited number of common longevity-associated variants, suggesting that rare genetic variants may play a role in human longevity. Ageing research in model organisms has implicated the insulin/insulin-like growth factor signalling (IIS) network in lifespan and longevity. A mild reduction in activity through genetic and pharmacological interventions in the IIS network, e.g. with rapamycin, have been shown to robustly extend lifespan. The mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) signalling pathway is one branch of the IIS network and has been implicated in lifespan in yeast, worms, flies and mice, through its genetic and pharmacological manipulation, e.g. with trametinib. Whether it also plays a role in human lifespan and longevity, remains unknown. My PhD thesis first explores the role of rare genetic variants in the MAPK/ERK signalling pathway observed in long-lived individuals belonging to the Leiden Longevity Study. After their identification using a bioinformatic pipeline, I aimed to functionally characterise these genetic variants *in vitro* and *in vivo* to determine their possible role in human longevity. Second, I investigated the geroprotective potential of a dual MAPK/ERK and PI3K/AKT/mTOR signalling pathway inhibitor, Rigosertib, in promoting healthy ageing in the fruit fly.

Chapter 3 of this thesis entails a novel pipeline to functionally characterise genetic variants in cells and different model organisms. For this purpose, I generated cells, flies and mice harbouring longevity-associated human variants and assessed their effect on cellular phenotypes, such as pathway activity and stress resistance. Two variants, $Nf1^{Phe1112Leu}$ and $Raf1^{Asp633Tyr}$, showed opposing effects on MAPK/ERK signalling pathway activity and resulted in extensive rewiring of the MAPK/ERK related proteome and metabolome in cells. The one variant that I introduced in the fly, $Nf1^{Phe1148Leu}$, led to shortened lifespan in the homozygous state, while it did not seem to affect lifespan in flies in the heterozygous state (in which it was

detected in the long-lived individuals). For the other variant, however, *Raf1*^{Asp633Tyr}, I was able to show an improved stress response, which was conserved in mouse embryonic fibroblasts, suggesting that this variant may have contributed to human longevity through improved stress resistance mechanisms.

In Chapter 4 of this thesis, I investigated the effects of Rigosertib treatment on health- and lifespan in the fruit fly. I could show that Rigosertib robustly extends lifespan in female and male flies. Somewhat surprisingly, the MAPK/ERK signalling pathway activity appeared downregulated *in vitro* and upregulated *in vivo*, highlighting context specific regulation of this signalling pathway. Furthermore, in order to understand how the drug extends lifespan in both sexes, I investigated gut health of Rigosertib-fed flies. I could demonstrate that Rigosertib treatment ameliorated age-related decline in gut homeostasis, suggesting that this at least partially contributes to improved health- and lifespan in flies.

1. Introduction

1.1 Targeting Ageing

1.1.1 Ageing as a public health challenge

Demographic ageing has been on the rise over the past decades and is recognized at present as a public health challenge. Population ageing affects individuals, societies and economies, making it one of the major challenges of the 21st century. In 2018, the United Nations reported for the first time in history that seniors >65 years of age outnumbered children <5 years of age (https://www.un.org/en/global-issues/ageing). Key driving forces of population ageing are declining fertility rates alongside increasing life expectancies, due to improved standards of living and medical care (Partridge et al., 2018). Unfortunately, increasing lifespan is often accompanied by the occurrence of age-related diseases, contributing to an increased number of years individuals live with disability. Ageing, defined as a decline in physiological function, is the major risk factor for age-related diseases, such as cardiovascular disease (CVD), cancer and neurodegenerative diseases (Niccoli & Partridge, 2012).

1.1.2 Ageing is malleable

In recent years, basic research and understanding of the ageing process has gained increasing attention. López-Otín et al. (2013) have compiled the current understanding in the 'hallmarks of ageing', which suggests that ageing is associated with nine main attributes including genomic instability, loss of proteostasis and de-regulated nutrient sensing, among others. Central to these hallmarks is the fact that most of their underlying processes are evolutionarily conserved, opening the possibility for in-depth study in model organisms, such as the nematode worm (*Caenorhabditis elegans*), the fruit fly (*Drosophila melanogaster*) and the mouse (*Mus musculus*), to discover mechanisms potentially relevant to human ageing. Environmental factors such as diet and physical activity have a substantial effect on the manifestation of age-related hallmarks and diseases. Regular exercise and a balanced diet in older age have been shown to prevent as well as ameliorate age-associated decline in humans (Evans, 1995; Fontana & Partridge, 2015). Observations in long-lived individuals (>100 years of age) have revealed that there is also a genetic component to ageing, and that these individuals often show a compression of morbidity (Ismail et al., 2016). Diet, exercise and genetics influence ageing through partially shared cellular mechanisms (Ghanemi et al., 2021; Longo & Anderson, 2022).

By studying and understanding these mechanisms, research into the biology of ageing aims to ultimately reduce the burden of late-life morbidity on individuals and societies.

1.2 The role of nutrient-sensing pathways in ageing

1.2.1 The insulin/insulin-like growth factor 1 signalling network

The insulin/insulin-like growth factor 1 signalling (IIS) network is a central regulator of proliferation and metabolism and is highly conserved between species. It is activated by growth factors and hormones, which bind to corresponding receptors located in the cell membrane and transmit these extracellular cues to the inner cell machinery through the recruitment and activation of downstream cascades, including the mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) and phosphatidylinositol 3-kinase/protein kinase B/mechanistic target of rapamycin (PI3K/AKT/mTOR) signalling pathways (Fontana et al., 2010b; Taniguchi et al., 2006) **(Figure 1.1)**. In addition to the regulation of energy homeostasis, survival and resistance to various stressors, a growing body of evidence has also established a role for IIS in lifespan, through its downstream effectors, including AKT, mTOR and forkhead box protein O (FoxO). Initial discoveries of genes involved in lifespan were made by Klass (1983) and confirmed by Friedman & Johnson in 1988 and showed that mutations in the *age-1* gene (mammalian orthologue of the PI3K catalytic subunit) decrease pharyngeal pumping and thus food intake, which, in turn, significantly increased lifespan of these animals. In 1993 findings by Kenyon et al. followed, showing that mutations in the *daf-2* gene (worm orthologue of the mammalian insulin/insulin-like growth factor 1 receptors) could lead to doubled lifespan and that this extension was dependent on *daf-16* (the single worm FoxO orthologue). These ground-breaking observations proved that dampened IIS signalling, through genetic and dietary interventions, can result in extended lifespan of various organisms. This was later found to hold true in flies (Partridge et al., 2011), mice (Holzenberger et al., 2002; Selman et al., 2008) and humans, in which genetic variations of IIS signalling have been found to be associated with longevity (Deelen et al., 2013; Flachsbart et al., 2009; Willcox et al., 2008).

1.2.2 The role of Ras proteins in the MAPK/ERK and PI3K/AKT/mTOR signalling pathways

Ras proteins are central, upstream-located regulators of both the MAPK/ERK and PI3K/AKT/mTOR signalling branches of the IIS network, and they function as binary switches, depending on their GDP- or GTP-bound state, which is governed by guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs) **(Figure 1.1)**. PI3K/AKT/mTOR signalling can be initiated in a Ras-independent manner, for example through receptor tyrosine kinases and G-protein-coupled receptors, stimulating the generation of phospha-tidylinositol (3,4,5) triphosphates (PIP3) by p110 catalytic and p85 regulatory subunits of PI3K (Vanhaesebroeck et al., 2011). At the cell membrane, PIPs then facilitate the activation of pleckstrin-homology domain containing proteins, such as PDK1, which in turn phosphorylate AKT. PTEN proteins function as important antagonists of the pathway and dephosphorylate PIP3, through which cellular homeostasis and context dependent regulation is ensured (Endersby & Baker, 2008). Full activation of AKT is subsequently achieved through phosphorylation by mTORC2 (Sarbassov et al., 2005). The three isoforms of AKT are known to have many downstream effectors such as mTORC1, GSK-3, CREB and FoxOs (Castellano & Downward, 2011), regulating cell growth, survival, stress responses and lifespan. PI3K/AKT signalling may also be activated in a Ras-dependent manner, in which GTP-bound Ras directly activates the catalytic p110 subunit of PI3K **(Figure 1.1)** (Orme et al., 2006). MAPK/ERK signalling is, in turn, initiated through the recruitment of the Grb2 adapter protein to the receptor, which then associates with the Ras guanylnucleotide exchange factor SOS. This Grb2-SOS complex at the plasma membrane facilitates Ras activation and downstream signalling through RAF, MEK and ERK (Chen et al., 1996).

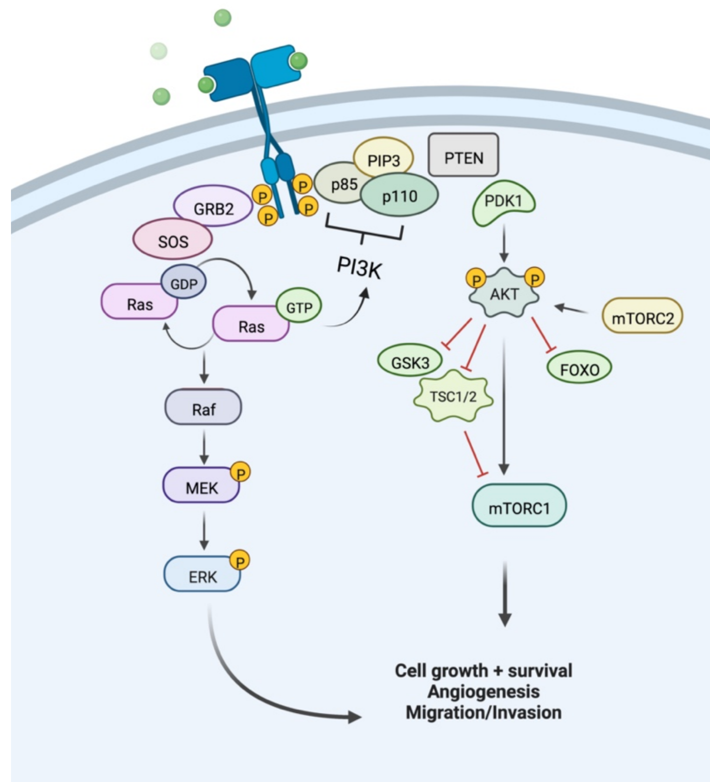

Figure 1.1 | Schematic of the MAPK/ERK and PI3K/AKT/mTOR signalling pathways as part of the IIS network.
Grb2, growth factor receptor-bound protein 2; SOS, son of sevenless; Ras, rat sarcoma; GDP, guanosine diphosphate; GTP, guanosine triphosphate; Raf, rapidly growing fibrosarcoma; Mek, mitogen-activated protein; Erk, extracellular-signal regulated kinase; PIP3, phospha-tidylinositol (3,4,5) triphosphate; PTEN, phosphatase and tensin homolog deleted on chromosome 10; PDK1, phosphoinositide-dependent kinase 1; Akt, protein kinase B; mTORC2, mechanistic target of rapamycin complex 2; mTORC1, mechanistic target of rapamycin complex 1; FoxO, forkhead box O; Tsc1/2, tuberous sclerosis complex 1 and 2; Gsk3, glycogen synthase kinase 3.

1.2.3 The PI3K/AKT/mTOR signalling pathway and its role in lifespan

The PI3K/AKT/mTOR signalling pathway is highly conserved and is a major regulator of cellular growth in the IIS network. Growth factor activated receptors recruit the PI3K complex to the cell membrane, which facilitates downstream signalling through AKT and mTOR complexes (Fresno Vara et al., 2004). TOR complexes encompass the mechanistic target of rapamycin complex 1 (mTORC1) and mechanistic target of rapamycin complex 2 (mTORC2), with mTORC1 predominantly regulating anabolic reactions such as protein and lipid synthesis and inhibiting autophagy. The rapamycin-insensitive mTORC2 complex is less studied and is mainly known for its role in cytoskeletal organisation (Jacinto et al., 2004; Zheng et al., 1995). mTORC1 is known to activate a variety of downstream located effectors (i.e. ribosomal protein S6 kinase and eukaryotic translation initiation factor 4E-binding protein) involved in lysosome

and mitochondrial biogenesis, glycolysis, protein turnover and stress responses. Controlling a wide range of cellular processes, PI3K/AKT/mTOR signalling has been implicated in many pathologies and is thus widely studied in the context of cancer, immunology and neurology. Activation of FoxO transcription factors downstream of the PI3K/AKT signalling pathway has been reported to underlie the observed longevity phenotype in various IIS mutant flies (Giannakou et al. 2004, Hwangbo et al. 2004, Slack et al. 2011, Zhang et al. 2011, Martins et al. 2016). Moreover, numerous mutations in these signalling hubs have been found to increase lifespan in different model organisms (Johnson et al., 2013) and the combined genetic variation in the TOR signalling pathway has been linked to longevity in humans (Passtoors et al., 2013).

1.2.4 The MAPK/ERK signalling pathway and its role in lifespan

MAPK/ERK signalling is another central growth and survival regulating branch of the IIS network **(Figure 1)**. This kinase cascade is activated through upstream located Ras proteins and transmits growth signals to the downstream located ERK1 and ERK2 proteins. Activated ERK proteins have a large repertoire of cytoplasmic and nuclear substrates that may differ depending on the cellular context and ultimately, they control survival, cell differentiation and migration (Yoon & Seger, 2006). The MAPK/ERK signalling pathway has also been implicated in lifespan, independent of the canonical IIS pathway, i.e. the PI3K/AKT/mTOR branch (Laskovs et al., 2022). In yeast, knockdown of *Ras2* was shown to extend replicative lifespan (Fabrizio et al., 2003), while in worms knockdown of *lin-45* (the homologue of human RAF1) using RNA interference reduced lifespan (Okuyama et al., 2010). Moreover, lifespan in fruit flies can be extended by overexpression of dominant negative Ras (Slack et al., 2015) and in mice by knockdown of *Rasgrf1* (Borrás et al., 2011). In addition to genetic manipulation, the role of MAPK/ERK in determination of lifespan is supported by the observation that long-lived caloric restricted mice as well as Snell dwarf mice show elevated basal phosphorylation of ERK (Ikeyama et al., 2002; Madsen et al., 2004). In humans, genetic variations in *HRAS1,* when occurring in combination with specific alleles of *APOE* and *LASS1,* have been associated with longevity, as reported by Jazwinski et al. (2010). However, these findings have thus far not been confirmed in additional populations and the exact role of the MAPK/ERK signalling pathway in human longevity is still largely unknown.

1.3 Genetics of human ageing

Observations in long-lived individuals have revealed that there is a genetic component to ageing. These long-lived individuals often show a compression of morbidity and longer healthspan, characterised by later onset of age-related diseases (Andersen et al., 2012; Christensen et al., 2008). In this thesis, we correspond to the general consensus on terminology and define healthspan as the number of years lived free of disease, lifespan as the total number of years lived, and longevity as survival to an exceptional old age, i.e. belonging to the top 10% longest lived of one's respective birth cohort (van den Berg, 2019). Early twin studies in Nordic countries have shown that the heritability of human lifespan lies between 12-25% (Herskind et al., 1996; Ljungquist et al., 1998; Mcgue et al., 1993; van den Berg et al., 2017). Heritability refers to the ratio of genetic components over the sum of other (e.g. environmental) components in a specific population (Tenesa & Haley, 2013). More recent estimates of the heritability of lifespan, based on large pedigrees, showed that the twin-based estimates are likely inflated due to assortative mating, as correlations were also found to be present in non-genetic relatives and thus suggest that the heritability of human lifespan is lower, at approximately 10% (Ruby et al., 2018; Kaplanis et al., 2018). For longevity, the heritability still remains unclear. A study by van den Berg et al. (2019) showed, however, that there is a genetic component to human longevity in two independent study populations when defined as top 10% survivors. This indicates that longevity can be transmitted as a quantitative genetic trait. The existence of a genetic basis for ageing is further supported by studies of other ageing-related phenotypes, such as osteoarthritis, Alzheimer's disease and cardiovascular disease, for which the heritability is estimated to lie between 45-79% (Gatz et al., 2006; Skousgaard et al., 2015; Zdravkovic et al., 2002).

Before whole exons and genomes could be sequenced, candidate gene studies looked at single genetic variants by comparing presumed longevity-associated loci between long-lived individuals and younger controls. These hypothesis-driven candidate studies can be used to study genes brought forward by research in model organisms, or age-related traits in humans, and have resulted in the identification of the *APOE* and *FOXO3* loci (Flachsbart et al., 2009; Schächter et al., 1994; Willcox et al., 2008), which have since been replicated in numerous studies (Morris et al., 2019). Progressions in DNA sequencing technology have made this an affordable and feasible tool and advanced our understanding of the role of human genetics in

ageing. Genome-wide association studies (GWAS) have enabled a hypothesis-free approach to identifying novel longevity-associated variants in the human genome. GWAS successfully identified a number of common lifespan, healthspan and longevity associated variants (i.e. annotated minor allele frequency (MAF) > 1%) (Melzer et al., 2020; Timmers et al., 2020). These studies offer a statistically powerful tool to correlate genetic variants with specific phenotypes and diseases by analysing many individuals and a large number of genetic variants at the same time (Manolio et al., 2008; Uffelmann et al., 2021). Unbiased GWAS, with the aim to decipher the role of genetic variation in human longevity, have thus far only identified one locus, APOE, which is successfully replicated between independent studies and populations (Deelen et al., 2019; Liu et al., 2021; Sebastiani et al., 2017). The small number of GWAS-replicated loci highlights a limitation of this study design, namely the large sample size required to detect statistical significance of identified variants. Moreover, in contrast to environmental factors, such as diet and healthcare infrastructure, the effect sizes of these rather common genetic variants are usually small and thus impede the association with longevity.

Gene-set analysis in candidate pathways offers another strategy to identify common genetic variation underlying human longevity. Previous findings, consolidated through research in animal models, have implicated the role of well-conserved cellular maintenance and signalling pathways in health and disease. These include the PI3K/AKT/mTOR signalling pathway and DNA repair mechanisms (Debrabant et al., 2014; Deelen, et al., 2013; Passtoors et al., 2013). Given that genetic approaches focussing on common variants have only been mildly successful, the field has now moved towards the possibility that rare variants, i.e. those with a MAF < 1%, may also contribute to longevity. To this end, whole-genome and exome sequencing approaches can be undertaken in combination with enrichment analysis or candidate pathway analysis (Lin et al., 2021; Nygaard et al., 2019). The identification of rare genetic variants through this approach is often hampered by a lack of suitable study cohorts of long-lived individuals or families and thus the lack of statistical power (Lee, 2014). Therefore, Lin et al. (2021) focussed on the combined effect of rare genetic variants located in the IIS network and AMPK signalling pathway and found these to be associated with human longevity and to protect against age-related diseases. However few studies have yet provided functional evidence *in vitro* or *in vivo*, which is needed to validate the role of rare variants in human ageing (Flachsbart et al., 2017; Tazearslan et al., 2011).

1.3.1 Functional characterisation of ageing-associated genetic variants

To investigate potential functional significance of variants brought forward by sequencing studies, their characterisation can be approached through *in silico*, *in vitro* and *in vivo* work, as we have recently reviewed elsewhere (Baghdadi et al., 2022). Many variants are located in intronic or intergenic regions and thus hamper the functional characterisation. *In silico* prediction tools can be used to estimate the effect of the variant by making use of annotated binding sites of interacting proteins or transcription factors and the variant's location in relation to regulatory protein elements. Selection criteria, such as using candidate gene or pathway approaches, molecular modelling and assessing the variant's frequency in open databases, can be further applied to increase *in silico* predictive power (Katsonis et al., 2022; Riahi et al., 2016). Cell lines and organoid culture offer valuable tools for the *in vitro* characterisation of longevity-associated variants. Since primary cell lines of long-lived individuals are challenging to obtain and culture, researchers instead commonly rely on induced pluripotent stem cells (iPSCs), highly proliferative cancer cell lines and the more advanced organoid culture (Kim et al., 2020; Zhu et al., 2011). These approaches allow for cell- and tissue specific modelling of the variant, and assessment of further phenotypes such as differential phosphorylation, stress resistance, gene expression and protein stability and can be adjusted based on molecular read-outs of the 'hallmarks of ageing' (López-Otín et al., 2013). *In silico* and *in vitro* experimental work should precede functional characterisation *in vivo*, in line with the 3R ethical principles to replace, reduce and refine animal experimental work (Kirschner, 2021). However, especially in the field of ageing research, the use of model organisms such as worms, flies, fish and mice, is often needed to validate the variants effect on ageing-related phenotypes. The obvious advantage of *in vivo* models is the possibility to perform longitudinal studies and to draw conclusions on health- and lifespan and possibly transfer these findings to humans (Ferrucci et al., 2020).

1.4 The role of pharmacological interventions in extending health- and lifespan

Better mechanistic understanding of ageing associated longevity pathways has led to the finding that some of the identified targets are druggable with already approved drugs, originally developed for other indications. Recent studies have indeed shown that some of these pharmaceuticals improve health- and lifespan in various species. One such example is the immunosuppressant rapamycin, which is in clinical use to prevent organ rejection after transplantation (Johnson et al., 2013). Rapamycin is a specific inhibitor of the mTORC1 protein complex, by binding to the FKBP protein and then occupying the active site cleft of mTORC1 kinase (Yang et al., 2013). mTORC1 inhibition reduces tumour formation and protein translation, while simultaneously increasing autophagy and extending lifespan (Bjedov et al., 2010; Harrison et al., 2009; Kapahi et al., 2004; Lamming et al., 2013). Recently, a geroprotective memory effect of brief rapamycin exposure in early adulthood in flies and mice has been reported (Juricic et al., 2022), which offers a possibility of circumventing the adverse side effects of continuous usage of this drug, such as immunosuppression, anaemia and hyperlipidaemia. Rapamycin has been shown to extend lifespan of healthy mice and disease mimicking mice, such as progeroid syndromes and various cancers (Christy et al., 2015; Hernando et al., 2007; Ramos et al., 2012).

Another interesting drug candidate that is being repurposed in ageing research, is metformin. Due to its hypoglycaemic properties and cardiovascular benefits, metformin is the most commonly used drug in the treatment of diabetes type 2 (Bailey, 2017; Sanchez-Rangel & Inzucchi, 2017). Its geroprotective effects have been established in a range of animal models (Piskovatska et al., 2018) and observational studies in humans have reported a reduced incidence of neurodegenerative diseases as well as various cancers, such as colorectal and breast cancer (Salvatore et al., 2020). The exact mechanism of action remains elusive, partly due to dosage inconsistencies between *in vitro* studies and those used in the clinical setting and the large number of molecular targets proposed. Adenosine monophosphate-activated protein kinase (AMPK)- and mTOR-dependent and -independent mechanisms and mitochondrial respiration have been reported (Bridges et al., 2014; Lamoia & Shulman, 2021). These include reduced mTOR and insulin signalling, which then attenuate different hallmarks of ageing by increasing autophagy, mitochondrial biogenesis and DNA repair among others (Kulkarni et al.,

2020). In healthy, elderly humans, small clinical trials have recently been initiated to study the effect of metformin on general health (https://www.afar.org/tame-trial, https://clinicaltrials.gov/ct2/show/NCT02432287).

In fruit flies, lifespan extending effects have also been observed for the MEK kinase inhibitor, trametinib, which is currently used in the treatment of MAPK/ERK driven cancers (Han et al., 2021). The inhibition of the MAPK/ERK signalling pathway alone (Slack et al., 2015), as well as combinatory effects with rapamycin and lithium, have been shown to significantly increase lifespan in *D.melanogaster* (Castillo-Quan et al., 2019). The geroprotective drugs like rapamycin, metformin and trametinib have a common target - the IIS network. The modulation of growth signalling pathways such as the MAPK/ERK and PI3K/AKT/mTOR branches have also been shown to be the root cause of longevity-promoting effects by caloric restriction, the current best described and reproducible, non-invasive intervention to slow the ageing process (Fontana & Partridge, 2015b). For this reason, this class of drugs is also referred to as 'caloric restriction mimetics'.

1.4.1 A novel Ras-mimetic: Rigosertib

Rigosertib (RGS), ON-01910, is a Ras-mimetic compound that is currently put to test in various cancer mouse models (Radke et al., 2021; Zhou et al., 2022) and in a range of human clinical trials for Ras driven cancers (https://clinicaltrials.gov/ct2/results?cond=rigosertib). RGS was first tested in the treatment of cancer in 2005 as an inhibitor of polo-like kinase 1 (PLK1) and belongs to the family of non-ATP-competitive compounds. PLKs and their inhibition have long been the focus of cancer research, as they have essential roles in cell cycle progression (Su et al., 2022). Initial *in vitro* experiments of RGS showed that this compound inhibits cell cycle progression, leading to apoptosis in a broad range of human tumour cell lines, with otherwise little or no toxicity in normal cell lines at the same doses (Gumireddy et al., 2005). Since then, different research groups have proposed different mechanisms of action of RGS, including microtubule-destabilisation, PI3K signalling inhibition, JNK signalling activation and a contaminant impurity, as responsible for the observed effects (Baker et al., 2020; Oussenko et al., 2011; Ritt et al., 2016; Twarog et al., 2016; Steegmaier et al., 2007). Most recently, RGS is being described as a Ras-mimetic, binding to the Ras-binding domain and thus blocking MAPK/ERK and PI3K/AKT/mTOR signalling transduction (Athuluri-Divakar et al., 2016a; Prasad et al., 2009). In an organoid neuroblastoma model, RGS treatment results in cell cycle arrest and decreased phosphorylation of AKT (Ser473) and ERK1/2

(Thr202/Tyr204) (Radke et al., 2021b), providing further evidence for a potential effect of this drug on regulation of IIS. The simultaneous inhibition of MAPK/ERK and PI3K/AKT/mTOR signalling, while well-tolerated and not toxic, makes RGS an interesting geroprotective drug candidate.

1.5 Rationale and aims of this thesis

Research has shown that there is a genetic component to lifespan and longevity in humans (van den Berg et al., 2019), but the exact estimate of the heritability remains elusive. Thus far, a few common genomic loci have been associated with longevity but only one locus in *APOE* could be replicated between different populations and studies (Deelen et al., 2019; X. Liu et al., 2021; Sebastiani et al., 2017). This offers the possibility that rare genetic variants contribute to longevity. The MAPK/ERK signalling pathway, part of the IIS network, has been implicated in lifespan of different organisms, such as yeast, worms, flies and mice (Borrás et al., 2011; Fabrizio et al., 2003; Okuyama et al., 2010; Slack et al., 2015). We hypothesised that rare genetic variants located in the MAPK/ERK signalling pathway may have contributed to the longevity phenotype in individuals belonging to the LLS. In order to investigate this hypothesis, we designed a novel pipeline with the aim to functionally characterize rare genetic variants linked to human longevity through CRISPR/Cas9 technology. Improved understanding of the role of genes and pathways in human longevity may ultimately contribute to the development of interventions targeting ageing-related phenotypes and an increased healthspan of the general population. In addition, this pipeline may facilitate and guide future studies that aim to assess functionality of human genetic variants *in vitro* and *in vivo*.

Chapter 3: Functional characterisation of rare genetic variants in the MAPK/ERK signalling pathway observed in long-lived individuals

- Identify variants residing in the MAPK/ERK pathway in long-lived families (done in collaboration with the group from Eline Slagboom at the LUMC).
- Create cell lines with the identified human variants through CRISPR/Cas9.
- Assess pathway activity and cellular phenotypes, i.e. stress resistance.
- In-depth functional characterization and follow-up in transgenic fruit flies and mice.

The development of geroprotective drugs may aid in alleviating the burden of demographic ageing on societies and individuals in the future. Based on findings that mild inhibition of the IIS network extends lifespan in different organisms (Barbieri et al., 2003) and on-going studies testing rapamycin and metformin as potential geroprotective drugs in humans (Lamming et al.,

2013; Piskovatska et al., 2018), we hypothesised that the reported dual inhibition of the MAPK/ERK and PI3K/AKT/mTOR signalling branches by Rigosertib may also extend lifespan (Athuluri-Divakar et al., 2016). We investigated this hypothesis in the fruit fly, in which the IIS network is well conserved and thus would allow for the translation of findings to other organisms and potentially humans.

Chapter 4: The effects of Rigosertib treatment on health- and lifespan in *Drosophila melanogaster*

- Does Rigosertib extend lifespan *in vivo* and both sexes? If so, at which doses?
- Identify possible underlying mechanism of action of Rigosertib treatment
    - Does Rigosertib inhibit the MAPK/ERK and PI3K/AKT/mTOR signalling pathways *in vitro* and *in vivo*?
    - What is the major tissue target of Rigosertib?

2. Methods

2.1 Identification and filtering of variants

Research in model organisms, such as yeast, worms, flies and mice, have implicated the MAPK/ERK signalling pathway in lifespan (Borrás et al., 2011; Fabrizio et al., 2003; Okuyama et al., 2010; Slack et al., 2015). To investigate whether this applies to human longevity, we designed the following study design. In this project, we used whole genome sequencing (WGS) data coming from the unique longevity-enriched Leiden Longevity Study (LLS) (van den Akker et al., 2016) and focused on the 183 long-lived individuals who showed the strongest evidence for genetic enrichment, as previously defined by van den Berg and colleagues. In this study, the researchers provide evidence that longevity, defined as belonging to the top 10% of longest-lived of a given birth cohort, is transmitted as a quantitative genetic trait in two independent large mortality data sets from the Netherlands and the United States in which off-spring survival increases with each long-lived relative, regardless of their sex (van den Berg et al., 2019). The variants from the LLS WGS data were filtered by Thies Gehrmann in the Department of Eline Slagboom (LUMC, Molecular Epidemiology) according to the following criteria (**Figure 3.1A**):

(1) The variants reside in a MAPK/ERK signalling-related gene (see **Table 3.1** for an overview of all analysed genes).
(2) The variants are protein-altering with a combined annotation-dependent depletion (CADD) score ≥20 (i.e. belonging to the 1% most deleterious variants in the genome) (Kircher et al., 2014). This machine learning based score incorporates genomic information from the surrounding sequence, gene model annotations and functional predictions and can thus be used to predict functionality of a variant (Rentzsch et al., 2019).
(3) The variants are absent or at very low frequency (MAF <0.001%) in the general population, as determined using publicly available reference databases, such as GnomAD (https://gnomad.broadinstitute.org/) and DiscovEHR and are also absent in the Dutch-specific controls previously used by van den Akker and colleagues (van den Akker et al., 2016). This filter was applied to ensure rarity and family-specificity of the genetic variants, as we hypothesised these to have contributed to the longevity of the individuals.

2.2 Biochemistry and molecular biology

2.2.1 genomic DNA preparation

Genomic DNA from mouse embryonic stem cells (mESCs) was obtained by harvesting cells using 0.1% Trypsin (Thermo Fisher) and subsequent centrifugation at 500 rpm for 5 min for pellet formation. The cell pellet was resuspended in Quick-Extract (Lucigen) according to the manufacturer's instructions. Genomic DNA was stored at 4°C for downstream applications or at -20°C until further usage.

To obtain genomic DNA from flies, a single fly or fly leg was lysed in Squishing buffer (Tris-HCL pH 8.2 20 mM, EDTA 1mM, NaCl 25 mM; Carl Roth) with freshly added Proteinase K (Promega) to a final concentration of 200 µg/ml. DNA was extracted by incubating the samples at 37°C for 30 min, followed by proteinase K inactivation at 95°C for 5 min.

Mouse ear clippings from founder animals were column purified with the NucleoSpin Tissue XS Kit (Macherey-Nagel) according to the manufacturer's instructions. Mouse ear clippings from animals used for backcrossing were purified using an in-house lysis buffer that contained 100 mM Tris-HCL pH 8.5, 5 mM EDTA pH 8.0, 0.2% SDS and 200 mM NaCl (Carl Roth). Samples were incubated in 500 µl lysis buffer with 5 µl Proteinase K (Promega) at 55°C overnight. Genomic DNA was precipitated by adding 500 µl Isopropanol and subsequent centrifugation at 13.000 rpm and 4°C for 20 min. The supernatant was removed and the pellet washed with 500 µl 70% EtOH by centrifuging it at 13.000 rpm and 4°C for 10 min. EtOH was subsequently poured carefully off and the samples dried at room temperature for 20 min. The final drying step was conducted in a thermoplate at 55°C for 20 min. The DNA pellet was resuspended in 100 µl deionized water and stored at 4°C until further usage.

2.2.2 Polymerase chain reaction (PCR)

For standard genotyping of cell lines and mice, GoTaq Master Mixes (Promega) were used according to the manufacturer's instructions. For standard fly genotyping PCRs, HotStarTaq Plus MasterMix (Qiagen) was used according to the manufacturer's instructions. Primers **(Table 2.1)** were ordered from Sigma-Aldrich.

Target	Species	Forward Primer	Reverse Primer
Raf1	mouse	TTAGATCATCTTCATGGTAGGCC	ACAGGGCCTTAACTTCATATTGC
Nf1	mouse	GTTGAAATTTTCCTTGTAATGAATC	TCCTTCCTCCTGGTTAATGG
Rras	mouse	GCAAGAGGAATTTGGTGCCA	CTTACTTCTTGCCTGCCTGC
Rps6ka4	mouse	GTGTATGACAACAGGCGCAG	GGGCACCCTTTATCTCCAGG
Ets2	mouse	CTGCCAAATGCTGGGATTAC	AAGACCGGGAGGGCATGT
Atf4	mouse	AGTTTAGAGCTAGGCAGTGAAGT	TTATTACAGCAAACACAGCAACA
Dusp3	mouse	TATGCTTGTTGTATGGAAGACTTTG	GGCCATGCACCCTGATAG
Rasgrf2	mouse	CCTGTTATTTGGGGAAAACTTGC	AGGATGAATCTGCGAGACCT
Shc2	mouse	CCCATGTGCATCTCTCCTGT	TGGTGCCCTGTCCAGAATAT
Raf1	drosophila	CAGAGGTGATTCGCATGCAG	ACCAAAGTGAAAGAGAGAGAGAG
Nf1	drosophila	ACAGGGCCTTAACTTCATATTGC	CTGGAAAACTGACCGCTCTG

Table 2.1 | Primers used in this study

2.2.3 Agarose gel electrophoresis

To analyse the size of DNA fragments or plasmids, 1x or 2x TAE (Tris base, acetic acid, ethylenediamintetraacetic acid; Promega) buffered agarose gel electrophoresis was used to separate DNA. DNA was stained with 10 µL Gel Red Stain (Invitrogen) per 100 mL agarose gel. Electrophoresis was performed with Sub Cell GT horizontal electrophoresis system (BioRad) for 30-60 min at 60-130 V. Hyperladder 50 bp or Hyperladder 1 kb (Bioline) served as DNA fragment size markers.

2.2.4 Cloning

Guide RNAs (gRNAs) targeting the desired locus **(Table 2.2)** were designed with Benchling (https://www.benchling.com) and ordered from Sigma Aldrich. For cloning, Phusion High Fidelity Master Mix with GC Buffer (NEB) was used. All restriction digest reactions were performed with enzymes provided by NEB according to their users' manual. T4 DNA Ligase (NEB) was used for ligation reactions. Two gRNAs were independently cloned into a vector containing the Cas9 D10A nickase mutant (pSpCas9n(BB)-2A-GFP), to minimize off target mutagenesis (Ran et al., 2013).

Chemically competent OneShot TOP10 *Escherichia coli* (LifeTechnologies) were used for transformation of ligation reactions or plasmids according to the manufacturer's instructions. For positive selection of transformants, a 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.
For subsequent plasmid purification from bacteria, the QIAprep Miniprep or Midiprep Kits (Qiagen) were used. Cloning success was verified with Sanger Sequencing at Eurofins Genomics (Germany).

Target	Backbone	gRNA1	gRNA2
NF1 Mutant 1	pSpCas9n(BB)-2A-GFP	GAAGATGAAAATGCACAAAC	CTTCACTACAGTCATTCAAA
NF1 Mutant 2	pSpCas9n(BB)-2A-GFP	CATAAATAATGTGAAGTACC	CTTCACTACAGTCATTCAAA
RAF1	pSpCas9n(BB)-2A-GFP	GCACGCTGACTACATCCCCA	AGTGTGAGCTGCCCGATGCA
RASGRF2	pSpCas9n(BB)-2A-GFP	TCCTGGTGAACGGTTTCCTC	CGTTCACCAGGATGTAAAGC
RRAS	pSpCas9n(BB)-2A-GFP	CTTCTCTTGCAGTTTCAATG	AACCTGCCCCGTGCTCAGCC
ATF4	pSpCas9n(BB)-2A-GFP	TAGAAGAGGTCCGTAAGGCA	TTTCAGATACTGGATCTCCT
RPS6KA4	pSpCas9n(BB)-2A-GFP	TGCCAGGAGTGCCATGATGC	CCGGGCCTGTATCCGGCACC
SHC2	pSpCas9n(BB)-2A-GFP	AAGGACCCCATCAACCAGAG	ATGGGGTCCTTGGCCACAT
DUSP3	pSpCas9n(BB)-2A-GFP	GTGAGGCAGAATCGTGAGAT	CCATCTTCTGCCGCATCATG
ETS2	pSpCas9n(BB)-2A-GFP	CAAAAGGAGCAACGGCGTCT	TGAAGGTGGCTTTTAAGGCT
Nf1 Fly	pOT2	ATTCCTCAGGCTGAAGGGGA	TTGCATCGATAGCTCCGAGG
Raf1 Fly	pOT2	TTGCGTCAAGTTTGGTTCAC	TCTGCCCAGCCCGAAAACGC

Table 2.2 | gRNAs used for cloning in this study.

2.2.5 CRISPR/Cas9 plasmid transfection

The created gRNA-containing vectors were subsequently transfected into the wild type cell line (i.e. mESC) using Lipofectamine 3000 (Thermo Fisher) together with a short (120 bp) single-stranded DNA oligonucleotide (ssODN) **(Table 2.3)**. The ssODN contained the mutation of interest, as well as a minimum of two silent protospacer adjacent motif (PAM) mutations that prevent the Cas9n protein from re-cutting after successful repair, and a restriction site for the screening of colonies.

Target	ssODN
NF1	AGGTCAGTCTCTGTATTTTCCAGGTACTTCACATTA**C**TTATGAATCTTTTGA ATGACTGTAGTGAAGTTGAAGACGAAAATGC**T**CAAACGGGTGGCAGGAAA CGTGGCATGTCTCGGAGG
RAF1	AAAATCAACAGGAGCGCCTCTGAGCCTAGCCTGCATCGGGCAGCTCACACT GAG**T**ACATCAATGCTTGCACGCTGACTACATCCCCAAGACTACCAGTCTTC TAGCTGATGATGTAGCTG
RASGRF2	AAACCAGATTGTGTTCACGATGGTGGAGGCAGAGACGGAGTATGTACACC AGCTTTACATCCTGGTGAACGGTTTC**A**TCAGACCTCTGCGCATGGCAGCCA GCTCCAAGAAGCCCCCCAT
RRAS	GGATGCTGGTGGGCCTGGCTGAGCACGGGGCAGGTTACTGCTAAGGTGGT **A**CCTCATGCTACTGACTTCTCTTGCAAATTCAATAAGGTGGGCAAGCTCTTC ACACAGATCCTCAGAGTC
ATF4	ATGAGGCTCTGAAAGAGAAGGCAGATTCTCTGGCAAAGGAGATCCAGTAT CTGAAAGACCTGATCGAAGAGGTC**T**GTAAGGCAAGAGGGAAGAAGAGAGT TCCGTAATAGGGTAGTCAGG
RPS6KA4	GACCACAACAATGCAGTGATGGCTGATGTACTGCAGGCACCGGGCGCCGG ATACAGACCTGGCAGGGCAGCAGTTG**T**CAGGAGTGCCATGATGCAGATGG GCTGGCCTGGGGGTGGAGGT
SHC2	CTGCCCCTCGAGGGAGCCGAACCCTGTTAGCGTCTTCTACACGTCTCTCTAG GACATGACTGATTACGTAGCCT**G**CGTAGCTAAGGACCCCATCAACCAGAG AGGTGAGCTGGATTGTGG
DUSP3	CAGCCGCTCCCCAACGCTAGTTATCGCCTATCTCATGATGCGTCAGAAGAT GGACGTCAAGTCTGCTCTGAGTACTGTGAGACAGAAT**T**GCGAGATCGGCCC CAACGATGGCTTCCTGGC
ETS2	CTTTGCTCACTCCCTGCAGCAAGGCAGTGATGAGTCAAGCCTTAAAAGCCA CCTTCAGCGGCTTCCAAAAGGAGCAATG**G**CGTTTGGGCATCCCCAAAAGTA AGTGCTAAGCTTTGAGGC

Table 2.3 | ssODNs used for CRISPR/Cas9 generation of transgenic cell lines.
Introduced variants are highlighted in red, silent PAM and restriction site mutations are highlighted in orange.

2.2.6 Fluorescence activated cell sorting (FACS)

The Cas9 we used co-expressed GFP, which we used a selection tool for transfected cells. Transfected mESCs were additionally stained with propidium iodide (Thermo Fisher) and Hoechst dye (Thermo Fisher) according to the manufacturer's instructions. One day after transfection, cells were single-sorted (for haploidy and GFP-positivity) on 96-well plates with the BD Facsaria Fusion at the FACS & Imaging Core Facility at the Max Planck Institute for Biology of Ageing (MPI Age). Cells were grown for 10 days after which the single clones were genotyped.

2.2.7 Protein extraction and western blotting

Cells (500.000), whole flies (n=25) and tissues from flies (n=12-15) and mice (20-30 mg) were counted or weighed in desired amounts. Before protein isolation, cells were washed twice with cold PBS (Sigma) after which 100-300 µl RIPA buffer was added (10 ml RIPA Buffer (Thermo Fisher), 1 cOMPLETE EDTA and 1 PhosSTOP tablet (Roche)) to the cells on ice. Cells were harvested using a cell scraper. The suspension was sonicated for 10 sec with 2% pulses and centrifuged at 14.000 rpm at 4°C. Supernatant was transferred to a 1.5 ml tube and stored at -80°C until usage.

Fly tissues or whole flies were homogenized in RIPA buffer using a hand homogenizer. Mouse tissues were pulverized with a tissue lyser (Qiagen) and resuspended in RIPA buffer. After centrifugation, the samples were sonicated in a water bath for 10 min and centrifuged at 13.000 rpm at 4°C. The supernatant was subsequently transferred to new vials.

Protein content was determined using the BCA Protein Assay Kit (Thermo Fisher) and samples were diluted accordingly. Laemmli Buffer (100 mM Tris pH 6.8, 20% glycerol, 4% SDS, (Carl Roth)) and 5% β- mercaptoethanol (Sigma) were added to the samples after which they were boileds at 95°C for 5 min.

Equal amounts of proteins (20-30 µg) were loaded and separated at 100-150 Volt for 90 min, using pre-stained SDS-PAGE gels (Bio-Rad). Stainfree gels were activated with UV light for 2.5 min with the ChemiDocImager (Bio-Rad) before transferring them onto a 0.45 µm nitrocellulose membrane (GE Healthcare) at 100 V for 1 h. After the transfer, stainfree images were captured using the ChemiDocImager (Bio-Rad). The membrane was blocked using 5% non-fat dry milk powder/TBST for 1 h and incubated with primary antibodies **(see Table 2.4** for an overview**)** at 4°C overnight. Afterwards, the membranes were washed three times for 15 min and the appropriate Horseradish peroxidase (HRP) coupled secondary antibody was added (ThermoFisher). For signal development, ECL Select Western Blotting Detection Reagent (GE Healthcare) was added and images were captured using the ChemiDocImager (Bio-Rad).

Antigen	Source	Dilution	Vendor
P42/44 MAPK	rabbit	1:1000	Cell Signaling #4370
42/44 MAPK	rabbit	1:1000	Cell Signaling #9102
Vinculin	rabbit	1:1000	Abcam #129002
P-Akt	rabbit	1:1000	Cell Signaling #9271
P-Akt	rabbit	1:1000	Phospho Solutions #p104-342
Akt	rabbit	1:1000	Cell Signaling #9272

Table 2.4 | Antibodies used for western blotting in this study.

2.3 Cell experiments

2.3.1 Cell culture

mESCs were cultured in high glucose Dulbecco's Modified Eagle Medium supplemented with 3,7 g/l Sodium bicarbonate, 13,7% FBS, 1% Pen-Strep, 1% L-Glutamate, 1% Sodium Pyruvate, 1% MEM Non-Essential amino acids, 0,1% beta-mercaptoethanol and 0,01% leukemia inhibitory factor (Millipore). Cells were grown in petri dishes as semi-adherent colonies at 37°C with 5% CO2.

Mouse embryonic fibroblasts (MEFs) were generated and cultured as described in Tan & Lei, 2019. In brief, heterozygous transgenic animals were intercrossed to obtain wildtype (WT), heterozygous and homozygous embryos. Plug-positive pregnant female mice were sacrificed on day 13.5 and embryos dissected under a laminar flow hood. MEFs were cultured in high glucose Dulbecco's Modified Eagle Medium supplemented with 10% heat-inactivated FBS and 1% Pen-Strep. MEFs were grown in T75 flasks as a semi-adherent monolayer at 37°C.

S2R+ cells were cultured in Schneider's medium supplemented with 10% heat-inactivated FBS and 1% Pen-Strep and 1% L-Glutamate. Cells were grown in T75 flasks as a semi-adherent monolayer at 30°C. For all experiments, cells were collected, counted with an automatic cell counter (Anvajo fluidlab R-300) and seeded in desired numbers. All reagents, if not stated otherwise, were purchased from Thermo Fisher.

2.3.2 Cell stress assays (tunicamycin and H202)

The WST-1 assay for cell proliferation and viability (Sigma) was used to measure normal cell growth and response to stressors, i.e. tunicamycin and H_2O_2. To this end, cells were plated at a confluence of 10.000 cells/96-well in quadruplicates and stressors were added in appropriate concentrations after allowing the cells to settle for 2 h. The next day, WST-1 labelling reagent was added according to the manufacturer's instructions and the spectrophotometric absorbance was measured at 492 nm with a plate reader Infinite 200 (Tecan). Cell viability was normalised to the respective untreated controls.

2.4 Fly work

2.4.1 Fly husbandry

Fly stocks **(Table 2.5)** were kept at 25°C on a 12 h light/dark cycle and fed a standard sugar/yeast/agar diet (1x SYA). The standard 1x SYA diet contained 5% sugar, 10% yeast, 1.5 % agar, 0.3% nipagin (10% in EtOH) and 0.03% propionic acid (MP Biologicals). Rigosertib or stressors were added to cooled down food in the desired concentration and directly dispensed. In all experiments, female and male flies were reared at controlled larval densities and allowed to mate for 48 h ("once-mated") before each experiment. All fly stocks were backcrossed for at least six generations into the wildtype *white Dahomey* (w^{Dah}) and *Wolbachia* positive background.

2.4.2 Generation of transgenic fly lines

For the generation of the *Nf1*^{Phe1148Leu} fly, two gRNAs targeting the *Nf1* locus **(Table 2.2)** were cloned in a *pCFD4* plasmid, along with a repair template containing the desired mutation with the Gibson Assembly kit (NEB). The *Nf1* donor construct contained the following elements: left homologous arm (512 bp); PAM sites (2) and the mutation of interest; right homologous arm (727 bp). These fragments were amplified from BAC clone (BACR02E09) and cloned into a *pOT2* vector. Embryos of flies expressing *Cas9* under the ubiquitous actin promoter were injected with 300 ng/µl DNA by Jaqueline Eßer at the MPI Age. PCR screening targeting the restriction site was used to identify positive flies and the correct introduction of the point mutation was confirmed with Sanger Sequencing. The positive *Nf1*^{Phe1148Leu} fly line was

backcrossed for 6 generations into the w^{Dah} background to get rid of possible off-target mutations.

Name	Source
w^{Dah}	Grönke et al., 2010
actin-Cas9	Bloomington #54590
NF1$NF1^{P1}$ **loss-of-function**	The et al., 1997
$NF1^{Phe1148Leu}$	This project

Table 2.5 | Trangenic fly lines used in this project.

2.4.3 Developmental assay and adult weight

For developmental time analysis, flies were allowed to lay eggs for 6 h, and these were then transferred to vials at a density of 25 per vial (n_{vials} = 8-10). The number of hatching flies was noted down 2-3 times a day. For body weight measurements of adult flies, 20 age-matched flies were anaesthetized with CO2 and weighed in pairs of two.

2.4.4 Lifespan and fecundity assay

For lifespan assays, once-mated flies were sorted into vials 48 h post eclosion (20 flies per vial, n_{vials} = 8-10). Every other day, flies were transferred to fresh vials and deaths scored. For fecundity assays, once-mated female flies were transferred into vials (5 flies per vial, n_{vials} = 8-10) and the number of eggs was counted using a hand counter once a week for the course of four weeks.

2.4.5 Stress assays

20 once-mated flies per vial (n_{vials} = 5) were transferred to 1x SYA food and aged for 10 days. Stress assays were started on day 10 with different diets:

- Starvation: 1% agar in water
- Paraquat: 20 mM in 1x SYA

2.4.6 Proboscis-extension assay

For each concentration of RGS tested, possible differences in feeding behaviour of flies were analysed (5 flies per vial, $n_{vials} = 5$). The evening before the assay, flies were tipped to fresh food and acclimatized in a separate room where the assay was performed. The number of flies extending their proboscis to the food was scored as 'feeding'. Feeding flies were scored for 10 times every 5 min.

2.4.7 Climbing assay

Half an hour prior to the experiment, flies were placed in plastic climbing assay tubes for acclimation. Subsequently, flies were transferred to vial one of the six-compartment climbing apparatus and tapped down to the bottom of the vial. In this apparatus, flies are allowed to climb to the upper vial ranging a distance of 15 cm for 20 sec. After 20 sec, flies in the upper vial are transferred into the second vial and tapped down. This procedure is repeated until the sixth vial is reached. The climbing index (CI) was calculated based on the number of flies in each vial (lowest = 0 = all flies in vial 1, highest = 1 = all flies in vial 6).

2.4.8 Immunostainings of fly tissues

2.4.8.1 pH3+ staining

Guts were dissected in PBS (Sigma) and fixed in cold 4% PFA (Paraformaldehyde; Carl Roth) for 30 min. Fixed guts were then washed in PBS (Sigma) with 0.1% Triton (PBST; Sigma) and blocked in PBST with 5% bovine serum albumin and 0.01% sodium azide (Carl Roth) for 1 h. Guts were incubated in primary antibody anti-pH3 (Cell Signaling) 1:500 at 4°C overnight, washed in PBST and incubated in the secondary antibody Alexa fluor 594 goat anti-rabbit 1:1000 for 1 h. Finally, the guts were mounted in VectaShield antifade mounting medium without DAPI (Vectorlabs) and imaged with a Leica SP5-X microscope.

2.4.8.2 Gut dysplasia staining

Guts were dissected in PBS (Sigma) and fixed with cold 4% PFA (Carl Roth) for 30 min. After washing in PBS (Sigma), guts were mounted in VectaShield antifade mounting medium with DAPI (Vectorlabs). Imaging was done with the Leica SP8-X confocal microscope. For each condition, three images per gut were taken and dysplastic regions were quantified in ImageJ, after which the average proportion for each gut was calculated.

2.4.8.3 Lysotracker staining

Flies were anaesthetized with CO2, dissected in 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 in PBS (Sigma), guts were mounted with VectaShield antifade mounting medium without DAPI (Vectorlabs) and immediately imaged on a Leica SP8-X confocal microscope. For each gut, three images were taken proximal to the proventriculus gut region.

2.5 Mouse work

All mouse experiments were performed in accordance with the recommendations and guideline of the Federation of the European Laboratory Animal Science Association (FELASA), with all protocols approved by the Landesamt für Natur, Umwelt und Verbraucherschutz, Nordrhein-Westfalen (Germany), Licence number AZ 2021.A407.

2.5.1 Generation of transgenic mice

The transgenesis facility of the MPI Age uses a protocol based on the purified Cas9 protein, crRNAs and a short (120 bp) single-stranded donor template. These components are injected or electroporated into mouse zygotes and subsequently implanted into pseudo-pregnant foster females. The offspring of these females (Founders) are genotyped, and the obtained transgenic mice are backcrossed for at least six generations with wildtype C57BL/6N mice before being intercrossed to obtain the required numbers of transgenic mice for the experiments.

2.5.2 Mouse maintenance

Animals used in this study were in the C57BL/6N background. For all mouse experiments, we used male and female mice. Up to 5 mice were housed in individually ventilated cages under specific-pathogen-free conditions with constant temperature of 21°C and 50–60% humidity at a 12 h light/dark cycle.

2.5.3 Tissue collection

Mice were sacrificed at 3-4 months of age by cervical dislocation. Dissected tissues (i.e skeletal muscle, white adipose tissue, liver, spleen, heart and brain) were snap frozen in liquid nitrogen and stored at -80°C until further usage. From whole blood, serum was collected by centrifugation at 2000x g for 10 min. Serum was transferred to a new tube and stored at -80°C until further usage.

2.6 Mass Spectrometry

2.6.1 LC-MS/MS Proteomics of cells and tissues

2.6.1.1 Protein extraction

~15 mg of tissue was lysed in a tissue lyser (Qiagen) and resuspended in 200 µl lysis buffer that contained 6 M of guanidinium chloride, 2.5 mM Tris-HCL, 0.25 M Tris(2-carboxyethyl) phosphine and 0.8M chloroacetamide dissolved in purified MilliQ (Merck) water. mESCs were grown at 500.000, pellets collected by trypsinisation and washed three times with PBS (Sigma). The cell pellet was resuspended in 20 µl lysis buffer. The mixture was heated to 95°C for 10 min and sonicated in a Bioruptor (Diagenode) (30 sec sonication, 30 sec breaks, 10 cycles). The lysate was centrifuged at 20.000x g for 10 min and transferred to new tubes. Protein concentrations were measured with NanoDrop (Thermo Fisher) and 300 µg diluted ten times with 20 mM Tris and digested with trypsin 1:200 overnight. The digestion was stopped the next morning by adding 100% formic acid (FA) and samples cleared by centrifugation at 20.000x g for 10 min.

2.6.1.2 Peptide cleaning

Peptide cleaning was done with house-made 30 µg C18-SD StageTips. In brief, these were wet with methanol, then 40% acetonitrile/0.1% FA and equibrilated with 0.1% FA by table centrifugations of 1-2 min without letting the tips dry in between these steps. The total protein digest was then loaded in 0.1% FA and washed with 0.1% FA. Final elution was done with 100 µl 40% acetonitrile/0.1% FA at 300x g for 4min and elutes dried in a SpeedVac at 45°C for 35

min. The digest was resuspended in 20 µl 0.1% FA and concentrations measured with NanoDrop (Thermo Fisher). 4 µg of peptides were used for subsequent TMT labelling.

2.6.1.3 Tandem Mass Tag (TMT) Pro labelling and fractionation

The peptides were reconstituted in 0.1 M triethylammonium bicarbonate buffer (Sigma Aldrich) and TMT Pro labelling done according to the manufacturer's instructions (Thermo Fisher). The ratio of peptides to TMT Pro reagent was 1:20. Labelled peptides were pooled, dried and resuspended in 200 µl 0.1% FA. These were then fractionated on a 1 mm x 150 mm ACQUITY column, packed with 130 Å, 1.7 µm C18 particles (Waters), using an Ultimate 3000 UHPLC (Thermo Fisher). Peptides were separated at a flow of 30 µL/min with an 88 min segmented gradient from 1% to 50% buffer B for 85 min and from 50% to 95% buffer A for 3 min. Buffer A contained 5% acetronitrile, 10 mM ammonium bicarbonate, buffer B contained 80% acetonitrile, 10mM ammonium bicarbonate. Fractions were collected every three minutes, pooled and dried in a SpeedVac.

2.6.1.4 LC-MS/MS analysis

Dried fractions were resuspended in 0.1% FA and separated on a 50 cm, 75 µm Acclaim PepMap column (Thermo Fisher) and analysed on a Orbitrap Lumos Tribrid mass spectrometer (Thermo Fisher) equipped with a FAIMS device (Thermo Fisher). The FAIMS device was operated in two compensation voltages, -50 V and -70 V. Peptide separations were performed on an EASY-nLC1200 using a 90 min linear gradient from 6% to 31% buffer. Buffer A contained 0.1% FA, buffer B contained 0.1% FA and 80% acetonitrile. The analytical column was operated at 50°C. Raw files were split based on the FAIMS compensation voltage using FreeStyle (Thermo Fisher). The final data was analysed using MaxQuant (Cox & Mann, 2008), differential expression analysis was performed using Limma (Ritchie et al., 2015) in R.

2.6.2 LC-MS/MS analysis of Rigosertib fed flies

2.6.2.1 Metabolite extraction

Flies (*n* = 4, 4 replicates) were treated with rigosertib for 10 days, their digestive system allowed to void for 1h and flies were then snap frozen in liquid nitrogen. Extraction buffer was freshly prepared in the ratio of 50:30:20 (v:v:v) of MTBE:methanol:water (Biosolve) and internal standards U-$^{13}C^{15}N$ amino acids, $^{13}C_{10}$ ATP, citric acid D_4 (Cambridge Isotope Laboratories) and EquiSPLASH LIPIDOMIX (Avanti) added. Frozen flies were homogenized

with a tissue lyser (Qiagen) at 25 Hz for 1min and resuspended in 1ml pre-cooled extraction buffer. This mixture was incubated at 4°C and 1500 rpm for 30 min and spun down at 21.000x g for 10 min. Cleared supernatant was transferred to a new tube, 200 µl MTBE and 150 µl LC-MS water (Optima-grade, Thermo Fisher) added and incubated at 15°C for 10 min. For phase separation, the mixture was centrifuged at 15°C and 16000x g for 5 min. The upper lipid phase was then carefully pipetted off and the lower polar metabolite phase dried in a SpeedVac at 20°C and 1000 rpm for 4-6 h. Metabolites were stored at -80°C until further analysis.

2.6.2.2 LC-MS/MS analysis

For the measurement, samples were resuspended in 25 µl water (ULC/MS grade, Biosolve) and thoroughly vortexed. Samples were passed through a 0.2 µm filter (Merck). Analysis was performed using a Xevo-TQs triple quadrupole mass spectrometer (Waters) coupled to an Acquity iClass liquid chromatography system (Waters). The LC was equipped with a BEH C18 column (100 x 2.1 mm, Waters). The mobile phase A contained UPLC-grade water (Biosolve), containing 10 mM ammonium acetate and 0.1% acetic acid (Sigma), while the mobile phase B contained acetonitrile: isopropanol 70:30 [v:v] (Biosolve). The LC-based separation was performed using a 6 min gradient starting with 300 µL/min of 25% mobile phase B. This flow was held constant for 0.5 min, before ramping to 100% mobile phase B within the next minute. This composition was held for the next 2 min, before ramping down within 0.1 min to the starting conditions of 25% mobile phase B. Finally, the column was equilibrated for 2.4 min (25% mobile phase B), before the next samples were injected. The MS parameters were set to capillary voltage 1.5 kV, desolvation temperature 550°C, desolvation gas flow 800 l/h, cone gas flow 50 L/h and collision cell gas flow 9 L/h. Rigosertib samples were analysed using a multiple reaction monitoring (MRM) method. For this purpose, a quantitative scan, where quadrupole 1 was set to m/z 452.03, while quadrupole 3 was set to m/z of 194.18. As a qualitative scan the quadrupole 1 was set to m/z 452.03, while quadrupole 3 was set to m/z 284.06. For the absolute quantification of rigosertib authentic standards were diluted to concentrations of 0, 1, 2, 5, 10, 50, 100, 500 ng/mL.
The LC-MS data was analysed using the TargetLynx software (Version 4.1, Waters).

2.6.3 Targeted Metabolomics of cells

2.6.3.1 Metabolite extraction of mESCs

Cells were seeded at 500.000 and extracted in freshly prepared 40:40:20 (v:v:v) LC-MS methanol:acetonitrile:water extraction buffer. U-$^{13}C^{15}N$ amino acids, $^{13}C_{10}$ ATP, $^{13}C_{10}$ $^{15}N_5$ AMP, $^{15}N_5$ ADP and citric acid D_4 (Cambridge Isotope Laboratories) were added as internal standards. Before extraction, cells were washed in 750 mM ammonium carbonate (Sigma) diluted 1:10 in LC-MS grade water (Optima-grade, Thermo Fisher), with an adjusted pH of 7.4 with glacial acetic acid. Cells were collected by trypsinization and polar metabolites extracted in 1 ml pre-cooled extraction buffer. Samples were sonicated with 3-5 pulses at 65% (Bandelin, Sonoplus Mini 20), incubated at 4°C and 1500 rpm in a thermomixer for 30 min and finally centrifuged at 21.000x g for 10 min. The supernatant was then transferred to a new Eppendorf tube and the polar metabolite fraction dried in a SpeedVac at 20°C and 1000 rpm for 4-6 h.

2.6.3.2 Targeted liquid chromatography-high-resolution mass spectrometry (LC-HRS-MS) based analysis of amine-containing metabolites

For LC-HRS-MS analysis, the extracts were resuspended in 200 µl LC-MS water (Optima-grade, Thermo Fisher) and incubated at 4°C for 15 min. The resuspension was then centrifuged at 4°C and 21.100x g for 5min and 50 µl mixed with 25 µl of 100 mM sodium carbonate (Sigma) and 25 µl 2% [v/v] benzoylchloride (Sigma) in acetonitrile (UPLC/MS-grade, Biosolve).

1 µl of the derivatized sample was injected onto a 100 x 2.1 mm HSS T3 UPLC column (Waters). The flow rate was set to 400 µl/min using a binary buffer system consisting of buffer A (10 mM ammonium formate (Sigma), 0.15% [v/v] formic acid (Sigma) in ULC/MS grade water (Sigma). Buffer B consisted of acetonitrile (Optima-grade, Thermo Fisher). The column temperature was set to 40°C, while the LC gradient was: 0% B at 0 min, 0-15% B 0- 4.1min; 15-17% B 4.1 – 4.5 min; 17-55% B 4.5-11 min; 55-70% B 11 – 11.5 min, 70-100% B 11.5 - 13 min; B 100% 13 - 14 min; 100-0% B 14 -14.1 min; 0% B 14.1-19 min; 0% B. The mass spectrometer (Q-Exactive Plus, Thermo Fisher) was operating in positive ionization mode recording the mass range m/z 100-1000. The heated ESI source settings of the mass spectrometer were: Spray voltage 3.5 kV, capillary temperature 300°C, sheath gas flow 60 AU, aux gas flow 20 AU at 330°C and sweep gas at 2 AU. The RF-lens was set to a value of 60.

2.6.3.3 Anion-Exchange Chromatography Mass Spectrometry (AEX-MS) based analysis of anionic metabolites

Metabolites were resuspended in 200µl LC-MS water (Optima-grade, Thermo Fisher) and incubated at 4°C for 15min, centrifuged at 4°C and 16000x g for 5min and 100 µl of the cleared supernatant transferred to polypropylene autosampler vials (Chromatography Accessories Trott). The samples were analysed using a Dionex ionchromatography system (Integrion, Thermo Fisher). 5 µL of polar metabolite extract were injected onto a Dionex IonPac AS11-HC column (2 mm × 250 mm, 4 µm particle size, Thermo Fisher) equipped with a Dionex IonPac AG11-HC guard column (2 mm × 50 mm, 4 µm, Thermo Fisher). The column temperature was held at 30°C, while the auto sampler was set to 6°C. A potassium hydroxide gradient was generated using a potassium hydroxide cartridge (Eluent Generator, Thermo Fisher), which was supplied with deionized water. The metabolite separation was carried at a flow rate of 380 µL/min, applying the following gradient conditions: 0-3 min, 10 mM KOH; 3-12 min, 10−50 mM KOH; 12-19 min, 50-100 mM KOH, 19-21 min, 100 mM KOH, 21-22 min, 100-10 mM KOH. The column was re-equilibrated at 10 mM for 4 min.

For the analysis of metabolic pool sizes the eluting compounds were detected in negative ion mode using full scan measurements in the mass range m/z 50 – 750 on a Q-Exactive HF high resolution MS (Thermo Fisher). The heated electrospray ionization (ESI) source settings of the mass spectrometer were: Spray voltage 3.2 kV, capillary temperature was set to 300°C, sheath gas flow 50 AU, aux gas flow 20 AU at a temperature of 330°C and a sweep gas glow of 2 AU. The S-lens was set to a value of 60.

Subsequent analysis of both amine-containing and anionic metabolites was conducted with TraceFinder software (Version 4.1, Thermo Fisher). Compound identity was validated with authentic reference compounds, which were run before and after every sequence. Peak areas of respective ions were extracted using a mass accuracy (<5 ppm) and a retention time tolerance of <0.05 min. Areas of the cellular pool sizes were normalized to the internal standards that were added to the extraction buffer and to the cell count of each individual sample.

2.7 Statistical analysis

All statistical analyses were performed using GraphPad Prism software 9. The applied statistical tests are mentioned in the appropriate figure legends. Significance was assigned based on the obtained the p-values: *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$.

3. Functional characterisation of rare variants in genes encoding the MAPK/ERK signalling pathway identified in long-lived Leiden Longevity Study participants

Author contributions:

Conception of the study was done by Helena Hinterding, Maarouf Baghdadi, Eline Slagboom, Joris Deelen, and Linda Partridge.

Execution of experiments was predominantly done by Helena Hinterding.

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Evaluation of data was done by Helena Hinterding and Joris Deelen.

Graphical representation was done by Helena Hinterding and Joris Deelen.

Writing of the manuscript was done by Helena Hinterding with substantial contribution by Joris Deelen, Maarouf Baghdadi and Linda Partridge.

Functional characterisation of rare variants in genes encoding the MAPK/ERK signalling pathway identified in long-lived Leiden Longevity Study participants

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3.1 Introduction

We have come to understand over the past years that ageing, generally defined as functional decline with advancing age, is malleable (Kenyon, 2010; Kirkwood, 2005). Underlying cellular and molecular changes (see, for example, the hallmarks and pillars of ageing, Kennedy et al., 2014; López-Otín et al., 2013) have been found to be highly evolutionarily conserved. Human lifespan and longevity are largely determined by lifestyle factors, but studies have shown that there is a genetic component to it as well. Genetic studies of ageing and longevity, defined as survival to an exceptional old age, have shown that the genetic component underlying human lifespan accounts for 10-25% (Herskind et al., 1996; Mcgue et al., 1993; van den Berg et al., 2017). However, Ruby et al. (2018) showed that many of these estimates are likely inflated due to assortative mating and suggest the heritability of lifespan to be approximately 10%. The genetic component has been shown to be of particular importance for increasing age at death (Christensen et al., 2006; Hjelmborg et al., 2006; Ljungquist et al., 1998). In the quest to unravel the role of genetics in human lifespan, GWAS successfully identified variants and/or loci correlating with lifespan and longevity (Melzer et al., 2020). The total number of longevity-associated loci identified through GWAS that was replicated across studies and populations is, however, rather small, offering the possibility that longevity may be at least partially determined by rare genetic variants. Rare genetic variants are commonly annotated with a MAF <1%, and are rarely picked up by GWAS, given that these studies mostly focus on variants with a MAF >5%. The identification of rare variants thus often relies on population

isolates and family studies, in which genetic bottlenecks have contributed to a less diverse genetic background (Auer & Lettre, 2015). Lifespan-associated pathways and genes, identified in model organisms are currently being investigated using candidate gene approaches to improve our understanding of the genetics of lifespan and longevity (Baghdadi et al., 2022; Gonzalez et al., 2022; Ryu et al., 2021; Simon et al., 2022; Zhang et al., 2020). For the current study, we have also used a targeted approach by focusing on rare, family-specific variants in genes that are part of the MAPK/ERK signalling pathway.

One of the most robust and highly conserved longevity-associated pathways coming from studies in model organisms, such as worms, flies, and mice, is the highly conserved nutrient-sensing IIS network (Fontana et al., 2010). Moreover, human studies have indicated a role for both common and rare genetic variation in this pathway in human longevity (Deelen et al., 2013; Lin et al., 2021), including in specific genes such as *FOXO3* (Flachsbart et al., 2009; Willcox et al., 2008) and *IGF1R* (Suh et al., 2008).

The MAPK/ERK signalling pathway is part of the IIS signalling network and is well known for its key role in cell proliferation and survival and metabolism (Cargnello & Roux, 2011; Raman et al., 2007). Within this pathway, the activated RAS proteins function as central signalling hubs that initiate a downstream phosphorylation cascade through RAF, MEK and, finally, ERK proteins, transmitting extracellular cues to the cytoplasm and, through the translocation of ERK, to the nucleus **(Figure 3.1 B)** (Wasylyk et al., 1998). Genetic and pharmacological interventions in the MAPK/ERK signalling pathway have been shown to extend lifespan in yeast, worms, flies and mice (Fabrizio et al., 2003; Okuyama et al., 2010; Slack et al., 2015; Borrás et al., 2011).

In this study, we investigated the effects of rare genetic variants in MAPK/ERK-related genes, identified in long-lived families from the Leiden Longevity Study (LLS) through whole-genome sequencing (WGS) (van den Akker et al., 2016). We subsequently used CRISPR/Cas9 to generate mESC lines and flies harbouring some of these variants to functionally characterize their potential effect *in vitro* and *in vivo*. By applying a number of stringent filters to the genetic variants analysed, we aimed to increase the chance of functional significance and similarly, the chance to be able to detect cellular phenotypes in the analysis. Through in-depth functional characterisation we ultimately aim to be able to judge whether these variants have a beneficial or detrimental effect. Our results show that two variants, located in *NF1* and *RAF1*, had opposite functional effects on MAPK/ERK pathway activity, accompanied by prominent changes at the proteomic level. Additionally, we found that the variant in *RAF1* improved

resistance to ER and oxidative stress *in vitro*. We also created transgenic fruit flies harbouring the variant in NF1. Homozygous *Nf1* mutant flies were short lived, while no significant effect on lifespan was observed for heterozygous mutant flies. Therefore, we conclude the heterozygous *Nf1* variant to have no lifespan-extending effect in the fly, while the *Raf1* variant shows a stress resistance phenotype *in vitro,* likely to have contributed to longevity phenotype of the individuals harbouring this variant.

3.2 Results

3.2.1 Identification of unique variants located in the MAPK/ERK signalling pathway in the longevity-enriched LLS cohort

Previous studies have shown that genetic manipulation of the MAPK/ERK signalling pathway by deletion or knockdown of single genes can extend lifespan in yeast, worms, flies and mice (Borrás et al., 2011; Fabrizio et al., 2003; Okuyama et al., 2010; Slack et al., 2015). To investigate whether the MAPK/ERK signalling pathway also plays a role in human ageing, we made use of WGS data coming from the unique longevity-enriched LLS (van den Akker et al., 2016).

For the purpose of this study, we only focused on the 183 long-lived individuals who showed the strongest evidence for genetic enrichment, as defined by van den Berg and colleagues (van den Berg et al., 2019). In this study, the researchers provide evidence that longevity, defined as belonging to the top 10% of longest-lived of a given birth cohort, is transmitted as a quantitative genetic trait in two independent large mortality data sets from the Netherlands and the United States in which off-spring survival increases with each long-lived relative, regardless of their sex (van den Berg et al., 2019). The variants from the WGS data were filtered according to the following criteria (**Figure 3.1A**):

(1) The variants reside in a MAPK/ERK signalling-related gene (see **Table 3.1** for an overview of all analysed genes).

(2) The variants are protein-altering with a combined annotation-dependent depletion (CADD) score ≥20 (i.e. belonging to the 1% most deleterious variants in the genome) (Kircher et al., 2014). This machine learning based score incorporates genomic information from the

surrounding sequence, gene model annotations and functional predictions and can thus be used to predict functionality of a variant (Rentzsch et al., 2019).

(3) The variants are absent or at very low frequency (MAF <0.001%) in the general population, as determined using publicly available reference databases, such as GnomAD (https://gnomad.broadinstitute.org/) and DiscovEHR and are also absent in the Dutch-specific controls previously used by van den Akker and colleagues (van den Akker et al., 2016). This filter was applied to ensure rarity and family-specificity of the genetic variants, as we hypothesised these to have contributed to the longevity of the individuals.

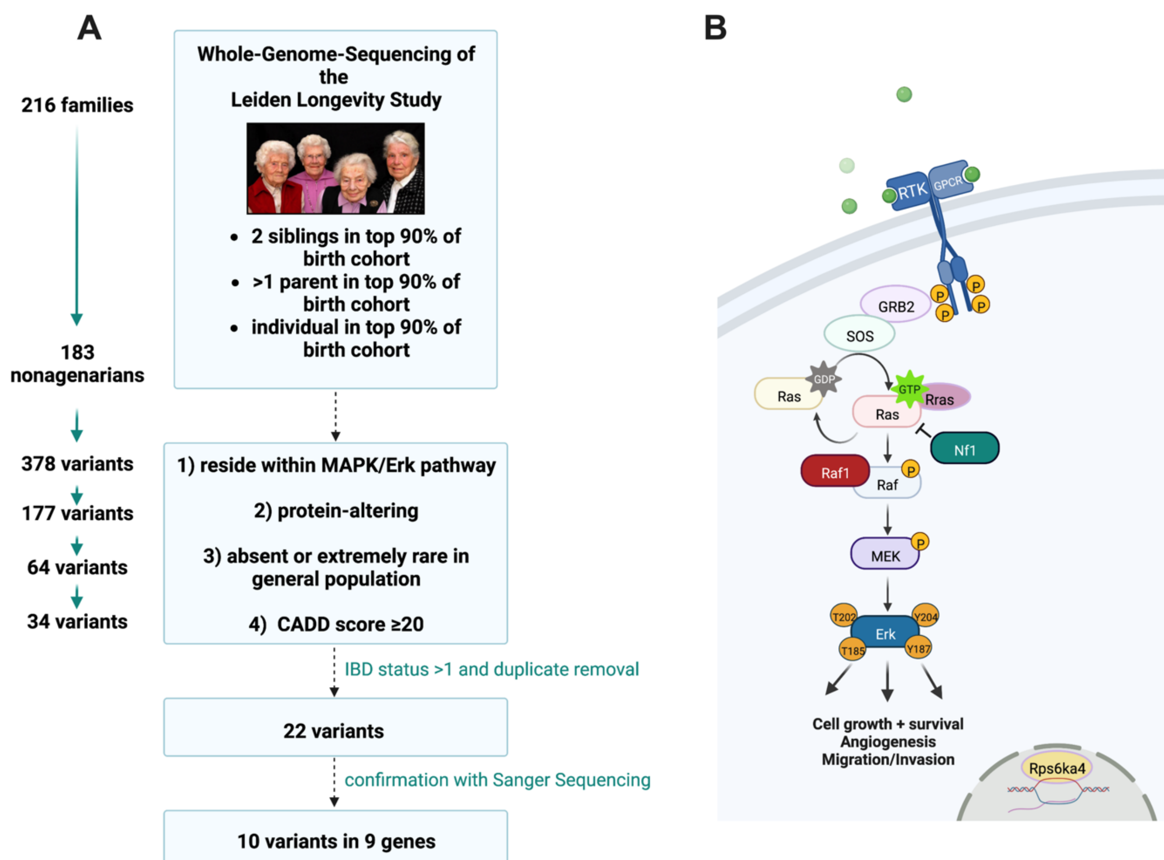


Figure 3.1 | Filtering pipeline of MAPK/ERK signalling pathway-associated rare genetic variants.

(A) The bioinformatic pipeline filtering whole-genome-sequencing data from the Leiden Longevity Study, which resulted in a list of 10 variants residing within genes encoding the MAPK/ERK signalling pathway that were confirmed through Sanger Sequencing **(B)** The MAPK/ERK signalling pathway transmits cues into the cytoplasm and consists of upstream located Ras proteins and a phosphorylation cascade of RAF-, MEK- and ERK-kinases. IBD, identity-by-descent; CADD, combined annotation-dependent depletion. *This figure was created with biorender.com.*

Gene	AA change in Human	AA change in Mice	CADD score	Position (GRCh37/hg19)	AA change in fruit flies	Allele frequency in Europeans
DUSP	Arg158Cys	Arg158Cys	35.0	17-41847063-G-A		0.00004399
RASGRF2	Leu270Ile	Leu270Ile	24.7	5-80369192-C-A		0
ATF4	Arg342Cys	Arg340Cys	33.0	22-39918575-C-T		0.00004537
SHC2	Tyr266Cys	Tyr244Cys	25.8	19-436409-T-C		0
RAF1	Asp633Tyr	**Asp633Tyr**	31.0	3-12626063-C-A	Asp713Tyr	0
ETS2	Arg235Trp	Arg95Trp	23.5	21-40186295-C-T		0.00002324
RPS6KA4	Ala394Val	**Ala394Val**	23.2	11-64135713-C-T		0
NF1	Ala1125Val	**Ala1127Val**	22.0	17-29559777-C-T		0.000008796
NF1	Phe1110Leu	**Phe1112Leu**	27.9	17-29559731-T-C	**Phe1148Leu**	0
RRAS	Glu118Lys	**Glu118Lys**	35.0	19-50139977-C-T	Glu118Lys	0.00006170

Table 3.1 | The 10 variants identified in long-lived individuals from the Leiden Longevity Study residing in genes encoding the MAPK/ERK signalling pathway.
Variants found in genes highlighted in bold were generated in mESCs and/or *Drosophila melanogaster* through CRISPR/Cas9. The corresponding amino acids of variants in *NF1*, *RAF1* and *RRAS* are conserved in the fruit fly. CADD; combined annotation dependent depletion, AA; amino acid. Allele frequencies are based on high quality genotypes of Europeans (non-Finnish) who are included in the GnomAD database.

3.2.2 Generation of transgenic cell lines with CRISPR/Cas9

To study the functional consequences of the LLS-derived variants, we used CRISPR/Cas9 gene editing (Ran et al., 2013) to generate mutant cell lines in haploid AN3-12 mESCs (Elling et al., 2017). Paired with a double-nicking strategy using the D10A Cas9 enzyme, these cells allow for precise genome editing of homozygous variants. We presumed functional effects of these variants, if present at all, to be more pronounced in homozygotes and thus used this cell line. We successfully generated cell lines for five of our identified variants: two variants in close proximity in *Nf1* (Ala1127Val and Phe1112Leu (two independent lines), one in *Raf1* (Asp633Tyr), one in *Rras* (Glu118Lys), and one in *Rps6ka4* (Ala394Val) (**Figure 3.2**). Due to the imperfect nature of the DNA repair process of CRISPR/Cas9 induced homology-directed repair, we also generated knockouts for *Rras* (end of exon 3 removed), *Nf1* (beginning of exon 26 removed) and *Raf1* (stop codon at position 603) in mESCs, which are, however, incomplete

gene knockouts. For this reason, we only used them in the initial analysis of pathway activity, to estimate the variant's effects on gain- or loss-of-function, and not in any of the follow-up experiments.

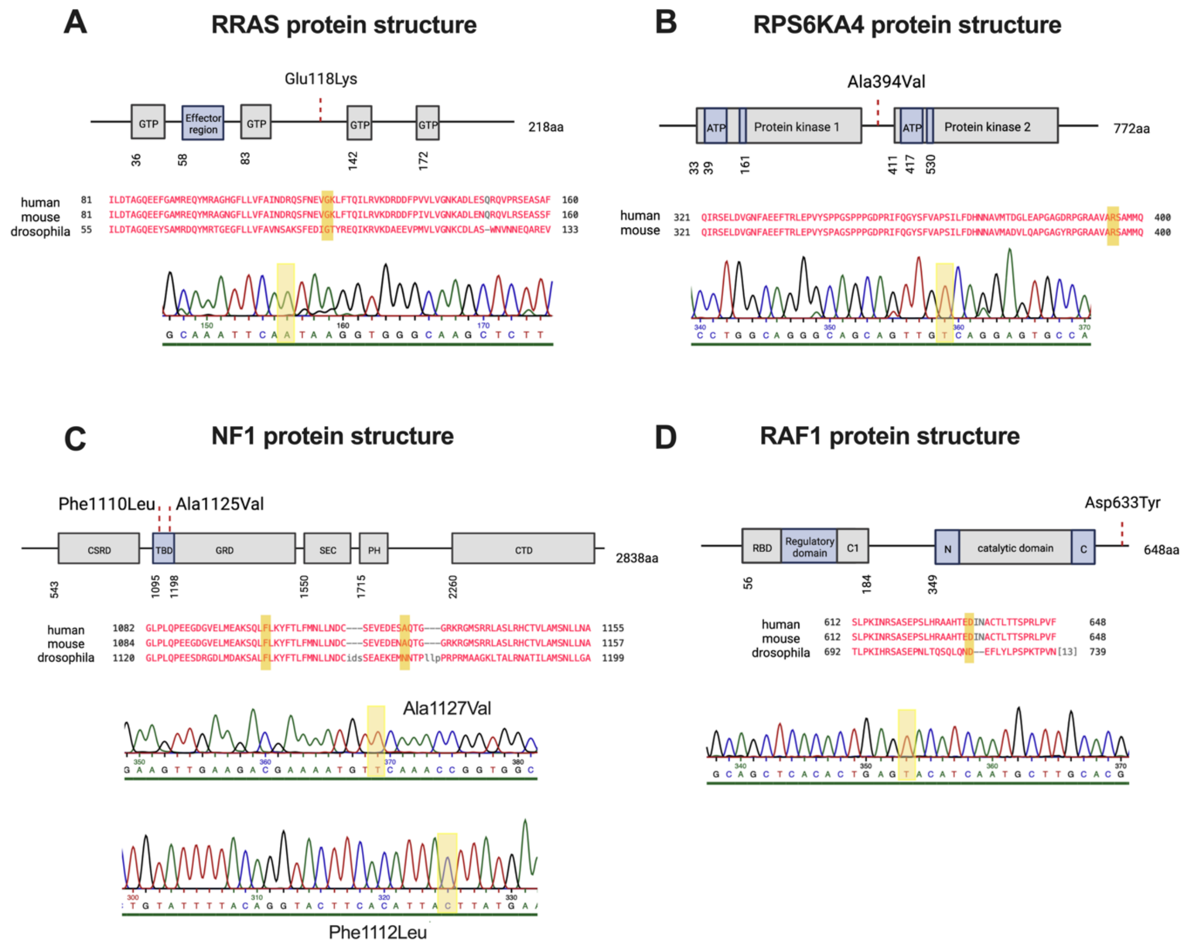


Figure 3.2 | Protein Schematics and Sanger Sequencing confirmation of introduced variants.
(A-D) Protein schematics highlighting features and domains of proteins that the identified variants reside in. Homology of human, mouse and fruit flies showed conservation of variant site for **(A)** Rras, **(B)** Rps6ka4, **(C)** Nf1 and **(D)** Raf1. CSRD, cysteine- and serine-rich domain; TBD, tubulin-binding domain; GRD, GAP-related domain; Sec, Sec14 homologous domain; PH, pleckstrin homologous domain; CTD, C-terminal domain; RBD, Ras-binding-domain; C1, cysteine-rich domain; GTP, Guanosine triphosphate binding sites; ATP, Adenosine triphosphate binding sites.

3.2.3 Stimulations of the MAPK/ERK signalling pathway

The MAPK/ERK signalling branch is known to transmit extracellular cues through different receptors, such as G-protein-coupled receptors and receptor-tyrosine-kinases, to the cytoplasm and nucleus. These cues include growth factors and cytokines and enable proliferation, survival and metabolism of cells (Yoon & Seger, 2006). In order to analyse changes in phosphorylation of proteins within the MAPK/ERK signalling pathway by western blotting, the pathway needs to be fully activated. Cytokines such as epidermal growth factor (EGF), platelet-derived growth factor (PDGF), insulin and FBS are commonly described in the literature to stimulate the MAPK/ERK signalling pathway (Jurek et al., 2009; S. Lee et al., 2016; Ley et al., 2003; Ramalingam et al., 2016). In preceding experiments, we found that the MAPK/ERK signalling pathway, measured by the ratio of phosphorylated and total ERK, is highly active in the AN3-12 mESCs that we used for our experimental work, when these cells are grown in normal medium. This is likely due to the high proliferative nature of these actively dividing stem cells. We could show that stimulations of wildtype mESCs with PDGF, EGF, insulin and serum did not seem to substantially increase the pathway's activity (**Figure 3.3**). We could, however, strongly elevate pathway activity by starving the cells overnight in serum-free medium, and subsequently stimulating them with FBS them for 20 min (acute). The transgenic cell lines, illustrated in the response of the *Raf1*^{Asp633Tyr} cell line **(Figure 3.3B)**, did not differ from wildtype cells in their response to the different stimulants tested. Since we found the MAPK/ERK signalling pathway highly activated in mESCs and the stimulants to have no effect, except the acute treatment, we decided to perform all subsequent experiments in the basal state of the cells to introduce as little as possible external factors.

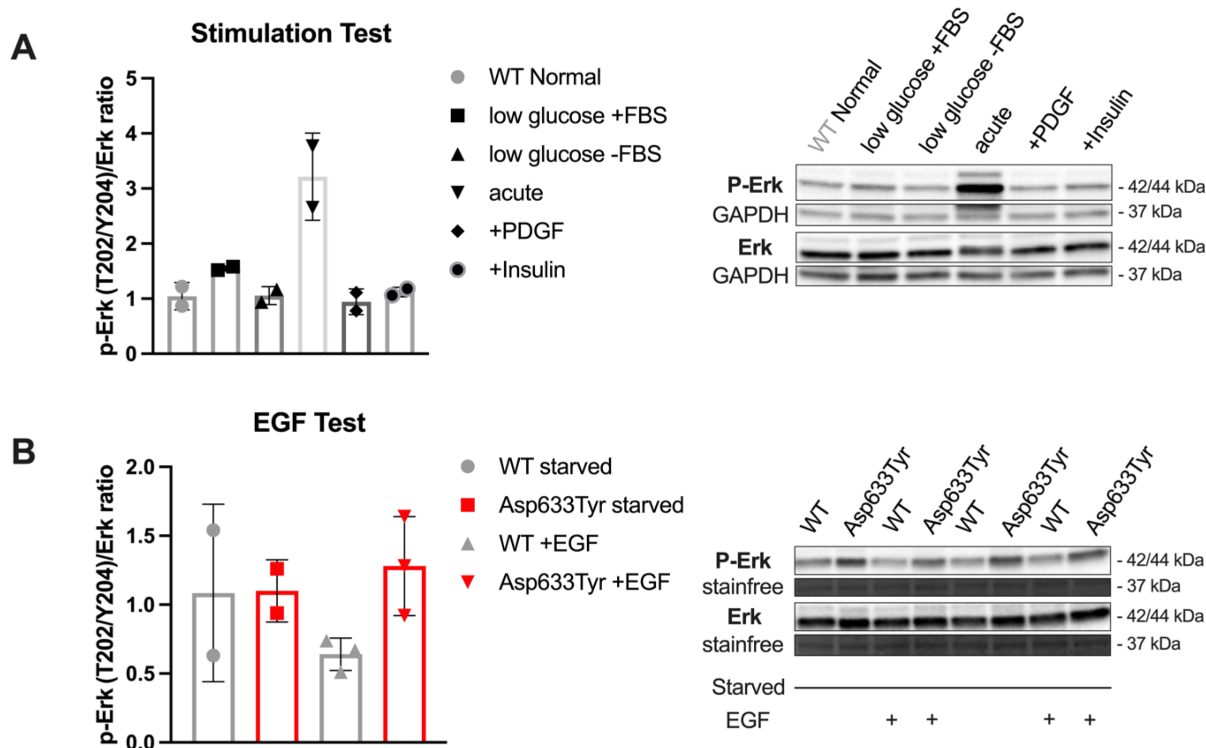


Figure 3.3 | Stimulations of the MAPK/ERK signalling pathway.
(A) stimulation of wildtype cells with FBS, PDGF and insulin did not elevate pathway activity. Acute stimulation with FBS for 20 min after over-night starvation in FBS free medium resulted in elevated pathway activity. Cells were normalised to 'wildtype normal' cells **(B)** stimulation with epidermal growth factor (EGF) did not significantly alter pathway activity. WT and *Raf1*^{Asp633Tyr} cells were normalised to their respective starved controls.

3.2.4 The *Nf1* and *Raf1* variants alter MAPK/ERK signalling pathway activity in opposite directions

Dual ERK phosphorylation at Thr202/Tyr204 (ERK1) and Thr185/187 (ERK2) is required for MAPK/ERK signalling pathway activation and signal transduction. To assess functional effects of the introduced variants on MAPK/ERK pathway activity, we measured ERK phosphorylation levels (Martinez-Lopez et al., 2013; Masaki et al., 2003; Mitic et al., 2015), i.e., the ratio of phosphorylated ERK over total ERK in protein lysates of the generated transgenic cell lines through western blotting.

Western blot analysis of pathway activity showed no change in the *Rras*^{Glu118Lys}, *Rps6ka4*^{Ala394Val} and *Nf1*^{Ala1127Val} variants in comparison to the wildtype mESCs (**Figure 3.4A-C**). Contrary, the *Raf1*^{Asp633Tyr} cell line showed an elevation in pathway activity (**Figure 3.4D**). In conditional mouse models of *Raf1*, ERK activation has been reported to be unimpaired, highlighting compensatory roles of the Raf1 kinase family *in vitro* (Hüser et al., 2001; Mercer et al., 2005; Yamaguchi et al., 2004). Human disease-causing gain-of-function mutations have, however, reported elevated MAPK/ERK signalling activity, suggesting improved functioning of the *RAF1* gene in these mESCs (Pandit et al., 2007; Razzaque et al., 2007).

The two independent cell lines harbouring the *Nf1*^{Phe1112Leu} variant displayed a significant reduction in MAPK/ERK signalling pathway activity (**Figure 3.4C**), consistent with improved functioning of the *NF1* gene and in line with previously reported elevations of pathway activity in *Nf1* knockout cell lines (Masgras et al., 2017) and conditional neuronal knockouts (Hegedus et al., 2007). Based on these results, we presume the *Rras*^{Glu118Lys}, *Rps6ka4*^{Ala394Val} and *Nf1*^{Ala1127Val} variants to have no functional effect *in vitro* and thus excluded them for further analysis. However, the *Raf1*^{Asp633Tyr} and *Nf1*^{Phe1112Leu} variants showed opposing effects on pathway activity in mESCs, pointing towards functionality of these genetic variants.

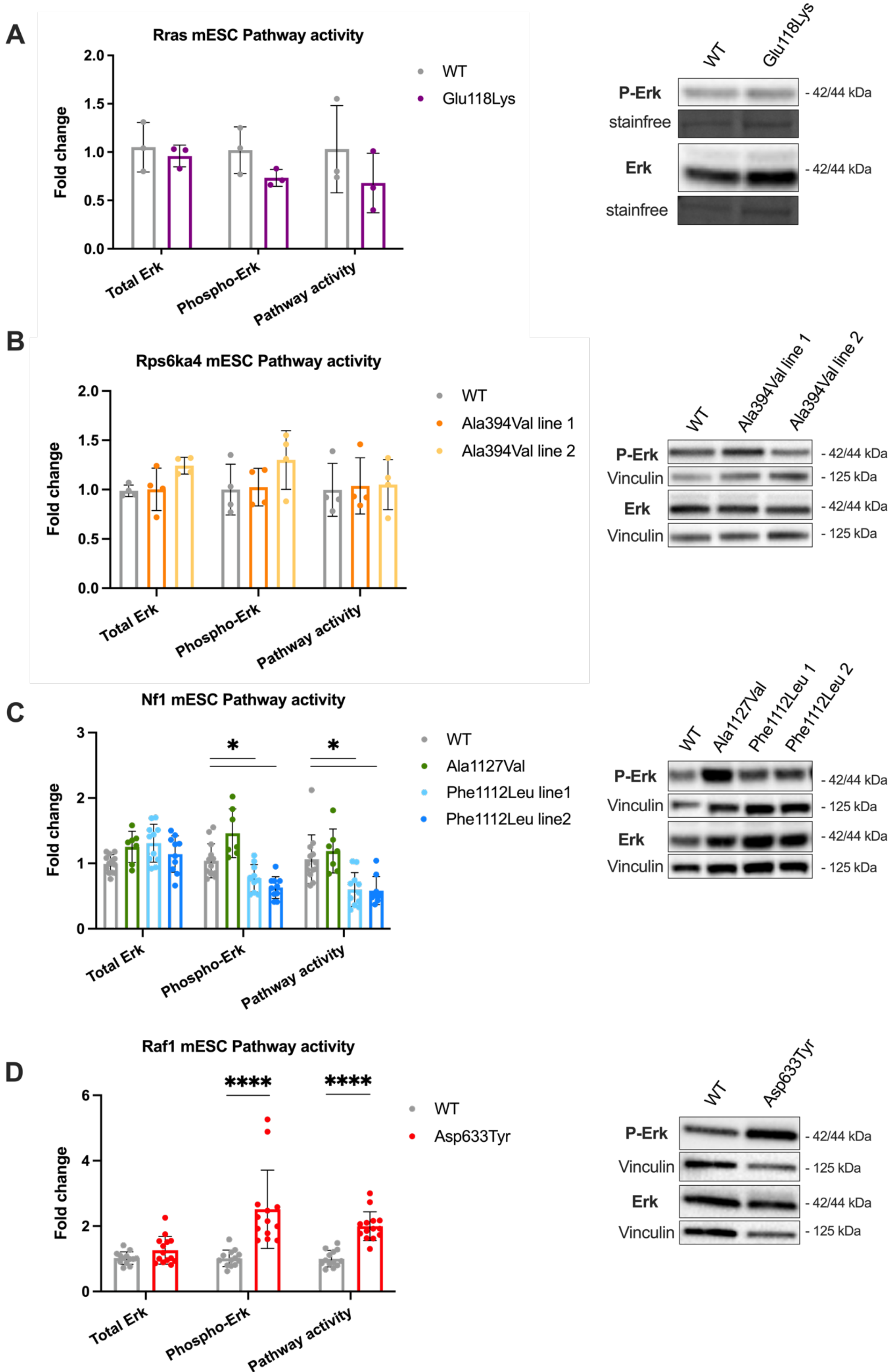

A
Rras mESC Pathway activity
Fold change
2.0
1.5
1.0
0.5
0.0
Total Erk
Phospho-Erk
Pathway activity
WT
Glu118Lys
P-Erk
stainfree
Erk
stainfree
- 42/44 kDa
B
Rps6ka4 mESC Pathway activity
WT
Ala394Val line 1
Ala394Val line 2
Vinculin
- 125 kDa
C
Nf1 mESC Pathway activity
*
Ala1127Val
Phe1112Leu line1
Phe1112Leu line2
Phe1112Leu 1
Phe1112Leu 2
D
Raf1 mESC Pathway activity
****
Asp633Tyr

Figure 3.4 | The effect of the variants in *Rras*, *Rps6ka4*, *Nf1*, and *Raf1* on MAPK/ERK signalling pathway activity.
(A-D) Pathway activity, represented by the ratio of phosphorylated over total ERK, in the cell lines harbouring **(A)** *Rras*^{Glu118Lys} (data shown of one western blot normalised to stainfree loading control, representative of two independent experiments) **(B)** *Rps6ka4*^{Ala394Val} (data shown of one western blot normalised to stainfree loading control, representative of two independent experiments) **(C)** *Nf1*^{Ala1127Val} and *Nf1*^{Phe1112Leu} (data shown of three pooled and independently vinculin normalised western blots), and **(D)** *Raf1*^{Asp633Tyr} (data shown of three pooled and independently vinculin normalised western blots). Dots represent technical replicates. Data was analysed with Student's t-test. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

3.2.5 The *Nf1* Phe1112Leu and *Raf1* Asp633Tyr variants show both overlapping and distinct proteomic signatures

To determine whether the differential regulation of ERK phosphorylation by the *Raf1*^{Asp633Tyr} and *Nf1*^{Phe1112Leu} variants led to altered expression of MAPK/ERK signalling pathway related proteins, we performed LC-MS/MS-based TMT-labelled quantitative proteomics of these cell lines, and of the cell line harbouring the *Nf1*^{Ala1127Val} variant. In total, we detected 7726 proteins. In line with our observations on pathway activity, principal component analysis (PCA) confirmed that the *Nf1*^{Ala1127Val} variant clustered with the wildtype mESCs, while the other two variants showed a distinct proteomic profile, with the two independent cell lines harbouring *Nf1*^{Phe1112Leu} clustering together (**Figure 3.5A**). The commonalities between the differentially expressed proteins (DEPs) in the cell lines harbouring the *Nf1*^{Phe1112Leu} and *Raf1*^{Asp633Tyr} variants were strongest in the upper part of the pathway (**Figure 3.5C-D**), characterised by significantly increased expression of epidermal growth factor receptor (Egfr), growth factor receptor-bound protein 2 (Grb2) and decreased expression of Ras-guanyl nucleotide-releasing protein (Rasgrp4), facilitating downstream signalling transduction to Ras proteins. The *Nf1*^{Phe1112Leu} and *Raf1*^{Asp633Tyr} protein expression profiles started to diverge at the level of the Ras protein family, with Rras, Hras, Rras2 and Kras all being upregulated in the *Nf1*^{Phe1112Leu} cell line. In general, we found more DEPs in the *Raf1*^{Asp633Tyr} variant **(Figure 3.5B)**. We previously showed elevated MAPK/ERK signalling pathway activity in the *Raf1*^{Asp633Tyr} variant (**Figure 3.4D**), which is mirrored in the expression of Erk1 in this proteomic dataset **(Figure 3.5C)**. The majority of DEPs in this cell lines are downregulated, suggesting negative feedback from Erk1 to upstream located proteins. Interestingly, Raf1 is downregulated in this cell line and Nf1upregulated, speaking for an overall reduced activity of the MAPK/ERK signalling pathway.

Similarly, reduced MAPK/ERK signalling pathway activity in the *Nf1*^{Phe1112Leu} variant (**Figure 3.4C**) is mirrored in the expression of Erk1 in this proteomic dataset **(Figure 3.5D)**.

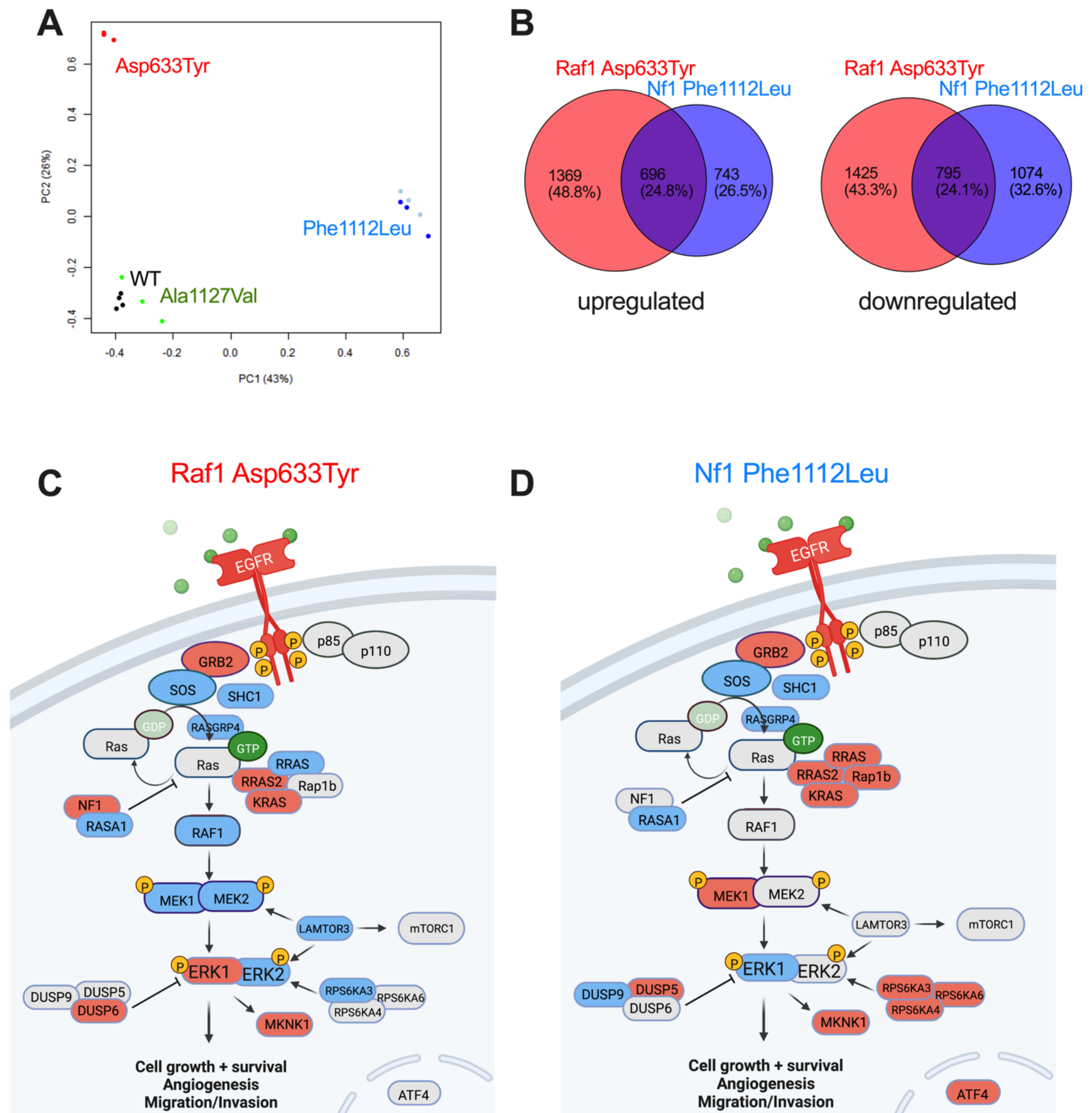


Figure 3.5 | Proteomics of the *Raf1*^{Asp633Tyr} and *Nf1*^{Phe1112Leu} variants.
(A) TMM normalized clustering confirms the two independent cell lines harbouring the *Nf1*^{Phe1112Leu} variant to be identical. The *Raf1*^{Asp633Tyr} cell line clusters different from wildtype and Nf1 cells. The *Nf1*^{Ala1127Val} variant is not different from wildtype cells **(B)** Venn diagrams representing the number and proportion of up- and downregulated proteins in the *Raf1*^{Asp633Tyr} and *Nf1*^{Phe1112Leu} variant **(C+D)** DEPs in the MAPK/ERK signalling pathway in the **(C)** *Raf1*^{Asp633Tyr} and the **(D)** *Nf1*^{Phe1112Leu} cell line (red=significantly upregulated DEPs; blue= significantly downregulated DEPs)

3.2.5.1 The *Raf1*^{Asp633Tyr} variant has lower expression of OXPHOS and metabolic pathways related proteins

To probe effects of the variants on biological processes, we performed overrepresentation analysis (ORA) using KEGG and MSigDB hallmark gene sets on the shared and cell line-specific DEPs of the *Nf1*$^{\text{Phe1112Leu}}$ and *Raf1*$^{\text{Asp633Tyr}}$ variants (**Figure 3.5**). Shared upregulated DEPs show an enrichment of several pathways, including the PI3K-Akt signalling pathway (**Table 3.2**) that shares growth factor activated receptors with the MAPK/ERK signalling pathway. We observed no enriched of pathways for the *Nf1*$^{\text{Phe1112Leu}}$ DEPs alone. However, for the *Raf1*$^{\text{Asp633Tyr}}$ downregulated DEPs, we detected enrichment of both oxidative phosphorylation and metabolic pathways, while the upregulated DEPs show enrichment for ribosome and endocytosis. The downregulation of metabolic pathways in the *Raf1*$^{\text{Asp633Tyr}}$ variant, that include the IIS network and MAPK/ERK signalling pathway, supports the hypothesis that negative feedback, outgoing from elevated Erk1 expression, leads to an overall dampened activity of metabolic signalling pathways in this cell line. Reduced activity of metabolic pathways has been shown to be at the root cause of lifespan extension in different organisms, and thus points towards longevity promoting functional effects in the *Raf1*$^{\text{Asp633Tyr}}$ variant (Laskovs et al., 2022; Partridge, 2010; Vellai et al., 2003).

	ID	Description	GeneRatio	BgRatio	p-value	p.adjust
Raf1$^{\text{Asp633Tyr}}$ Down	mmu01100	Metabolic pathways	295/1044	451/1834	1.56E-05	0.00490648
	mmu00190	Oxidative phosphorylation	42/1044	50/1834	3.57E-05	0.005629671
	mmu03030	DNA replication	16/1044	16/1834	0.00011565	0.012143302
Raf1$^{\text{Asp633Tyr}}$ Up	mmu03010	Ribosome	52/791	57/1834	1.17E-14	3.52E-12
	mmu04512	ECM-receptor interaction	20/791	23/1834	1.62E-05	0.002433457
	mmu04514	Cell adhesion molecules	18/791	21/1834	6.76E-05	0.006697431
	mmu04144	Endocytosis	55/791	87/1834	8.90E-05	0.006697431
	mmu05412	Arrhythmogenic right ventricular cardiomyopathy	19/791	25/1834	0.000816626	0.049160865
Raf1$^{\text{Asp633Tyr}}$ & Nf1$^{\text{Phe1112Leu}}$ Up	mmu05412	Arrhythmogenic right ventricular cardiomyopathy	12/259	25/1834	4.63E-05	0.010225616
	mmu05414	Dilated cardiomyopathy	10/259	19/1834	7.63E-05	0.010225616
	mmu05410	Hypertrophic cardiomyopathy	9/259	18/1834	0.000296041	0.026446364
	mmu04151	PI3K-Akt signalling pathway	22/259	77/1834	0.000565852	0.037912079
	mmu04510	Focal adhesion	18/259	59/1834	0.00077546	0.041564634
Raf1$^{\text{Asp633Tyr}}$ & Nf1$^{\text{Phe1112Leu}}$ Down	mmu04966	Collecting duct acid secretion	9/315	11/1834	4.69E-06	0.001378406
	mmu05323	Rheumatoid arthritis	10/315	14/1834	1.03E-05	0.001509726

Table 3.2 | Overrepresentation analysis with KEGG of the *Raf1*^{Asp633Tyr} and *Nf1*^{Phe1112Leu} variants. KEGG enrichment analysis revealed oxidative phosphorylation and metabolic pathways to be downregulated in the *Raf1*^{Asp633Tyr} variant, while ribosome and endocytosis related processes are upregulated.

3.2.6 Metabolomics recapitulate the proteomic signature of the *Raf1*^{Asp633Tyr} and *Nf1*^{Phe1112Leu} variant

Next, we tested if the biological processes by proteomic signatures were also present in metabolomic signatures. For this, we performed targeted metabolomics of TCA cycle intermediates and amino acids for the *Raf1*^{Asp633Tyr}, *Nf1*^{Phe1112Leu} and *Nf1*^{Ala1127Val} variants and compared their relative abundance to those of wildtype cells. Again, the *Nf1*^{Ala1127Val} cell line clustered together with the wildtype cells, while the *Raf1*^{Asp633Tyr} and *Nf1*^{Phe1112Leu} cell lines were clearly distinct from each other and the wildtype cells (**Figure 3.6+3.7A**). After false discovery rate (FDR) adjustment, a number of TCA cycle metabolites were found to be differentially abundant in the *Raf1*^{Asp633Tyr} and *Nf1*^{Phe1112Leu} cell lines, with the strongest effects observed in the *Nf1*^{Phe1112Leu} variant (**Figure 3.6C**). FDR adjustment resulted in a limited number of significantly altered metabolites in the *Raf1*^{Asp633Tyr} variant (**Figure 3.6B**). Although the recapitulation of the reduction in OXPHOS and metabolic pathways related proteins at metabolite level was weaker in this variant, it was mirrored in a reduction of TCA cycle and glycolysis related metabolites, such as galactose-6-phosphate and 3-hydroxyglutaric acid (**Figure 3.6B**).

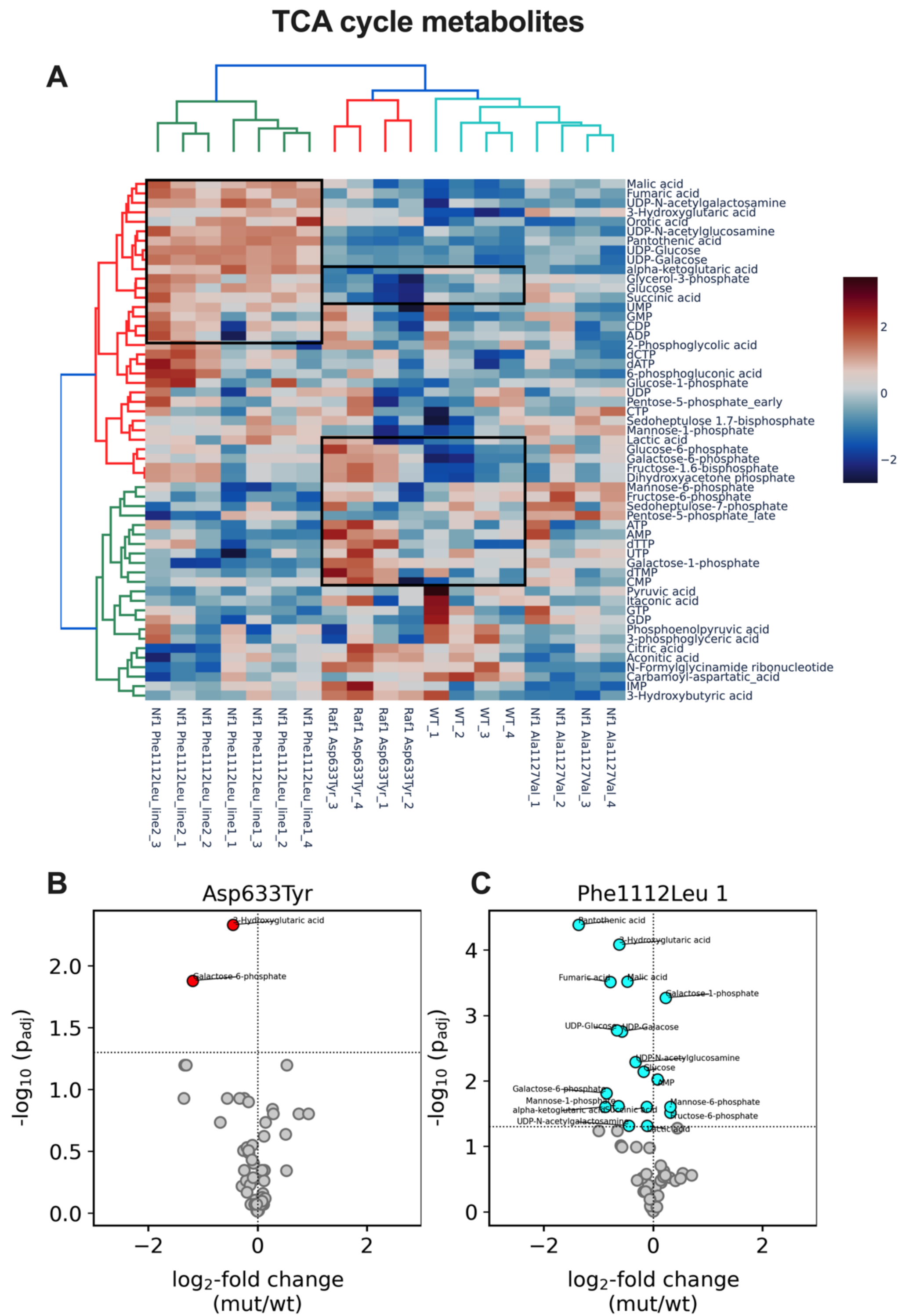


Figure 3.6 | Targeted metabolomics of TCA cycle metabolites of the *Raf1*^{Asp633Tyr} and *Nf1*^{Phe1112Leu} variants.
(A) Heatmap clustering confirms the *Nf1*^{Ala1127Val} variant to cluster with wildtype cells. The two independent cell lines harbouring the *Nf1*^{Phe1112Leu} variant cluster together. The *Raf1*^{Asp633Tyr} and *Nf1*^{Phe1112Leu} cell lines cluster distinct from each other and wildtype cells **(B+C)** FDR corrected differentially expressed TCA cycle metabolites and intermediates of the **(B)** *Raf1*^{Asp633Tyr} and **(C)** *Nf1*^{Phe1112Leu} variant relative to wildtype cells. All raw values were normalised to total intensity quantified (TIQ) and internal standards used.

We also observed reduced relative abundance of several amino acids in the *Raf1*^{Asp633Tyr} variant, such as cystathionine, aspartic acid, serine and threonine. The only upregulated amino acid in this cell line was S-adenosyl-L-homocysteine (SAH) (**Figure 3.7B**).

In the *Nf1*^{Phe1112Leu} cell lines, TCA cycle related metabolites, especially monosaccharides, appeared predominantly downregulated (**Figure 3.7C**). Interestingly, cystathionine was strongly downregulated in the *Raf1*^{Asp633Tyr} variant and appeared among the upregulated amino acids in the *Nf1*^{Phe1112Leu} variant together with glutathione, glycine and aspartic acid (**Figure 3.7C**). These findings confirm clearly differential metabolomic signatures of the *Nf1*^{Phe1112Leu} and *Raf1*^{Asp633Tyr} variants in mESCs, partially recapitulating those revealed by proteomic analysis. SAH and cystathionine belong to the transsulfuration pathway, pointing towards altered metabolite flux of this pathway in these cell lines.

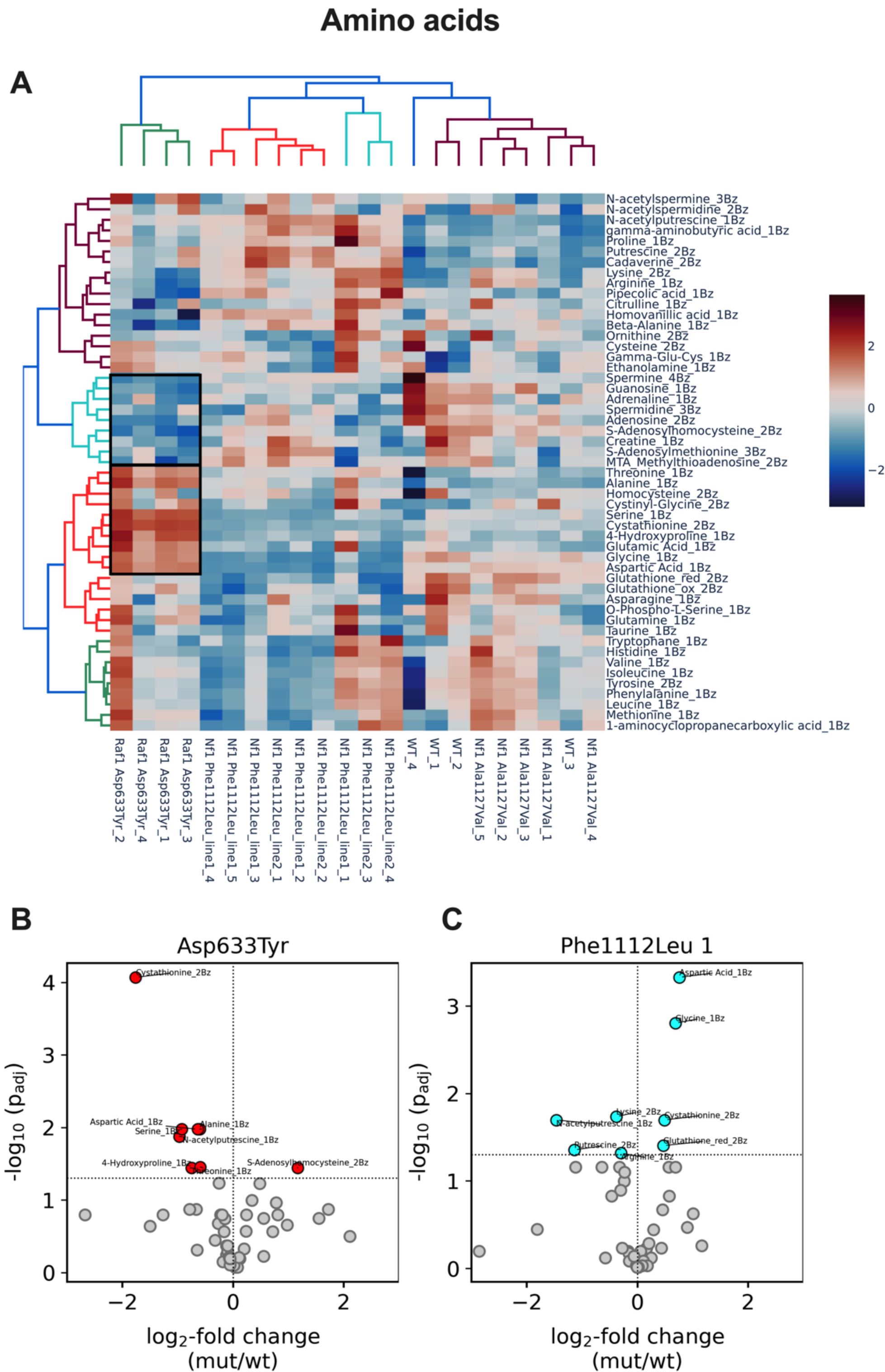


Figure 3.7 | Targeted metabolomics of amino acids of the *Raf1*$^{\text{Asp633Tyr}}$ and *Nf1*$^{\text{Phe1112Leu}}$ variants.

(A) Heatmap clustering confirms the *Nf1*$^{\text{Ala1127Val}}$ variant to cluster with wildtype cells. The two independent cell lines harbouring the *Nf1*$^{\text{Phe1112Leu}}$ variant cluster together. The *Raf1*$^{\text{Asp633Tyr}}$ and *Nf1*$^{\text{Phe1112Leu}}$ cell lines cluster distinct from each other and wildtype cells **(B+C)** FDR corrected differentially expressed amino acids and intermediates of the **(B)** *Raf1*$^{\text{Asp633Tyr}}$ and **(C)** *Nf1*$^{\text{Phe1112Leu}}$ variant relative to wildtype cells. All raw values were normalised to total intensity quantified (TIQ) and internal standards used.

3.2.7 The *Raf1*^{Asp633Tyr} variant shows improved resistance to oxidative and endoplasmic reticulum stress in mESCs

The MAPK/ERK signalling pathway is activated in response to increased endoplasmic reticulum (ER) and oxidative stress to maintain survival (Darling & Cook, 2014; Guyton et al., 1996; Rezatabar et al., 2019; Son et al., 2011). In order to assess the response to ER and oxidative stress of the *Nf1*^{Phe1112Leu} and *Raf1*^{Asp633Tyr} variants in comparison to wildtype mESCs, we stressed these cell lines with tunicamycin, an antibiotic that induces ER stress, and hydrogen peroxide (H202), inducing oxidative stress. We showed that two independent cell lines of the *Nf1*^{Phe1112Leu} variant do not differ from wildtype cells in response to tunicamycin and H202 (**Figure 3.8A-B**) induced stress, suggesting that the proteomic and metabolic rewiring in this variant is uncoupled from the cellular stress response in these cells for the stressors tested. We could show, however, that the *Raf1*^{Asp633Tyr} cell line had a significantly increased resistance to both stressors, when compared to wildtype mESCs (**Figure 3.8C-D**). These findings in the *Raf1*^{Asp633Tyr} cell line suggest coupling of elevated MAPK/ERK signalling pathway activity and stress response mechanisms, potentially facilitated through proteomic and metabolomic rewiring.

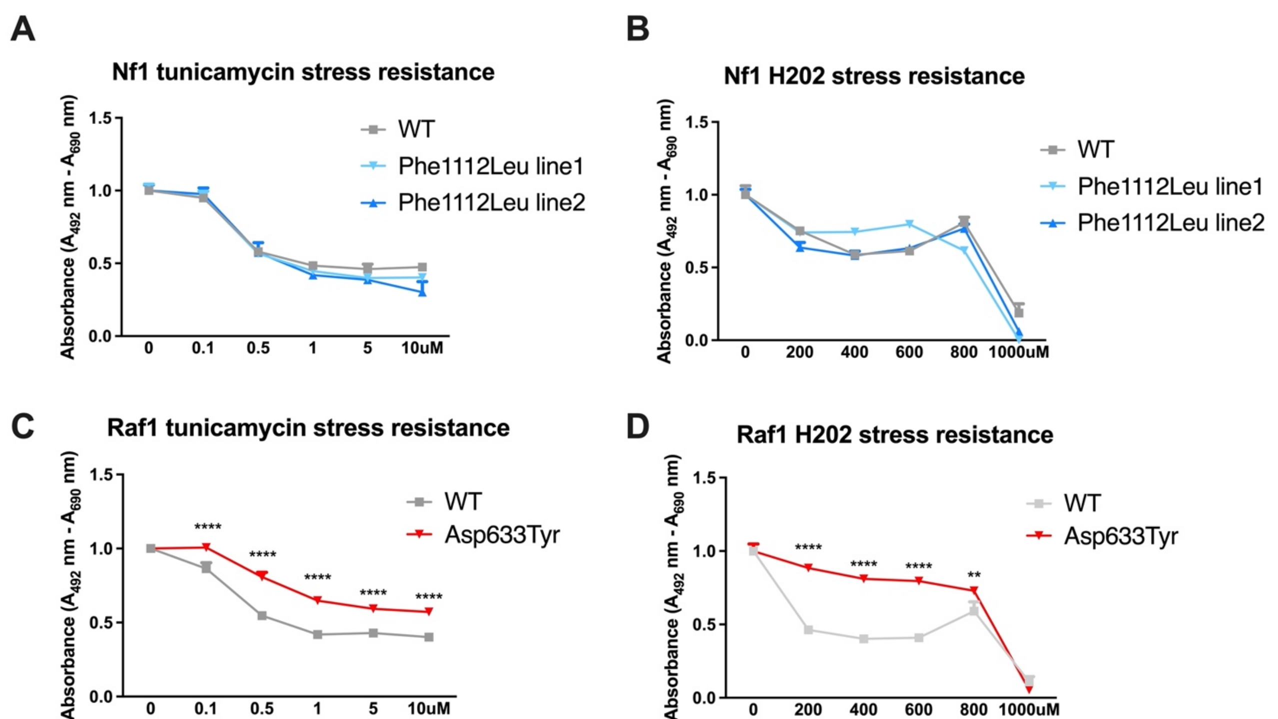


Figure 3.8 | The *Raf1*^{Asp633Tyr} variant shows elevated stress resistance in mESCs.
(A-D) Absorbance-based detection of formazan through the reduction of tetrazolium salt WST-1 by dehydrogenases, in metabolically active cells after being stressed with tunicamycin and H202. The **(A)**

Nf1^{Phe1112Leu} cell lines did not show any deviation from wildtype cells in response to ER stress (tunicamycin) and **(B)** oxidative stress (H2O2) **(C)** The *Raf1*^{Asp633Tyr} variant showed significantly elevated stress resistance in response to ER stress (tunicamycin) and (**D**) oxidative stress (H2O2) (Values were normalised to respective untreated controls and analysed with 2-way ANOVA) *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$

3.2.8 Generation of transgenic flies harbouring the *Nf1*^{Phe1112Leu} variant

3.2.8.1 The heterozygous *Nf1*^{Phe1112Leu} variant does not increase lifespan in *Drosophila melanogaster*

Next, we aimed to assess whether we could recapitulate the effects on pathway activity and stress resistance *in vivo*. The amino acids of the *Raf1*^{Asp633Tyr} and *Nf1*^{Phe1112Leu} variant are conserved in *D.melanogaster,* but not in *C.elegans*. We therefore aimed to generate transgenic fly lines harbouring these variants, however, we did not succeed in generating a transgenic Raf1^{Asp713Tyr} fly line, likely because this variant leads to embryonic lethality. We did succeed in generating a transgenic fly line harbouring the *Nf1*^{Phe1148Leu} mutation, which we subsequently backcrossed for six generations into the *wDah* background (**Figure 3.9**). Importantly, this was the first time point at which we could analyse the functional effect of the heterozygous variant. To determine whether the *Nf1*^{Phe1148Leu} mutation results in a gain- or loss-of-function phenotype, we also analysed the previously generated *Nf1*^{P1} loss-of-function mutants (LOF) (The et al., 1997), backcrossed for six generations into the *wDah* background, and trans-heterozygous flies (i.e. *Nf1*$^{Phe1148Leu/P1\ LOF}$) in terms of their pathway activity. Both trans-heterozygous and *Nf1*^{P1} LOF mutants were viable and fertile. As reported by The et al. (1997), homozygous *Nf1*^{P1} LOF mutants were significantly smaller than wild type flies. Also, female homozygous *Nf1*^{Phe1148Leu} flies were significantly smaller than *wDah* flies (**Figure 3.9A**). First, we performed western blotting using protein lysates of fly heads to assess MAPK/ERK signalling pathway activity. In line with previous findings that loss of *Nf1* results in elevated MAPK/ERK signalling pathway activity (Xu et al., 1992; Legius et al., 1993; Shannon et al., 1994), western blotting revealed significant elevations in the trans-heterozygous and homozygous *Nf1*^{P1} LOF mutants in males (**Figure 3.9B-C**). We did not observe functional effects on MAPK/ERK signalling pathway activity for the heterozygous and homozygous *Nf1*^{Phe1148Leu} flies, allowing no judgement on the functional effect of this variant in the fly based on this read-out.

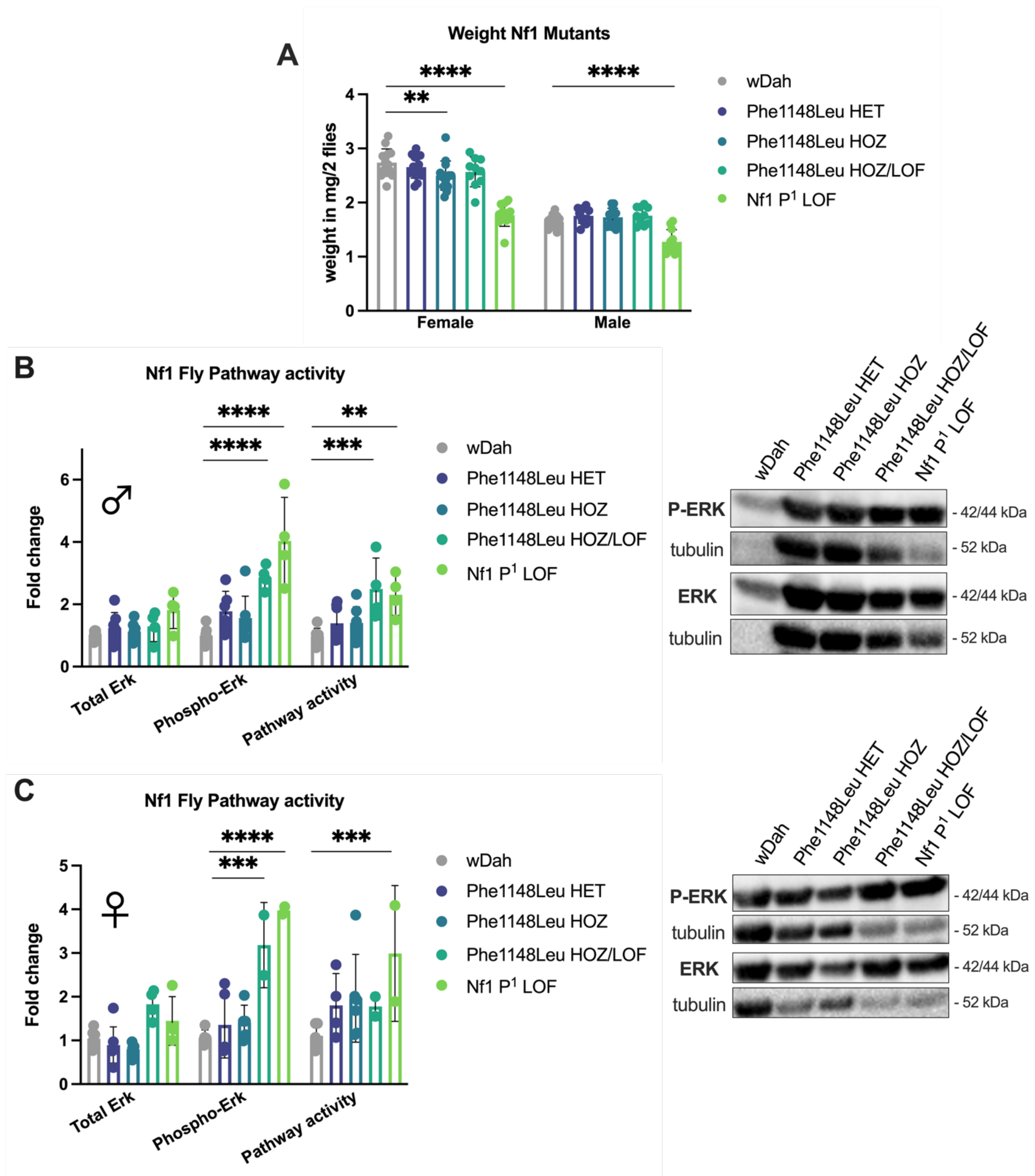


Figure 3.9 | The effect of the *Nf1*^{Phe1148Leu} variant on MAPK/ERK signalling pathway activity.

(A) Wet weight of female and male *Nf1* mutants. Homozygous female *Nf1*^{Phe1148Leu} flies and *Nf1*^{P1} LOF mutant flies were smaller than *wDah* flies (dots represent 2 flies, *n = 20)* (data was analysed with 2way ANOVA and Sidak's multiple comparisons test) **(B+C)** Western blotting showed a significant upregulation of phosphorylated ERK and pathway activity in trans heterozygous and *Nf1*^{P1} LOF mutants in **(B)** males and **(C)** females (Western blots were analysed with 2way ANOVA and Dunnett's multiple comparisons test). wDah, white Dahomey (wildtype); HET, heterozygous; HOZ, homozygous; LOF, loss-of-function.

We continued the functional characterization of the *Nf1*$^{\text{Phe1148Leu}}$ fly line and assessed developmental time and climbing ability of heterozygous and homozygous transgenic flies. It has long been known that environmental and genetic manipulations can influence development in the fruit fly (Alpatov, 1930; Burcombe & Hollingsworth, 1970; VanHook, 2012). By manipulating environmental factors such as population density, food and temperature, it was shown that developmental time correlates positively with longevity in flies (Alpatov, 1930; Hollingsworth, 1969; Miller & Thomas, 1958). In heterozygous and homozygous flies carrying the *Nf1*$^{\text{Phe1148Leu}}$ variant, we found a small, but significant developmental delay of flies hatching from synchronised eggs (**Figure 3.10A**). Next, we looked at climbing ability of adult transgenic flies which is a commonly used read-out for locomotor activity, which is known to deteriorate with increasing age (Rhodenizer et al., 2008). Here, we could show that heterozygous and homozygous *Nf1*$^{\text{Phe1148Leu}}$ female flies have reduced climbing ability, while in male flies only homozygous flies performed worse than *wDah* flies (**Figure 3.10B-C**). This indicates that the variant leads to reduced locomotor activity in flies.

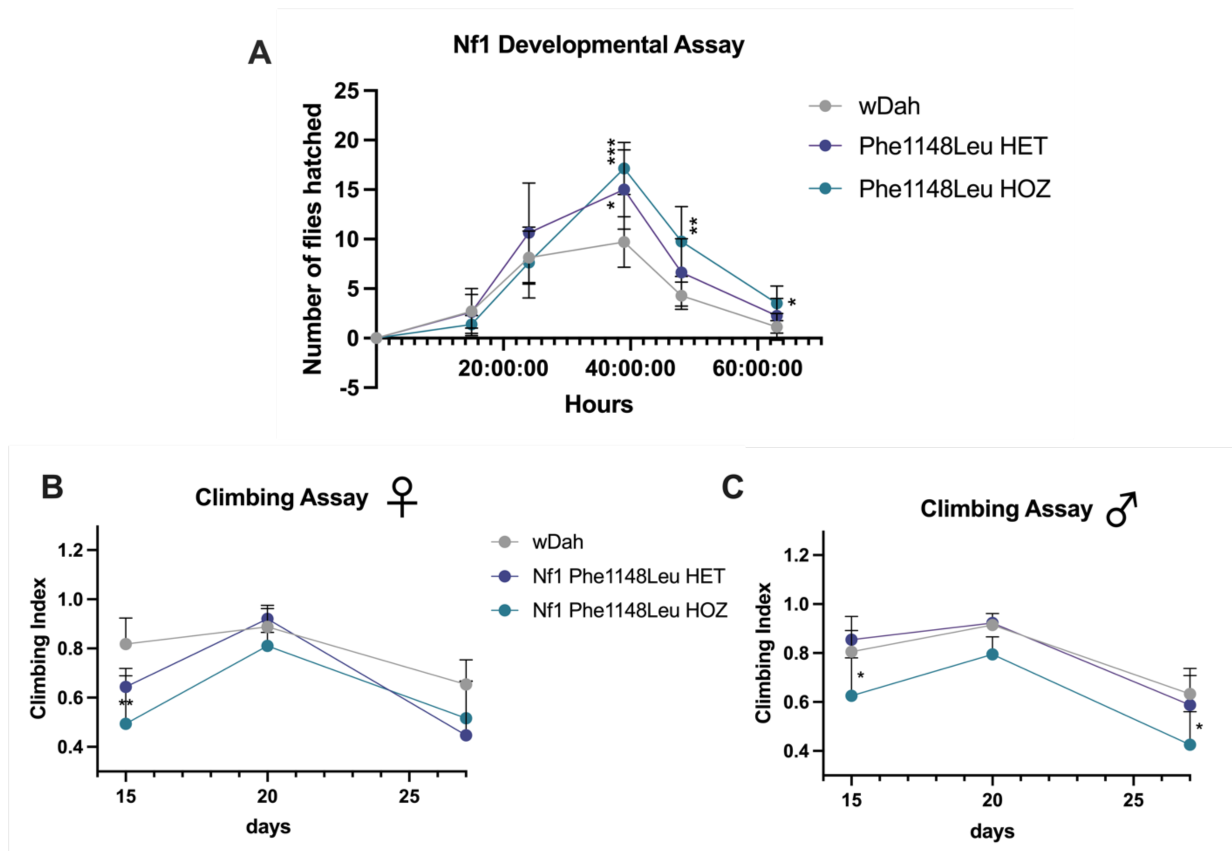


Figure 3.10 | *Nf1*$^{\text{Phe1148Leu}}$ mutants show delayed development and reduced climbing ability.

(A) developmental assay shows the homozygous *Nf1*^{Phe1148Leu} flies to have a small but significant developmental delay **(B+C)** climbing ability is reduced in **(B)** heterozygous and homozygous female flies and in **(C)** homozygous male flies (n= 80 flies) (2-way ANOVA with Kruskal-Wallis multiple comparisons test) $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

Overexpression of *Nf1* in the fruit fly has been shown to extend lifespan, improve fecundity and increase stress resistance to oxidative and thermal stress (Tong et al., 2007). To determine if the *Nf1*^{Phe1148Leu} variant has an effect on lifespan, due to improved or reduced activity of the *Nf1* gene, we subjected once-mated, heterozygous and homozygous transgenic flies of both sexes to a lifespan assay. The results clearly show that the homozygous *Nf1*^{Phe1148Leu} variant substantially reduced lifespan, while heterozygous animals showed no effect, indicating that the presence of the wildtype allele may protect against the deleterious effect of the mutant allele (**Figure 3.11A-B**). Lifespan modulations in flies often occur at the expense of fecundity (Fowler & Partridge, 1989; Partridge & Barton, 1993). We therefore assessed fecundity, but did not observe any significant difference between *Nf1*^{Phe1148Leu} and wildtype flies (**Figure 3.11C**). Based on these findings, we conclude the *Nf1*^{Phe1148Leu} variant to be deleterious in the homozygous state in the fruit fly, indicating a functional locus at this position in the *Nf1* gene, and that developmental delay is uncoupled from longevity of these flies.

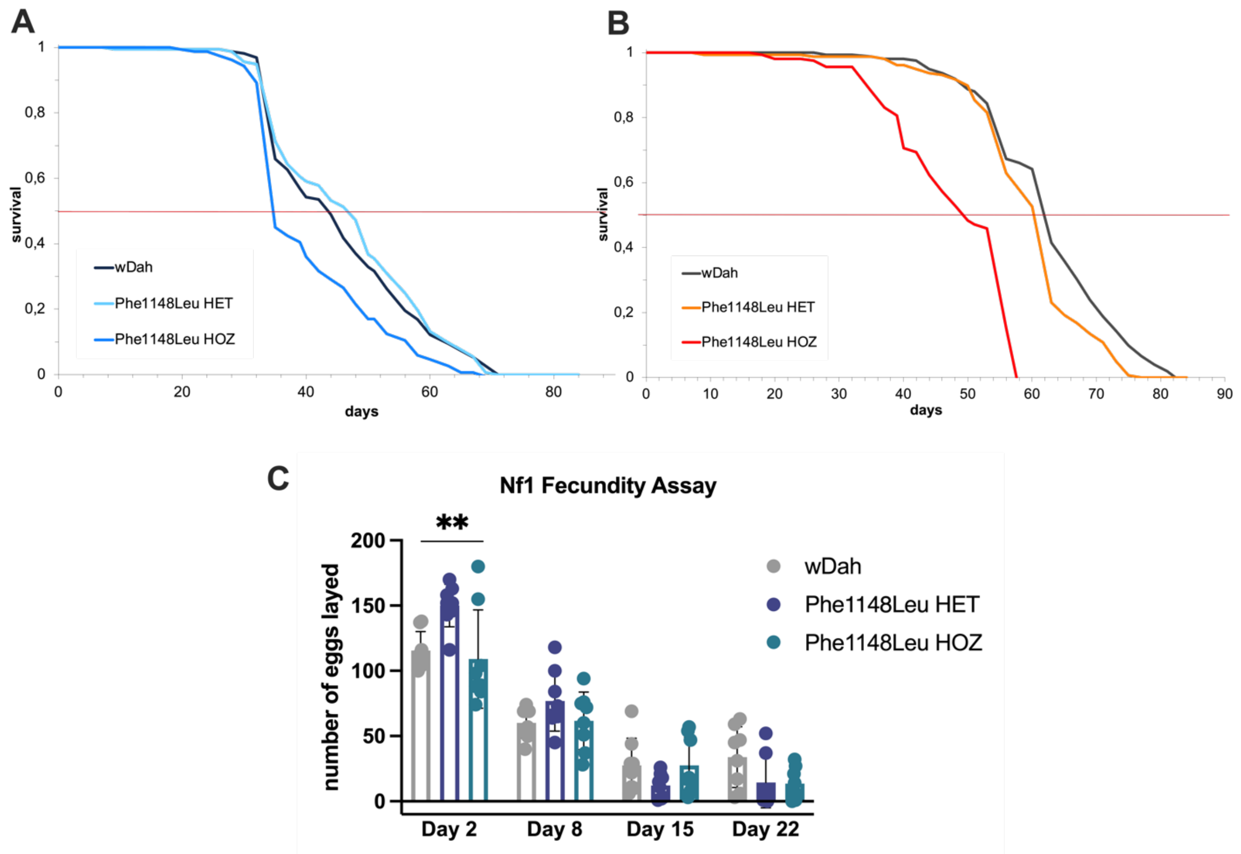


Figure 3.11 | The homozygous *Nf1*^{Phe1148Leu} variant reduces lifespan in *D.melanogaster*. **(A+B)** lifespan assay of wildtype (black), heterozygous and homozygous *Nf1*^{Phe1148Leu} flies of **(A)** male and **(B)** female flies. Lifespan of heterozygous animals did not differ significantly from wildtype flies, while homozygous animals showed significantly reduced lifespan (n = 160 flies per condition, $p < .001$, data was analysed with log-rank test) **(C)** Fecundity assay of once-mated female flies over a time course of three weeks. No differences in egg laying were detected in the *Nf1*$^{\mathrm{Phe1148Leu}}$ mutant flies (n = 50) (data was analysed with 2way ANOVA and Dunnett's multiple comparisons test) *P < 0.05, **P < 0.01, ***P < 0.001. wDah, white Dahomey (wildtype); HET, heterozygous; HOZ, homozygous.

3.2.9 Transgenic mice

3.2.9.1 Mouse embryonic fibroblasts of the *Raf1*^{Asp633Tyr} and *Nf1*^{Phe1112Leu} variants recapitulate mESC ER stress phenotype

Given the functional effects *in vitro* of the *Raf1*^{Asp633Tyr} and *Nf1*^{Phe1112Leu} variants, we decided to generate transgenic mouse lines harbouring these variants to follow them up *in vivo*. In line with the 3R principles, i.e. replace, reduce and refine, in experimental biomedical research with laboratory animals, we aimed to functionally characterise and prioritise the genetic variants observed in long-lived individuals first *in vitro* before we opted for the generation of mouse lines. The generation of transgenic mouse lines was for the *Raf1*^{Asp633Tyr} variant, however, the best option to assess the effect of the heterozygous variant, which is how they are present in the long-lived individuals from the LLS. To this end, we collaborated with Ingo Voigt from the Transgenesis Facility at the MPI Age, who performed the *in-vitro* fertilization of the CRISPR/Cas9 constructs.

We successfully generated transgenic mice harbouring the *Raf1*^{Asp633Tyr} and *Nf1*^{Phe1112Leu} variants (confirmed with Sanger Sequencing) and backcrossed them with wildtype C57BL/6 mice to reduce the occurrence of possible off-target mutations. We did not observe any adverse phenotypes in both transgenic mouse lines. As a first step, we analysed mouse MEFs of both variants to see whether we could recapitulate the functional effects observed in mESCs.
At Day 13.5 of gestation, we isolated and dissected embryos to obtain MEF cell lines of mice harbouring the *Raf1*^{Asp633Tyr} and *Nf1*^{Phe1112Leu} variants. To assess potential functional effects on MAPK/ERK signalling pathway activity, we isolated proteins of the respective MEF cell lines for western blotting. Pathway activity analysis of MEFs harbouring the *Raf1*^{Asp633Tyr} variant revealed reduced pathway activity in the homozygous *Raf1*^{Asp633Tyr} cell lines (**Figure 3.12A**), opposing to our findings in mESCs. Similarly, pathway activity analysis of the *Nf1*^{Phe1112Leu} variant in MEFs of heterozygous and homozygous animals revealed upregulated pathway activity, contrary to the downregulation of such as observed in mESCs (**Figure 3.12B**).

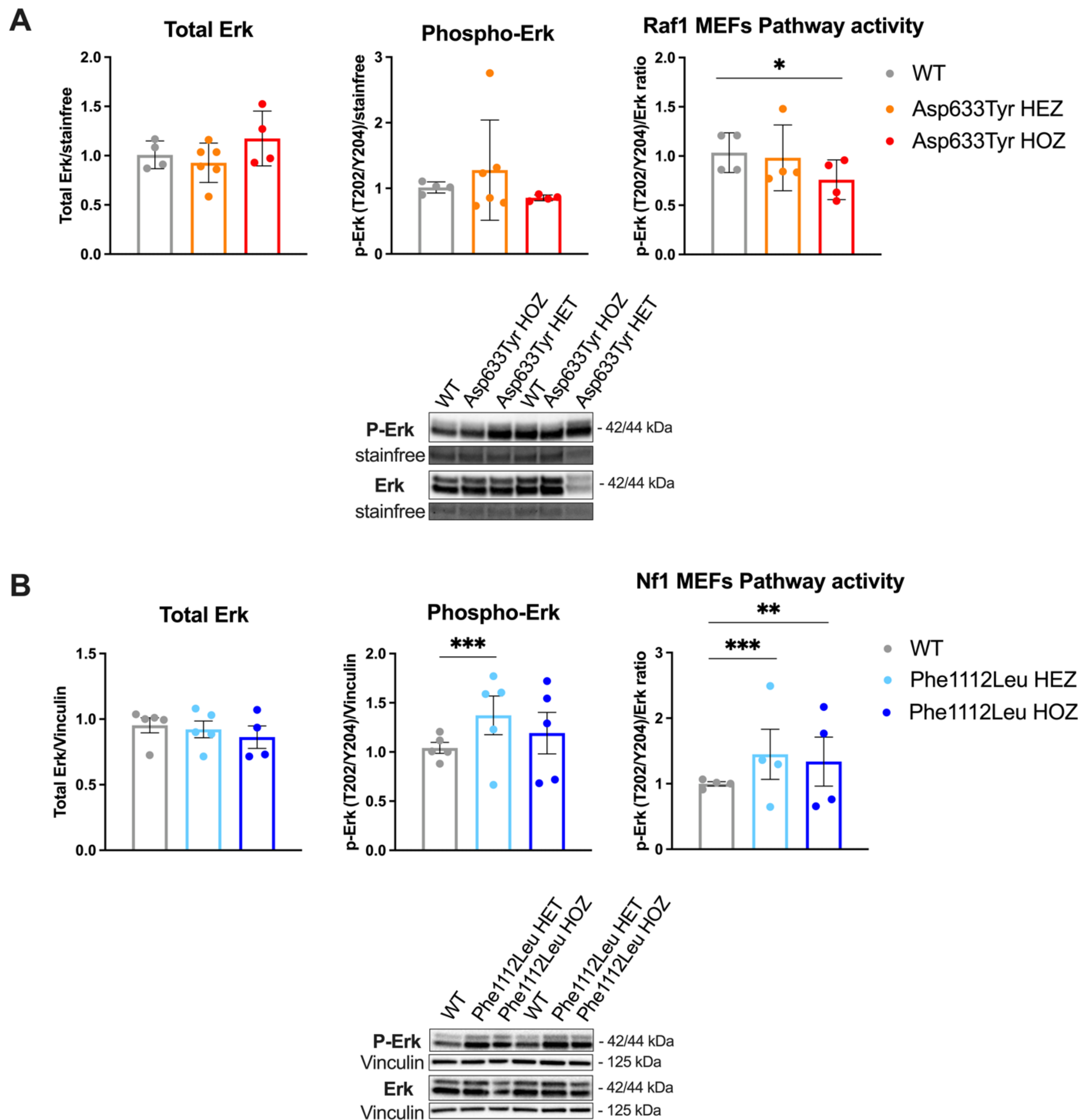


Figure 3.12 | The effect of the *Nf1*^{Phe1112Leu} and *Raf1*^{Asp633Tyr} variant on MAPK/ERK signalling pathway activity in MEFs.

(A+B) western blotting of **(A)** *Raf1*^{Asp633Tyr} MEFs shows reduced pathway activity in homozygous cells and an elevation in heterozygous and homozygous **(B)** *Nf1*^{Phe1112Leu} MEFs. Dots represent biological replicates with two technical replicates probed (data was analysed with 2way ANOVA and Dunnett's multiple comparisons test) *P < 0.05, **P < 0.01, ***P < 0.001

Next, we performed stress assays of these MEF lines with tunicamycin. The results of both MEF lines recapitulate previous findings in mESCs, i.e. we observed no effect in cells harbouring the *Nf1*^{Phe1112Leu} variant and an improved stress response in cells harbouring the *Raf1*^{Asp633Tyr} variant. The heterozygous MEFs appeared to be a milder phenotype of the homozygous MEFs (**Figure 13.3 A-B**).

Taken together, these findings in MEFs suggest that the *Raf1*^{Asp633Tyr} variant also exerts effects *in vivo*. Noteworthy, we found opposing results of MAPK/ERK signalling pathway activity in mESCs and MEFs, indicating that this pathway is differentially regulated in different cell types and the effects observed in one, cannot be directly transferred to another.

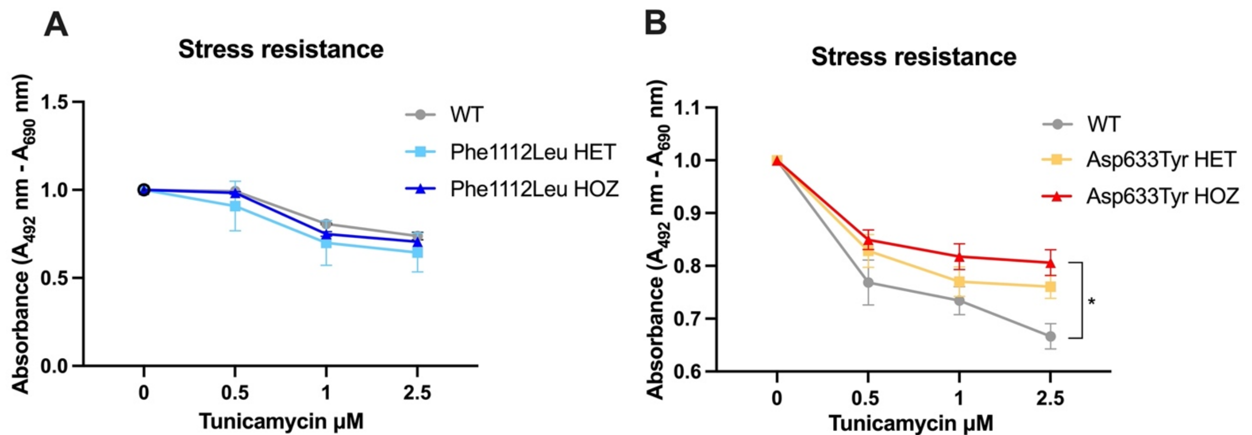


Figure 3.13 | The homozygous *Raf1*^{Asp633Tyr} variant shows elevated stress resistance in MEFs.

(A+B) tunicamycin stress resistance of MEFs harbouring the **(A)** *Nf1*^{Phe1112Leu} and **(B)** *Raf1*^{Asp633Tyr} variant. The *Raf1*^{Asp633Tyr} cell line shows elevated stress resistance to tunicamycin. Heterozygous MEFs show a milder phenotype of the homozygous MEFs (data was analysed with 2way ANOVA and Dunnett's multiple comparisons test) *P < 0.05, **P < 0.01, ***P < 0.001

3.2.9.2 The *Raf1*^{Asp633Tyr} and *Nf1*^{Phe1112Leu} mouse tissues show no functional effects at 3 months of age

To address whether the functional effects that we observed in mESCs and MEFs of the *Raf1*^{Asp633Tyr} and *Nf1*^{Phe1112Leu} variants are conserved *in vivo*, we continued backcrossing these two transgenic mouse lines for 6 generations into the C57BL/6 background to get rid of possible off-target mutations. Animals of all genotypes and sexes continued to show no obvious abnormalities and no differences in weight (**Figure 3.14**).

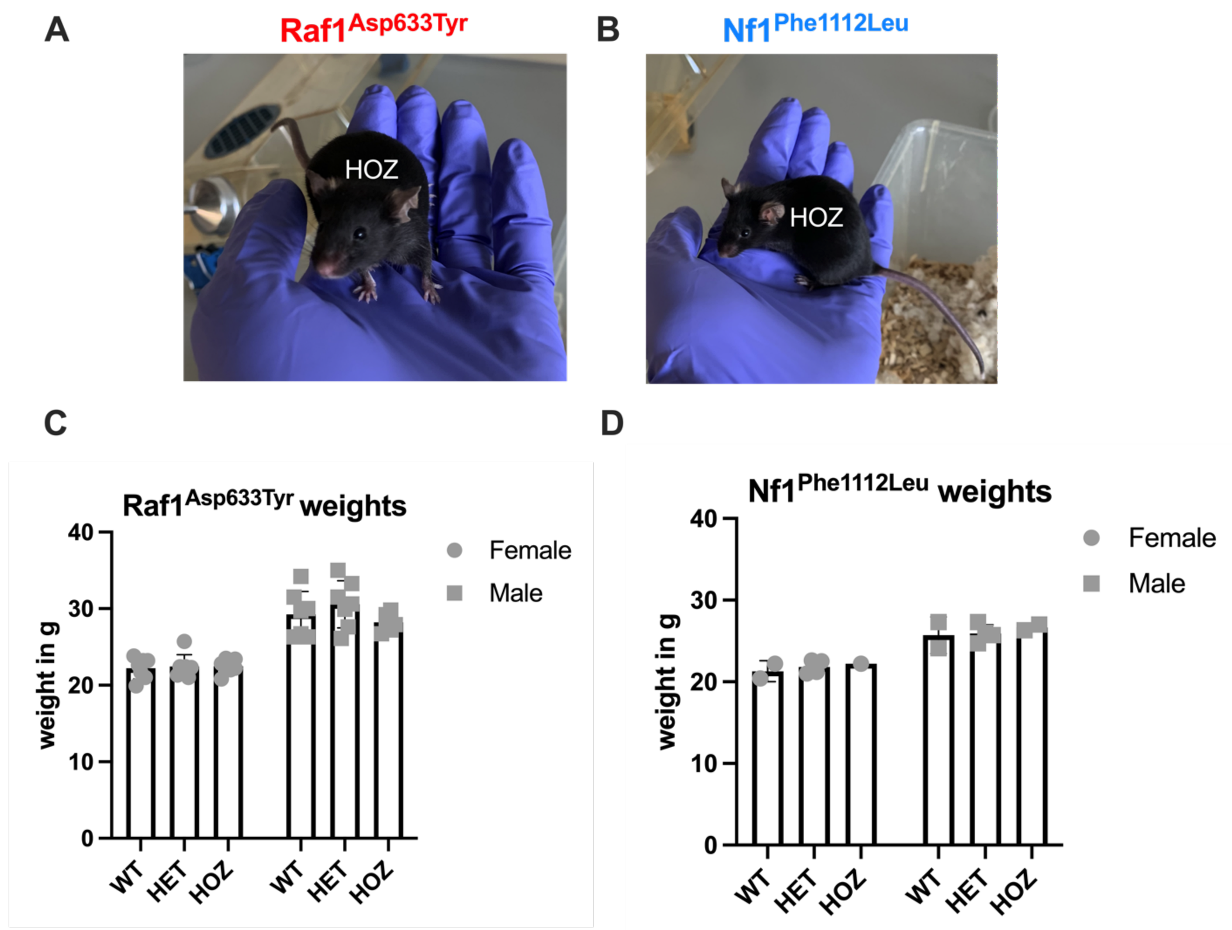


Figure 3.14 | *Raf1*^{Asp633Tyr} and *Nf1*^{Phe1112Leu} transgenic mice.
(A-B) Transgenic mice harbouring the homozygous **(A)** *Raf1*^{Asp633Tyr} and **(B)** *Nf1*^{Phe1112Leu} variants do not show obvious phenotypes at the age of three months. The weight of these animals was within normal range **(C+D)**. WT, wildtype; HET, heterozygous; HOZ, homozygous.

Based on the preceding experimental work, we prioritized the *Raf1*^{Asp633Tyr} variant. At three months of age, we sacrificed 7 animals per genotype and sex and collected skeletal muscle, white adipose tissue, liver, spleen, heart and brain, being highly metabolically active tissues. Raf1 protein is ubiquitously expressed in the mouse, however, based on its highest expression in the heart, brain and liver, we first subjected protein lysates of these tissues to western blotting. In all tissues analysed, however, we did not observe significant differences in MAPK/ERK signalling pathway activity in heterozygous (HET) and homozygous (HOZ) animals of both sexes when compared to wildtype animals (**Figure 3.15 & Figure 3.16**). This lack of findings could implicate several things: first, the effect of the variant might be tissue specific and we have not yet looked into the correct tissue. Secondly, the effect of the variant might only come into effect at later stages in life when age-related pathologies occur. Potential protective effects of the genetic variants, such as improved stress resistance, might only become apparent as such once cellular function is impaired. Thirdly, the functional effects that we observed in mESCs and MEFs might be cell type specific, simply not conserved in the mouse or we have not used correct read-outs to study potential minor functional effects. Follow-up experiments at later points in life of these animals are likely to answer some of these points. We have also started a longitudinal lifespan analysis of heterozygous and homozygous *Raf1*^{Asp633Tyr} animals, including 53 animals of both sexes, which will answer the question whether this variant contributes to longevity in mice.

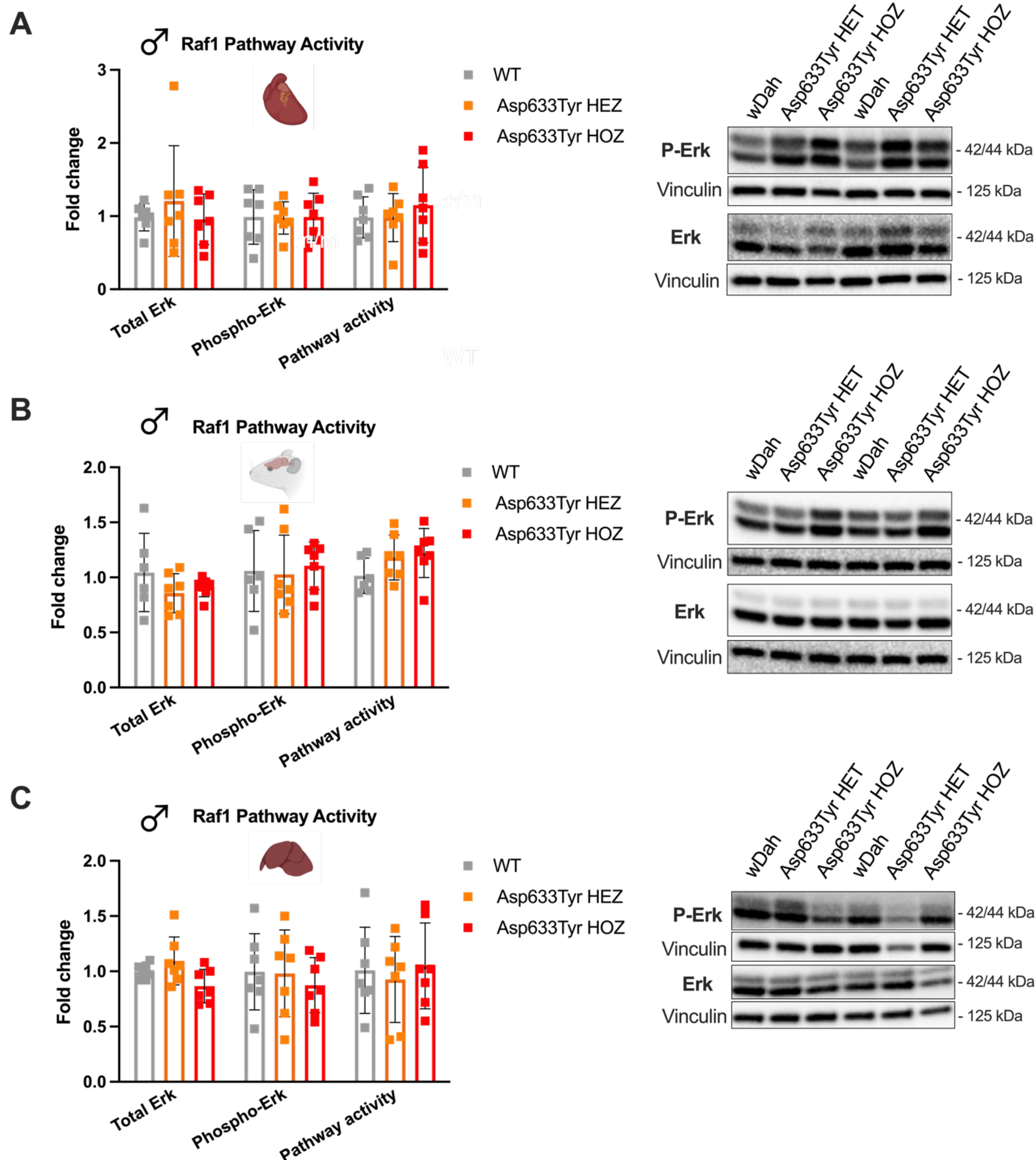


Figure 3.15 | Western blot analysis of heart, brain and liver tissues of three-month-old *Raf1*^{Asp633Tyr} male mice.
(A-C) western blotting of **(A)** heart protein lysates, **(B)** brain protein lysates and **(C)** liver protein lysates showed no effect on MAPK/ERK signalling pathway activity. Dots represent biological replicates (data was analysed with 2way ANOVA and Dunnett's multiple comparisons test) WT, wildtype; HET, heterozygous, HOZ, homozygous. *P < 0.05, **P < 0.01, ***P < 0.001

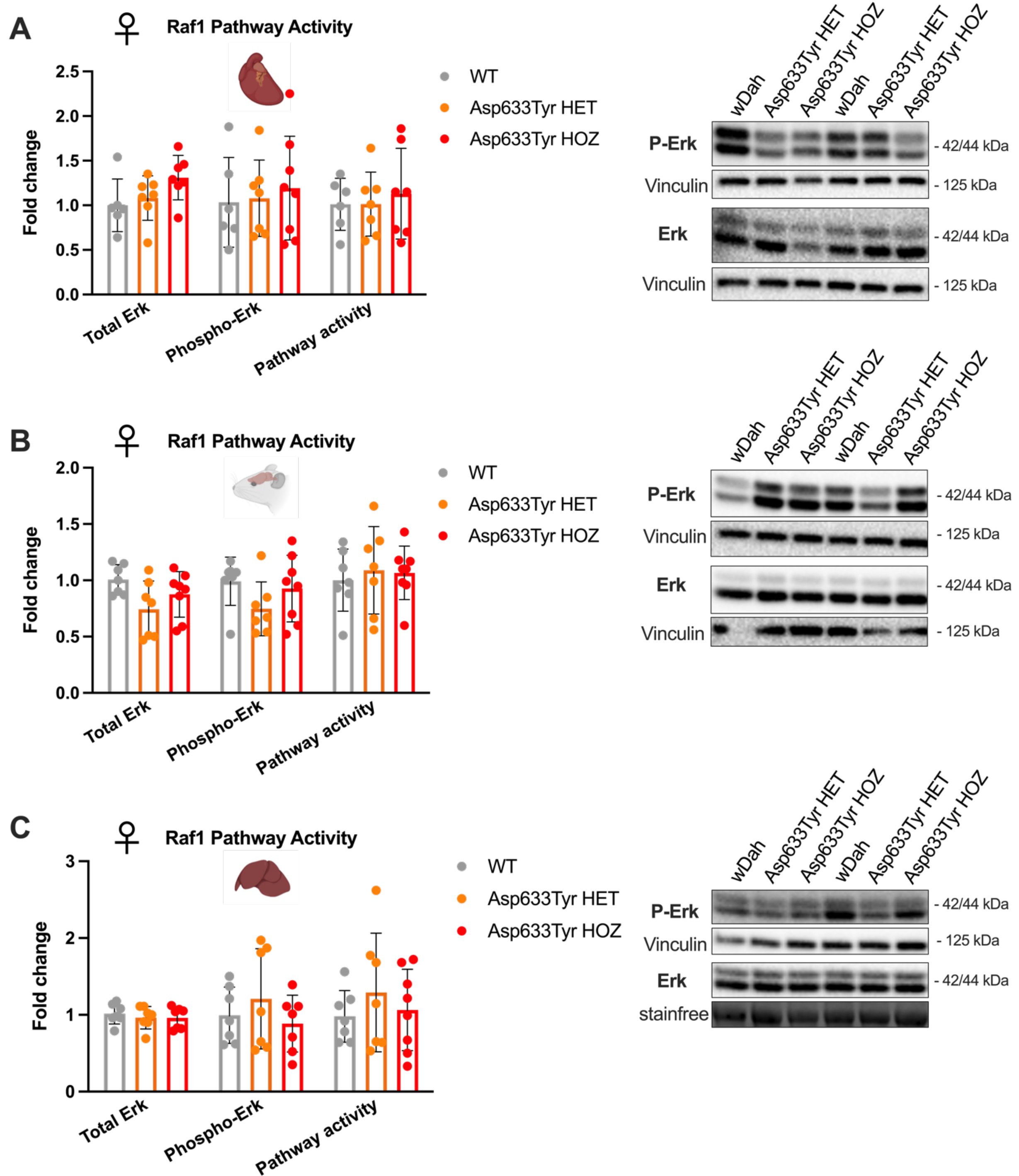


Figure 3.16 | Western blot analysis of heart, brain and liver tissues of three-month-old Raf1^{Asp633Tyr} female mice.

(A-C) western blotting of **(A)** heart protein lysates, **(B)** brain protein lysates and **(C)** liver protein lysates showed no effect on MAPK/ERK signalling pathway activity. Dots represent biological replicates (data was analysed with 2way ANOVA and Dunnett's multiple comparisons test) WT, wildtype; HET, heterozygous, HOZ, homozygous. *P < 0.05, **P < 0.01, ***P < 0.001

We also performed a preliminary functional analysis in mice harbouring the *Nf1*^{Phe1112Leu} variant. Due to the prioritization of the *Raf1*^{Asp633Tyr} variant, the number of analysed *Nf1*^{Phe1112Leu} animals is lower and should be repeated with a greater number. Nf1 protein is ubiquitously expressed in the mouse, however, based on its highest expression in the brain and muscle, we decided to first subject protein lysates of these tissues to western blotting. In females, we detected no functional effects on MAPK/ERK signalling pathway activity in either tissue (**Figure 3.17A+C**). Similarly, no effects were apparent in male brain lysates. In male skeletal muscle samples, however, it appeared as if homozygous animals displayed a reduction of pathway activity, while heterozygous animals showed an elevation of pathway activity (**Figure 3.17D**). These results point towards a potential sexual dimorphic response and tissue specificity of the *Nf1*^{Phe1112Leu} variant *in vivo*. However, the marginal number of *Nf1*^{Phe1112Leu} animals hampers any deeper interpretation of the results and needs to be repeated with a greater *n* number in the future.

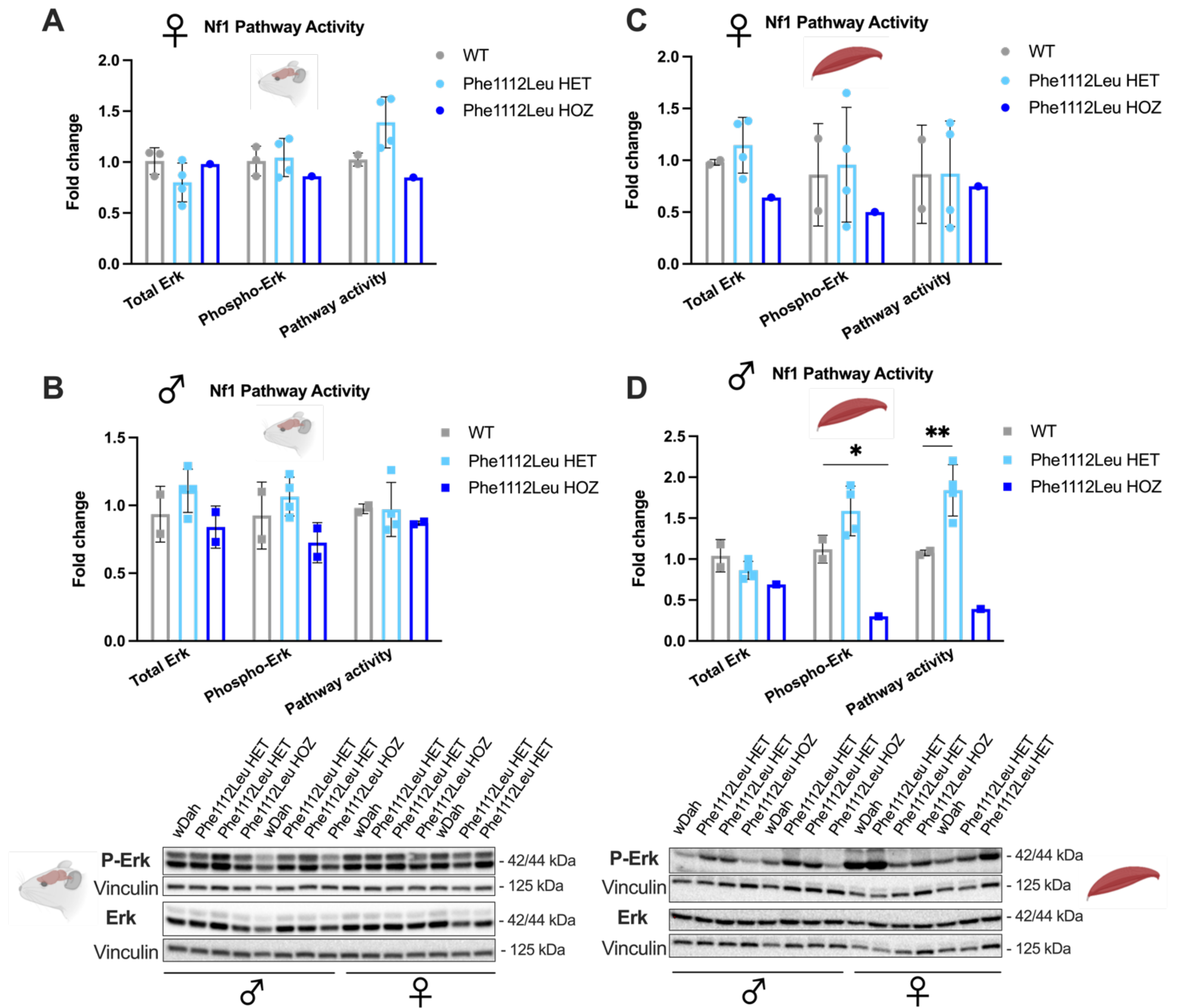


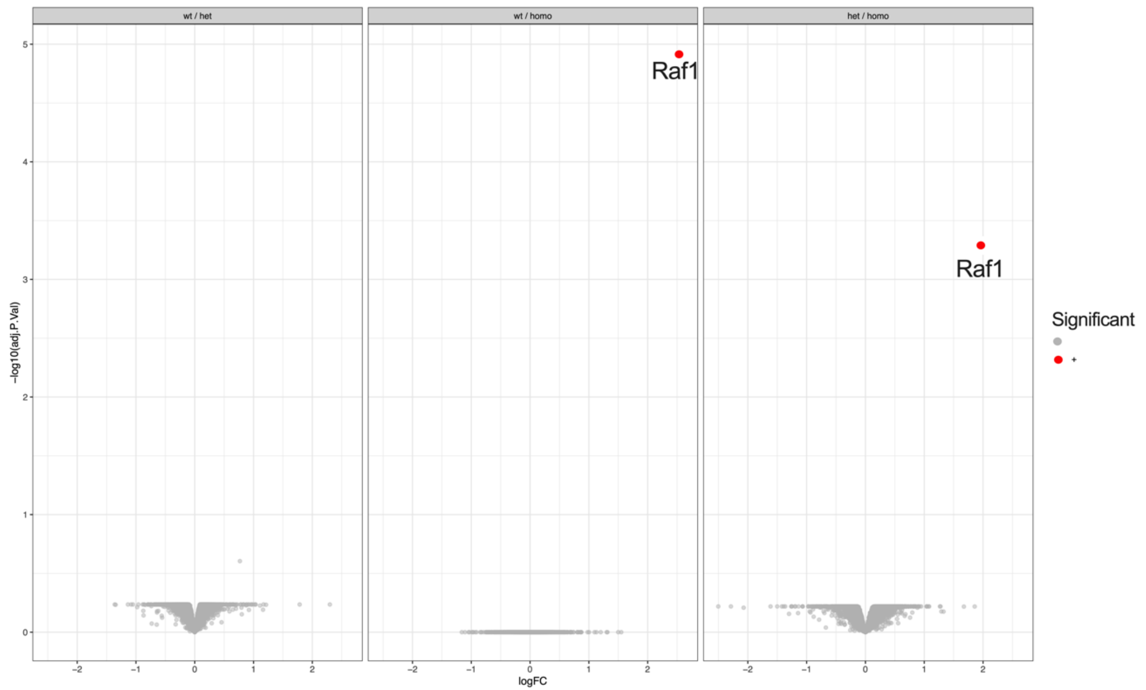
Figure 3.17 | Western blot analysis of brain and skeletal muscle tissues of three-month-old *Nf1*$^{\text{Phe1112Leu}}$ mice.

(A+B) western blotting of **(A)** female *Nf1*$^{\text{Phe1112Leu}}$ brain protein lysates and **(B)** male brain protein lysates showed no functional effect on pathway activity **(C+D)** western blotting of **(C)** female *Nf1*$^{\text{Phe1112Leu}}$ skeletal muscle protein lysates showed no effect on pathway activity **(D)** Pathway activity appeared upregulated in male, heterozygous (HET) animals and reduced in homozygous animals (HOZ) Dots represent biological replicates (data was analysed with 2way ANOVA and Dunnett's multiple comparisons test) *P < 0.05, **P < 0.01, ***P < 0.001

3.2.9.3 Raf1 protein is reduced in the liver of *Raf1*^{Asp633Tyr} mice

Our findings in mESCs and MEFs revealed that MAPK/ERK signalling pathway activity is highly variable depending on its cellular environment. It is not surprising that functional effects on pathway activity are less straightforward to be detected in a more complex *in vivo* model. To better understand underlying molecular mechanisms and possibly recapitulate the signature observed in mESCs, we performed whole proteome analysis of 3-month-old *Raf1*^{Asp633Tyr} liver samples at the Proteomics Facility at the MPI Age. Analysis of such proved that only Raf1 itself was significantly downregulated in heterozygous and more so in homozygous females when compared to wildtype animals (**Figure 3.18**). In males, no proteins appeared significantly differentially expressed and Raf1 itself was too low expressed in the samples, hampering its detection. These results implicate a much more centred effect of the *Raf1*^{Asp633Tyr} variant *in vivo* and confirm reduced expression of Raf1 *in vitro* and *in vivo*. These findings show that this pipeline to study rare genetic variants through CRISPR/Cas9 in different organisms, generally works. We show that some functional read-outs, such as MAPK/ERK signalling pathway activity, are context specific, while others, such as protein expression, are conserved in cells and mice.

A



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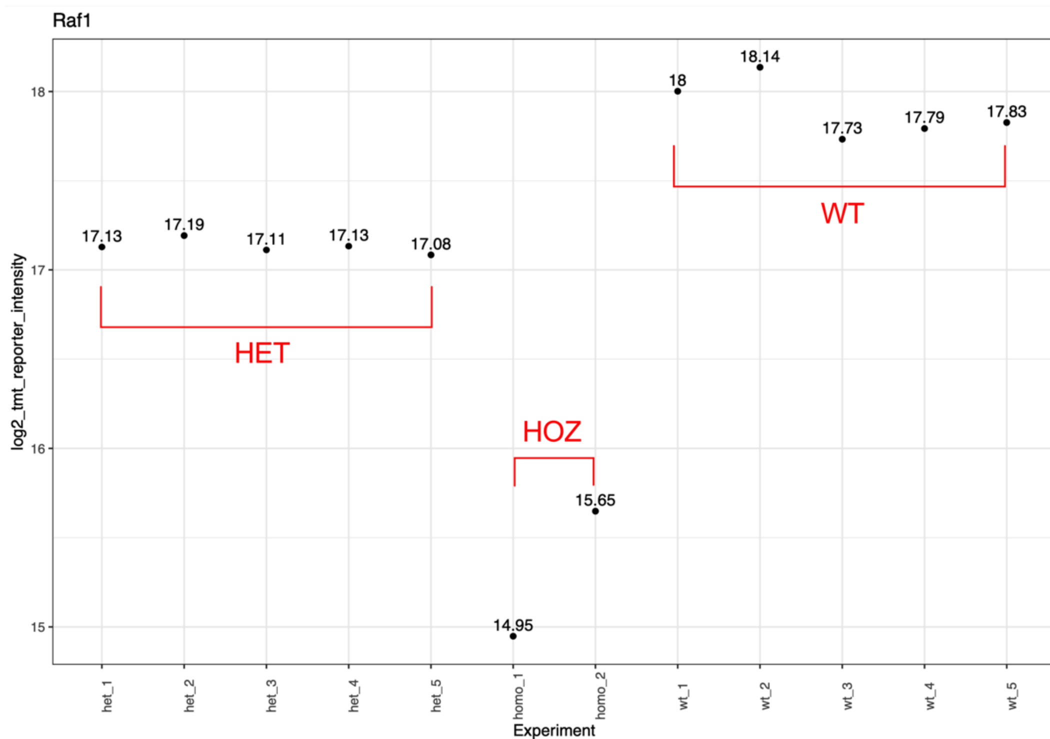


Figure 3.18 | Proteomics of liver tissues of *Raf1*$^{\mathrm{Asp633Tyr}}$ female mice reveal Raf1 specific downregulation.

(A) Volcano plots of differentially expressed proteins in female liver samples of 3-month-old mice harbouring the *Raf1*$^{\mathrm{Asp633Tyr}}$ variant. Raf1 is the only differentially expressed protein **(B)** TMT reporter intensities of Raf1 protein are reduced in heterozygous and homozygous liver samples, compared to wildtype samples. WT, wildtype; HET, heterozygous; HOZ, homozygous.

3.3 Discussion

In this study, we present a novel pipeline for the functional characterization and potential validation of candidate variants involved in human longevity. We generated cell, fly and mouse lines through CRSIPR/Cas9 technology, harbouring unique genetic variants observed in long-lived individuals that belong to the longevity-enriched LLS cohort, with the aim to decipher if and how they contributed to the longevity phenotype. Genetic and pharmacological interventions in the MAPK/ERK signalling pathway have been shown to extend lifespan in different organisms. These studies reported both dampened and elevated MAPK/ERK signalling pathway activity (Bjedov et al., 2010; Slack et al., 2015). We therefore used this read-out, alongside other cellular read-outs such as stress resistance and proteomic and metabolomic signatures, to assess functionality of the introduced variants. Out of the five generated mESC lines, two variants located in the *Raf1* gene (Asp633Tyr) and *Nf1* gene (Phe1112Leu) showed opposing effects on these cellular read-outs.

Elevated MAPK/ERK signalling pathway activity in the *Raf1*^{Asp633Tyr} variant pointed towards improved functioning of the *Raf1* gene, however, proteomic analysis revealed that negative feedback from Erk1 likely results in overall dampened pathway activity in this variant. Reduced pathway activity as a result of treatment with the Mek kinase inhibitor, trametinib, has been shown to extend lifespan in fruit flies (Slack et al., 2015). In MEF cell lines harbouring this variant, MAPK/ERK signalling pathway activity appeared reduced, supporting the hypothesis of negative feedback loops being present. Strikingly, both mESCs and MEFs of this variant had an improved stress response, likely mediated through proteomic and metabolomic rewiring and thus altered MAPK/ERK signalling pathway activity. Elevated stress response mechanisms are known to mediate lifespan and longevity (Harshman et al., 1999; Johnson et al., 2002; Lithgow & Walker, 2002; Murakami, 2006). Even though we were thus far not able to recapitulate these findings *in vivo*, we showed that the reduced expression of *Raf1* is conserved and therefore speculate other phenotypes to become apparent at later time points. Follow-up experiments will show whether these animals indeed present an improved stress response that might contribute to longevity.

Reduced MAPK/ERK signalling pathway activity in the *Nf1*^{Phe1112Leu} variant pointed towards improved functioning of the *Nf1* gene in mESCs, however, proteomic analysis revealed an overall elevated expression of MAPK/ERK related DEPs and no effect on *Nf1* itself. Strikingly, pathway analysis in MEFs of this variant, revealed an upregulation of pathway activity, in line

with the proteomic analysis and pointing towards reduced functioning of *Nf1*. Indeed, when we introduced this variant in the fruit fly, we could show that it reduces lifespan in the homozygous state and had no effect in the heterozygous state. One study reported lifespan extension in the fly by overexpressing *Nf1*. In line with these findings, we conclude the *Nf1*^{Phe1112Leu} variant to result in reduced functioning of *Nf1* in the fly and likely not to have contributed to the longevity phenotype of the original individuals that it was observed in.

The role of the MAPK/ERK signalling pathway in lifespan in different model organisms has been recognized before. We provide further evidence of its role in stress response mechanisms and potentially underlying human longevity. We show that this pathway is regulated in a highly context specific manner and cannot rule out the possibility that the observed functional effects are cell type or organism specific.

4. The effects of Rigosertib treatment on health- and lifespan in *Drosophila melanogaster*

The second project of my PhD focused on the pharmacological manipulation of the MAPK/ERK and PI3K/AKT/MTOR signalling pathway in *Drosophila melanogaster.*
Parts of this study have also served as a Master thesis project for Salim Caglar Avci. I supervised Caglar from January 2021 to March 2022 and have indicated in the figure legends which experiments were conducted by him.

4.1 Introduction

Ageing is until today not officially classified as a disease by health authorities such as the Food and Drug Administration (FDA) and European Medicine Agency (EMA), because of its vast spectrum of manifestations and thus lack of defined endpoints. It is argued by some that this hampers the development of novel drugs of age-related diseases and ageing per se. However, this has opened the door to use already existing and approved drugs to treat age-related diseases. Metformin and rapamycin are examples of potential geroprotective drugs that are currently put to test in model organisms and early human clinical trials (Kulkarni et al., 2020; Nielsen et al., 2022). Simultaneously, the number of potential geroprotective compounds tested in animal models is steadily increasing. Promising starting points for such interventions include modulation of nutrient-sensing pathways and the microbiome and the removal of senescent cells (Partridge et al., 2020). In this Thesis, we tested the potential of RGS treatment, mildly inhibiting the MAPK/ERK and PI3K/AKT/mTOR signalling pathways, as a geroprotective drug in *D.melanogaster*.

RGS has been reported to inhibit Ras signalling through the MAPK/ERK and PI3K/AKT/mTOR signalling branches (**Figure 4.1A**). Preceding experiments, conducted by a former postdoc in the Partridge department, Luke Tain, showed that RGS is well tolerated in flies and extended lifespan in male flies. We performed MS-based analysis of flies fed with RGS to determine to what extent the drug is taken up by female and male flies (**Figure 4.1B**). We were able to show an expected, dose-dependent increase in whole fly lysates with increasing concentrations of the drug. Moreover, it revealed that systemic levels of RGS in female flies were much higher than in males, suggesting a higher food intake in female flies. Sex differences in feeding behaviour of *D.melanogaster* have been described before and are thus likely to explain these findings (Deshpande et al., 2014; Wong et al., 2009). Based on

these Mass-Spectrometry results, we accordingly adjusted the dosing range of RGS in male and female flies in order to find the optimal treatment range in both sexes and determine if the drug also extends lifespan in female flies.

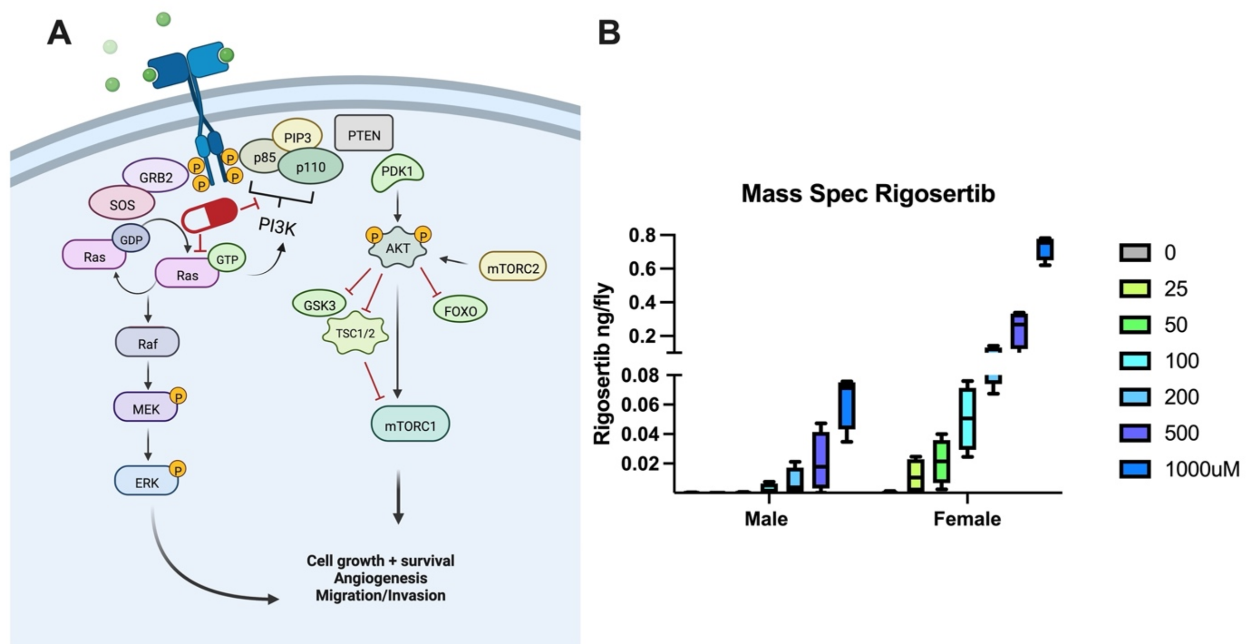


Figure 4.1 | Rigosertib inhibits the MAPK/ERK and PI3K/AKT/mTOR signalling pathways.

(A) RGS allosterically binds to the Ras-binding-domains of PI3K and Raf, thereby inhibiting downstream signalling through the MAPK/ERK and PI3K/AKT/mTOR signalling pathways (**B)** Mass-Spectrometry-based analysis of 10-day old flies revealed that systemic levels of RGS are significantly higher in females than in males ($n_{vials} = 4$, 10 flies per replicate)

4.2 Results

4.2.1 Rigosertib inhibits MAPK/ERK signalling in a dose-dependent manner *in vitro*

We first tested the efficacy of RGS in *Drosophila* Schneider's S2+ cells, which we treated with different doses of the drug. Subsequent western blotting of cell lysates confirmed a dose-dependent decrease in ERK phosphorylation (**Figure 4.2**). We did not obtain quantifiable results of AKT phosphorylation, which is likely due to lacking additional stimulation with growth factors in order to detect AKT phosphorylation with western blotting (Molgaard et al., 2016). We included trametinib as a positive control in this experiment, which proved to be a much more potent inhibitor of the MAPK/ERK signalling pathway than RGS. Physiologically, this is highly relevant as it could imply the occurrence of less side effects and a more modest inhibition of these signalling branches compared to other potential geroprotective drugs.

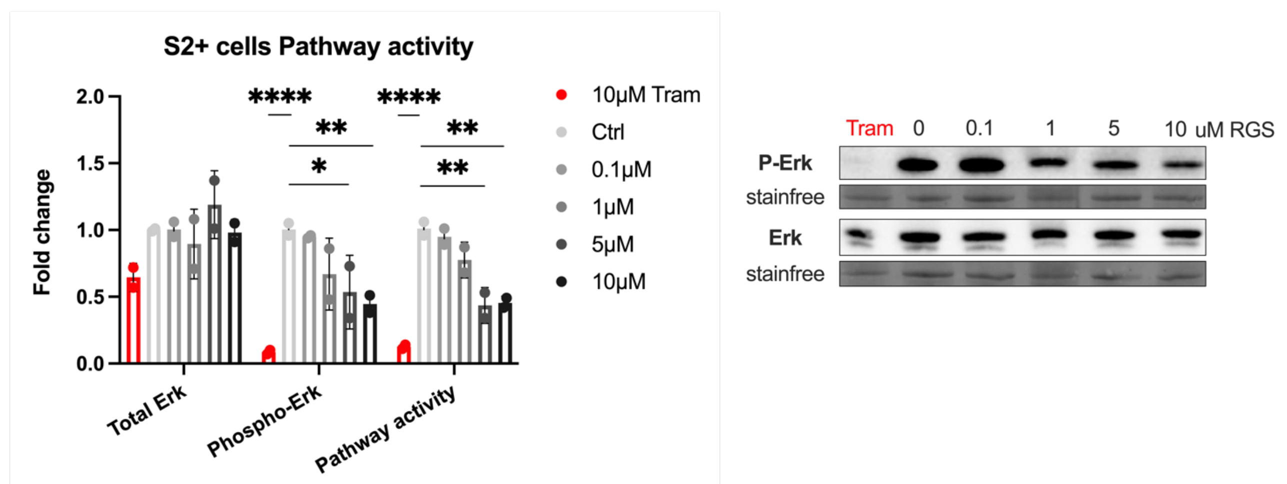


Figure 4.2 | Effect of Rigosertib treatment *in vitro*.
Western blotting of S2+ *Drosophila* cells showed that the MAPK/ERK branch is inhibited in a dose-dependent manner. Trametinib served as a positive control. (data was analysed with 2way ANOVA and Dunnett's multiple comparisons test) $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ *Mass spectrometry analysis was conducted by Luke Tain and the western blot was done by Salim Caglar Avci.*

Orally administered drugs naturally first pass through the gastrointestinal system. Therefore, we repeated this western blot analysis with dissected guts to see whether we could recapitulate the effects of RGS *ex vivo*. Surprisingly, we detected significantly elevated MAPK/ERK signalling pathway activity at 50 µM RGS treatment, and no effect on AKT phosphorylation **(Figure 4.3A-B)**.

These findings confirm differential MAPK/ERK signalling pathway regulation in different organisms, in line with our findings in **Chapter 3** of this study. It is possible that RGS acts in a tissue- and organism-specific manner and that the MAPK/ERK signalling pathway might be subject to negative feedback regulation *in vivo*. Further tissue-specific analysis will answer these questions. Contrary to previous findings that RGS inhibits the PI3K/AKT/mTOR signalling branch, we show that this does not seem to be the case in RGS treated fly guts.

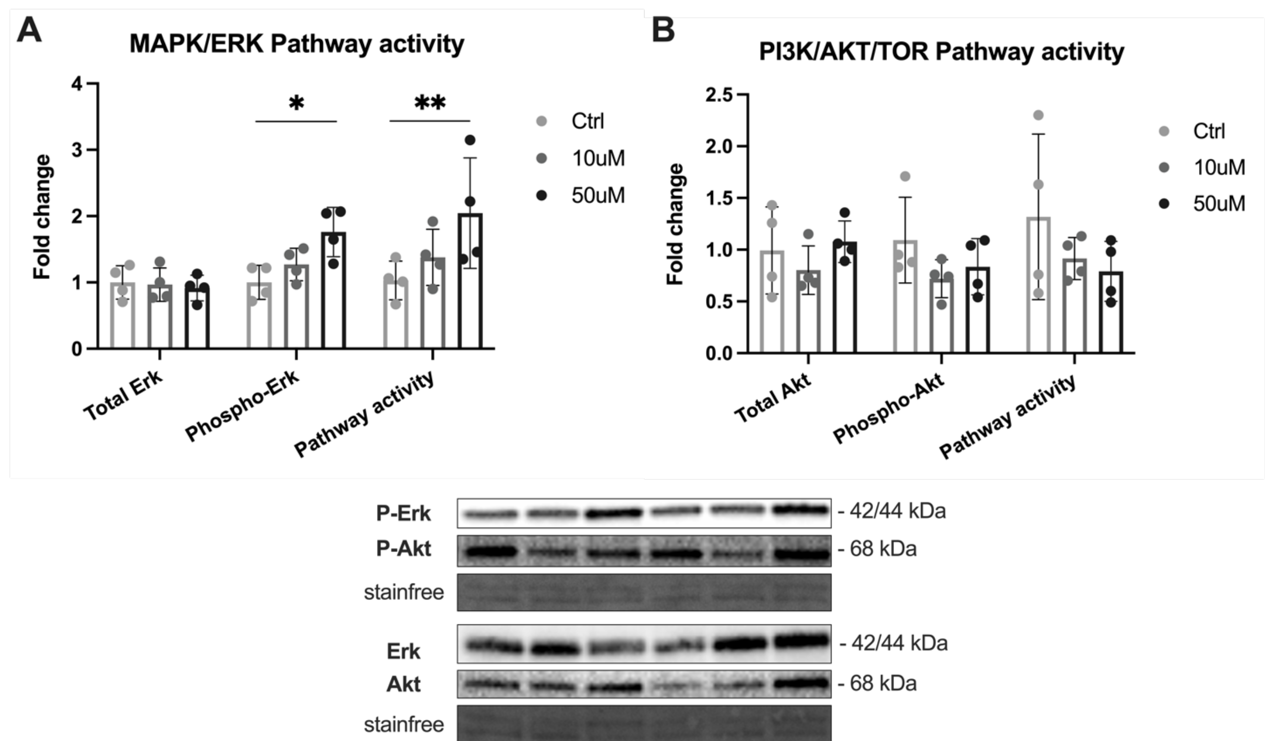


Figure 4.3 | Effect of Rigosertib treatment in fly guts.
(A+B) Western blots analysis of fly guts of 10-day old flies fed 0, 10 and 50 µM RGS show **(A)** significantly upregulated ERK activity at 50 µM **(B)** and no effect on Akt activity (n=4, 12 guts each; data was analysed with 2way ANOVA and Dunnett's multiple comparisons test) $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$

4.2.2 RGS extends lifespan in male and female flies, without affecting feeding behaviour and fecundity

Given the high safety profile of the drug in flies and the initial lifespan extensions observed in male flies, we repeated the lifespan assays with adjusted doses based on the MS findings in male and female flies. For this, we prepared normal 1x SYA food containing different doses of RGS, as indicated in the figure legends.

We could confirm previous lifespan extending effects in male flies and showed that RGS treatment in the range of 50-200 µM significantly increased lifespan. Also in female flies, we were able to show significant lifespan extension when feeding *wDah* flies 5-15 µM of RGS. The average median lifespan extension was 6-12% in three repeated experiments (**Figure 4.4A-D**).

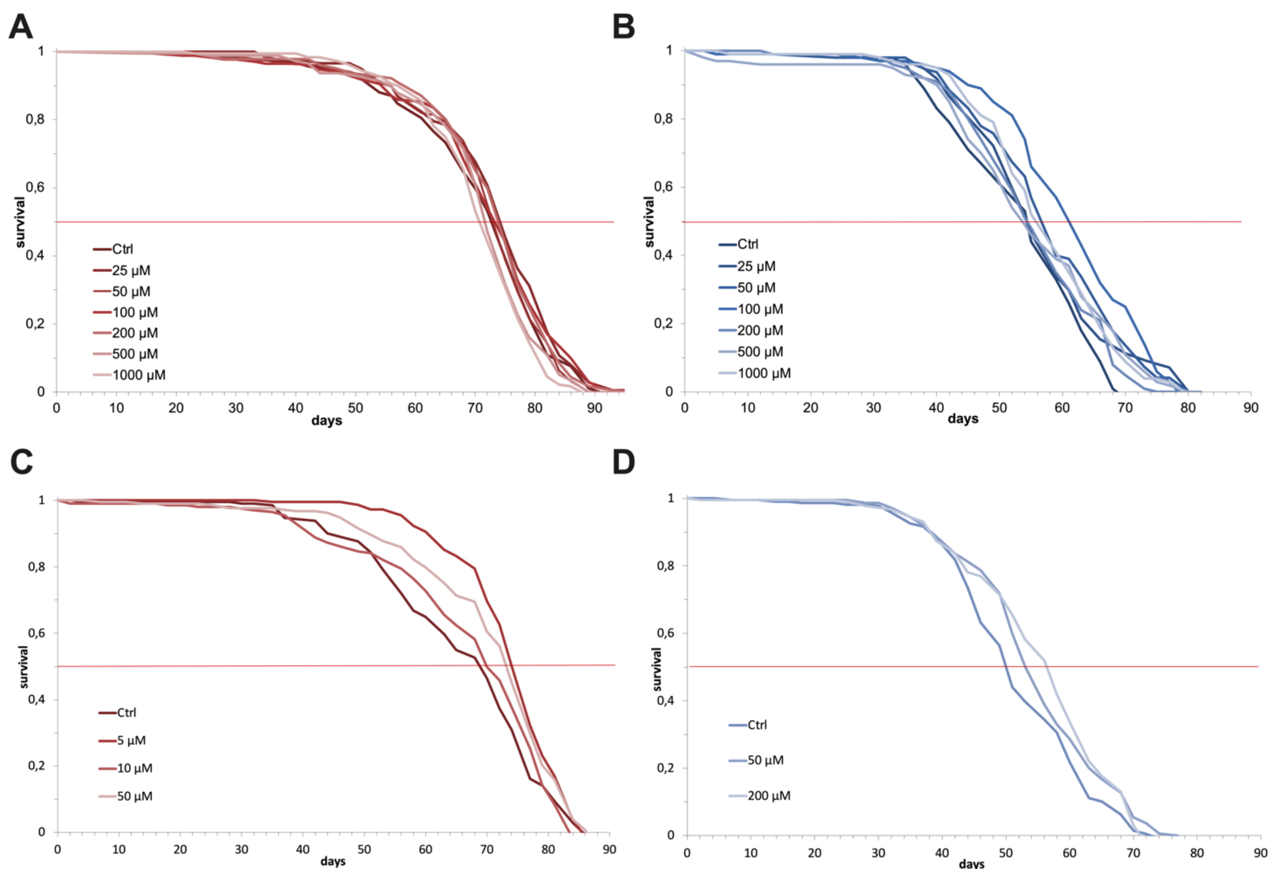


Figure 4.4 | Rigosertib extends lifespan in male and female *Drosophila*.
(A+B) initial lifespan analysis by Luke Tain revealing that 50-200 µM Rigosertib has **(A)** no effect on female flies **(B)** and extends lifespan in male flies. RGS doses until 1000 µM do not cause toxicity in both sexes **(C)** adjusted doses of RGS (5, 10, 50 µM) extend lifespan in female flies (**D**) repeated lifespan analysis in males showed significant lifespan extension in males at 50 and 200 µM (n=160; lifespan data was analysed with log-rank test).

Previous research in *D.melanogaster* has shown that male flies are more affected by systemic inflammation when compared to females, which often leads to the occurrence of “sticky food” in lifespan experiments (Regan et al., 2016). This makes male health- and lifespan analysis highly variable. After having established that RGS treatment extends lifespan in both sexes, we thus decided to perform all follow-up experiments in female flies only.

Dietary restriction (DR) is until today the best studied intervention to extend lifespan and improve metabolic health across species (for review see Green et al., 2022; Partridge et al., 2005). DR is defined as the reduction of caloric intake without malnutrition and laboratory animals this usually entails a 10-50% reduction of *ad libitum* food intake. To rule out a possible DR effect underlying the observed lifespan extension in flies kept on RGS, we performed a feeding assay. In this proboscis-extension assay, we counted the number of flies that are eating in a given time window and could show that RGS did not negatively affect feeding behavior at doses conducive to lifespan extension (**Figure 4.5B**).

Next, we assessed whether the observed lifespan extension comes at the expense of fecundity, which is a commonly observed phenomenon (Kirkwood, 1977). Other drugs that inhibit MEK (trametinib (Slack et al., 2015)) or TORC1 (rapamycin (Bjedov et al., 2010)), have been reported to significantly reduce fecundity in flies. In the case of RGS, we could show that the drug does not decrease fecundity in female flies in the lifespan extending range of 5-15 μM. Only at a dose of 200 μM, we observed a significant reduction in egg laying, suggesting a small effect of the drug, affecting fecundity without reducing lifespan (**Figure 4.5A**). RGS proves to be well tolerated in flies.

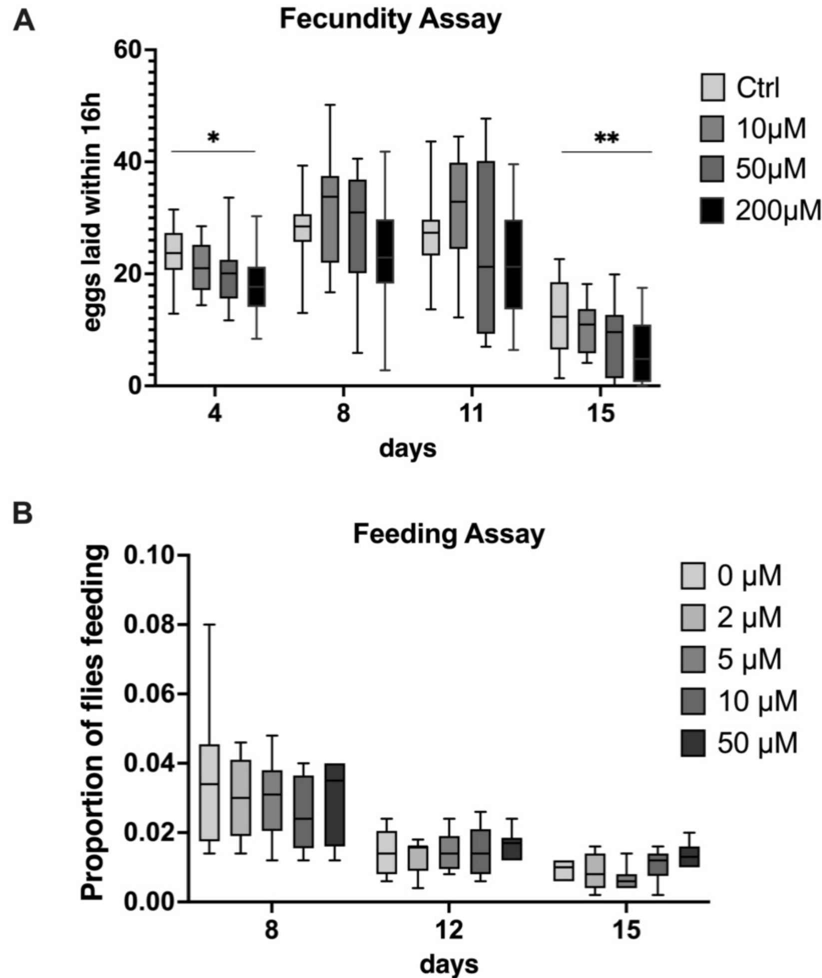


Figure 4.5 | Rigosertib does not affect feeding behaviour and fecundity at the lifespan extending dose.
(A) fecundity analysis, recorded as the number of eggs laid within 24h, showed a significant reduction at 200 µM on day 15 (data was analysed with one-way ANOVA) **(B)** proportion of females feeding, recorded as extending proboscis, showed no differences at three different time points (data was analysed with 2way ANOVA and Turkey's multiple comparisons test) *P < 0.05, **P < 0.01, ***P < 0.001

4.2.3 RGS treatment does not improve stress resistance or climbing ability

Stress resistance has been reported to positively correlate with lifespan and longevity (Deepashree et al., 2019). We hypothesized that one of the mechanisms explaining the lifespan extension through RGS treatment could be due to an improved stress response to oxidative or starvation stress, for instance through improved antioxidant defence mechanisms or lipid metabolism. To test the effect of RGS treatment on stress resistance, we subjected 10-day old flies to oxidative stress, by adding paraquat to RGS containing food. We found that flies fed with RGS did not show an improved resistance to paraquat induced oxidative stress **(Figure 4.6A-B)**. Similarly, we did not observe any beneficial effect of RGS treatment in the response

to starvation stress, by switching ten-day RGS pre-treated flies to only agar containing vials **(Figure 4.6C-D)**. These findings highlight that an improved stress resistance to oxidative stress is likely not causing the observed lifespan extension and that RGS treatment does not improve fat mobilization, which is known to underlie an improved starvation stress response (Beller et al., 2010).

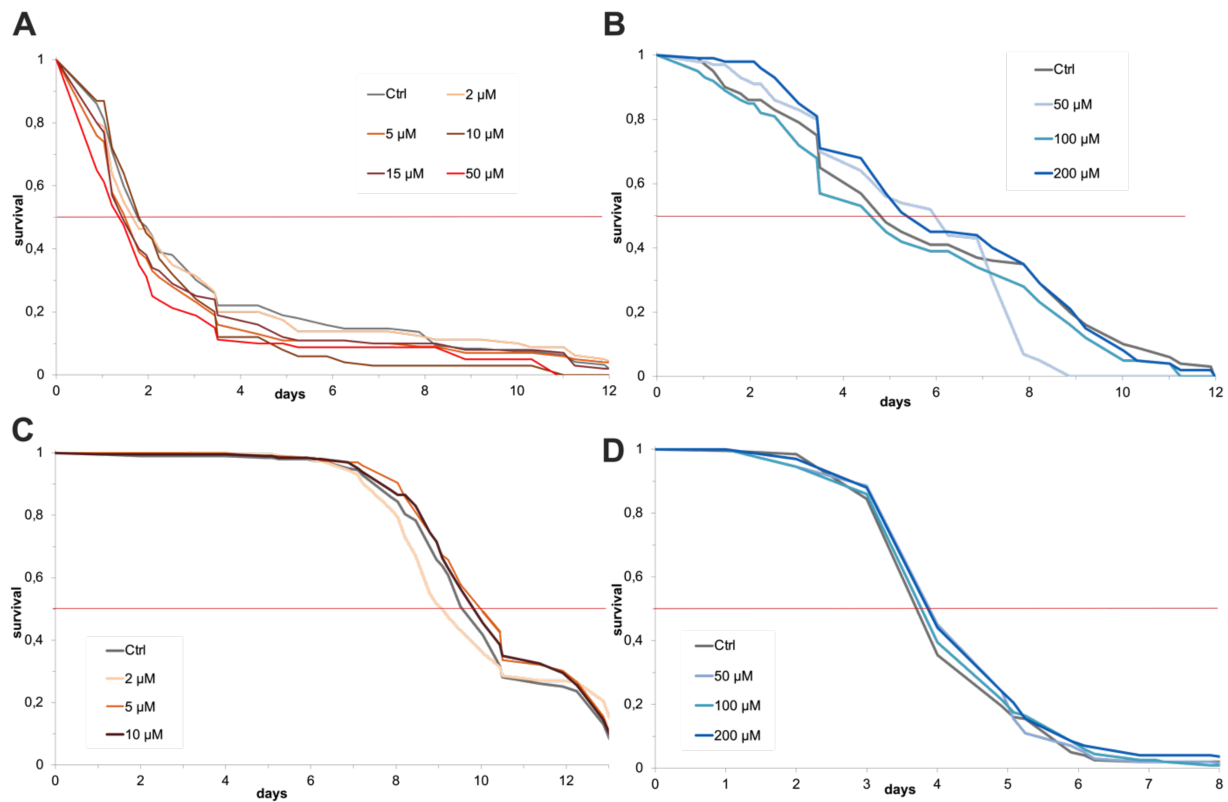


Figure 4.6 | RGS treatment does not improve stress resistance.
(A+B) RGS treated 10-day old **(A)** female flies and **(B)** male flies do not show elevated resistance to oxidative (paraquat) stress **(C+D)** RGS treated 10-day old **(C)** female flies and **(D)** male flies do not show elevated starvation resistance (n=100, data was analysed with log-rank test). *Stress assays were performed by Salim Caglar Avci and Helena Hinterding.*

Next, we were interested to see whether RGS treatment would improve health parameters, such as locomotor function. Locomotor function, measured by vertical climbing ability, is known to deteriorate with increasing age in *Drosophila* (Gargano et al., 2005). For this, we subjected flies kept on varying doses of RGS, to a climbing assay. We did not find significant improvements in terms of locomotor ability over a course of three weeks of continuously RGS treated flies **(Figure 4.7A-B)**. Taken together, these experiments suggest that RGS treatment

does not sustain or improve locomotor ability of flies with advancing age, nor that elevated stress resistance to oxidative and starvation stress contribute to the observed lifespan extension.

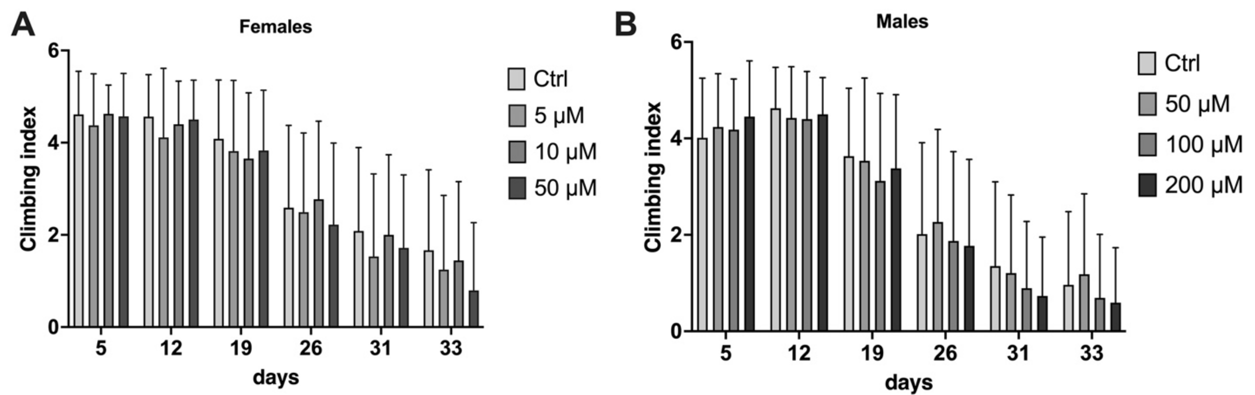


Figure 4.7 | RGS treatment does not improve climbing ability.
(A+B) climbing ability is not improved with increasing age under RGS treatment in **(A)** female and **(B)** male flies (data was analysed with 2way ANOVA and Dunnett's multiple comparisons test) *P < 0.05, **P < 0.01, ***P < 0.001. *This climbing assay was conducted by Salim Caglar Avci.*

4.2.4 RGS treatment improves female gut homeostasis in *D.melanogaster*

In order to answer the question if RGS treatment has a beneficial effect on fly gut health and thus contributes to lifespan extension, we first looked at intestinal stem cell (ISC) proliferation in middle-aged flies. Previous research in fly gut homeostasis and aging has established that ISCs tend to over-proliferate with increasing age, leading to dysplasia (Biteau et al., 2008). Importantly, we only analysed female flies for these experiments, as it is known that male flies lack many of age-related gut phenotypes that occur with increasing age (Regan et al., 2016). To determine ISC proliferation, we dissected 35-day old flies and stained their midguts in the R2 region **(Figure 4.8A-B)** for phosphohistone H3 (PH3+), a marker of chromosome condensation and thus commonly used as a read-out for meiosis and mitosis (Hendzel et al., 1997; van Hooser et al., 1998). Quantitative analysis of positive PH3+ punctae revealed their number to be significantly lower in RGS treated flies compared to untreated control flies **(Figure 4.8B)**. In line with these findings, we wondered if RGS could also reduce the formation of dysplasia, i.e. uncontrolled cell proliferation and tumorigenic phenotypes. These pre-tumorous formations are known to only occur late in life (Ryu et al., 2008). Thus, we dissected

female guts at the age of 65 days, stained the nuclei and imaged them with confocal microscopy to analyse the structure of the gut epithelium. For the quantitative analysis, we measured the proportion of dysplasia in the luminal region and could show that RGS treatment significantly reduces the occurrence of dysplasia in old age **(Figure 4.8C)**.

We used lysotracker to stain acidic autolysosomes in 12-day old flies and could show an increased number of lysotracker stained punctae in RGS treated flies, indicating elevated autophagy in the gut under RGS treatment **(Figure 4.8D)** (Lu et al., 2021). To confirm that autophagy is indeed elevated under RGS treatment, this should be confirmed with Cyto-ID co-staining, specifically labelling early autophagosome formation. The ratio of autophagosomes and autolysosomes can be used to give insights on the autophagic flux. In summary, these data suggest RGS treatment to ameliorate age-related loss in gut homeostasis in *D.melanogaster* through reduced ISC proliferation, dysplasia formation and elevation of autophagy.

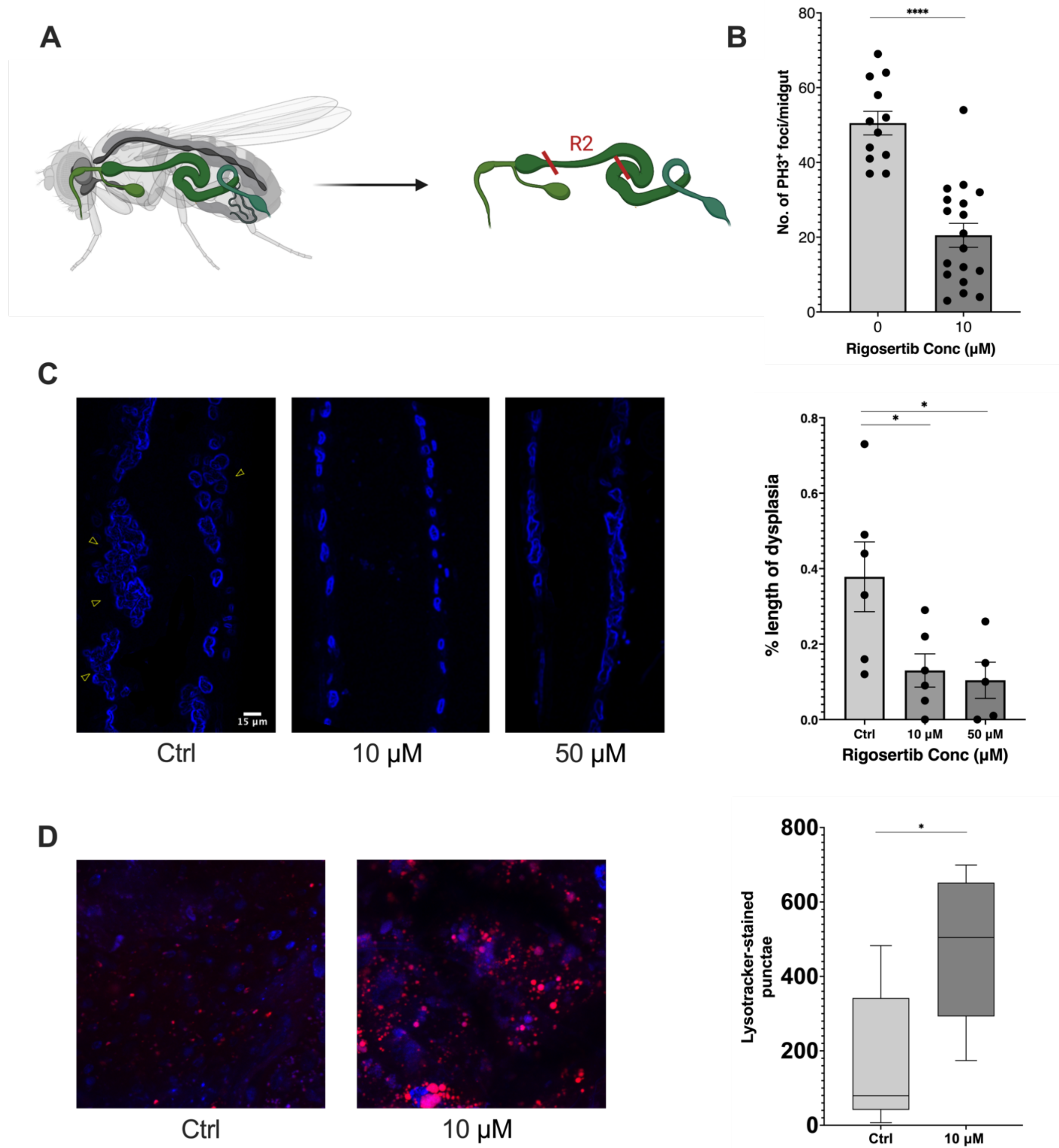


Figure 4.8 | Rigosertib reduces ISC proliferation and dysplasia and elevates autophagy in the female gut.

(A) schematic representation of the *Drosophila* gut. Gut analysis focused on the R2 region **(B)** The number of positive phosphohistone punctae in the R2 gut region was significantly reduced in 50-day old females treated with 10 µM RGS (data was analysed with Student's t-test) **(C)** dysplasia formation in the R2 region (indicated by yellow arrows) in 70-day old females was significantly reduced at 10 and 50 µM Rigosertib treatment (n=6, 3 representative images per gut analysed, data was analysed with 1-way ANOVA) **(D)** autophagy, measured by lysotracker stained punctae, in the R2 region was significantly elevated in 50 µM Rigosertib treated 12-day old females (n=6, data was analysed with Student's t-test) *Gut dissections and stainings have been conducted by Caglar Avci and Helena Hinterding, microscopy imaging was done by Salim Caglar Avci. The graphic representation of the fly gut was created with biorender.com.*

4.3 Discussion

In this study, we tested RGS treatment in *D.melanogaster* as a potential translational geroprotective drug. Previous studies in flies and mice have shown that the Mek kinase inhibitor, trametinib, and the TORC1 inhibitor, rapamycin, extend lifespan (Bjedov et al., 2010). We hypothesised that the novel, dual inhibitor of the MAPK/ERK and PI3K/AKT/MTOR signalling branches, RGS, may also exert health and lifespan extending effects. When we tested the efficacy of the drug *in vitro* and *in vivo*, we observed a dose-dependent inhibition of the MAPK/ERK signalling pathway, while it was upregulated in fly guts. Our analysis did not confirm the previously reported inhibition of the PI3K/AKT/mTOR branch. While drugs like trametinib are described to extend lifespan in flies through the inhibition of the MAPK/ERK signalling pathway (Slack et al., 2015), one study recently reported elevated pathway activity in the context of extended lifespan as a result of red ginseng treatment in flies (Hou et al., 2020). In line with our findings, this suggests that a mild elevation of MAPK/ERK pathway activity *in vivo* may have beneficial effects for the organism. Further, we show that the MAPK/ERK signalling pathway is differentially regulated in different cells and/or organisms. A more detailed and tissue-specific analysis is needed to understand its regulation *in vivo*. In order to decipher the mechanistics of RGS treatment in the observed lifespan extension in flies, we analysed gut health in aged, RGS treated flies. Age-related decline in gut health has been shown to be ameliorated through rapamycin and trametinib treatment (Fan et al., 2015; Regan et al., 2018). In a similar manner, we showed that gut homeostasis was maintained under RGS treatment in female flies. We conclude that maintained gut health likely contributes to extended lifespan in these flies, however, due to lifespan extension in both sexes, it does not seem to be the only underlying factor. A more extensive and tissue-specific analysis is needed to determine the mechanism of action of RGS treatment in flies. Importantly, this analysis should be expanded to different organisms to reveal whether these effects are conserved and thus may drive RGS forward as a potential geroprotective drug candidate.

5. General Discussion

The MAPK/ERK signalling pathway has been implicated in modulating lifespan in various organisms, including yeast, worms, flies and mice (Borrás et al., 2011; Fabrizio et al., 2003; Okuyama et al., 2010; Slack et al., 2015). Its role in human lifespan and longevity, however, remains unclear. Studies in the field of ageing research have showed that there is a genetic component to human longevity and that this heritability can partially be explained by rare genetic variants (Lin et al., 2021; Melzer et al., 2020). We hypothesised that rare genetic variants in the MAPK/ERK signalling pathway may contribute to the longevity phenotype of some individuals and thus applied a novel approach to functionally characterise variants identified in the longevity-enriched LLS cohort.

In the first part of this thesis (**Chapter 3**), we investigated the role of rare genetic variation in the MAPK/ERK signalling pathway on human longevity, through the functional characterisation of human genetic variants in cells, fruit flies and mice. To this end, we generated mESC lines harbouring the identified variants and assessed their functional effects on MAPK/ERK signalling pathway activity and stress resistance. We showed that two variants, located in the *RAF1* and *NF1* genes, altered pathway activity of the MAPK/ERK signalling pathway in opposing directions. Both variants led to extensive proteomic and metabolomic rewiring, while enrichment analysis showed a significant downregulation of metabolic pathways for the variant located in the *RAF1* gene. Additionally, we found an improved response to ER and oxidative stress for this variant. Follow-up experiments of the variant in *RAF1* in MEFs showed that this stress resistance phenotype was also present in these cells, suggesting that this variant might contribute to longevity through enhanced stress response mechanisms and elevated MAPK/ERK signalling. For the variant located in the *Nf1* gene, we were able to additionally generate fruit flies carrying this variant. We found no effect on lifespan in the heterozygous state in these flies, while it reduced lifespan in the homozygous state, suggesting that this variant may not have contributed to the longevity phenotype of the long-lived individuals carrying it.

In the second part of the thesis (**Chapter 4**), we investigated the role of RGS treatment on life- and healthspan in fruit flies as a potential novel geroprotective drug candidate. RGS is described as a dual inhibitor of the MAPK/ERK and PI3K/AKT/mTOR signalling pathways and is being tested in preclinical and early clinical trials as a tumour inhibitor. The main aim

of this project was to determine if, and at what doses, RGS extends lifespan and whether the effects are conserved in both sexes. We were able to show that RGS treatment significantly increased lifespan in both female and male flies without negatively affecting feeding behaviour and fecundity. We subsequently investigated the underlying mechanism of action and possible tissue targets. While MAPK/ERK signalling was inhibited *in vitro,* we showed that it was upregulated in the fly gut. Additionally, we showed that gut homeostasis was improved in old female flies treated with RGS, suggesting that elevated MAPK/ERK activity likely contributes to maintained gut health and increased lifespan in these flies.

5.1 The role of the MAPK/ERK signalling pathway in life- and healthspan *in vivo*

The MAPK/ERK signalling pathway is best known for its role in cancer. An estimated 10% of all human cancer patients carry mutations in the MAPK/ERK signalling pathway, most commonly occurring in *RAS (K-RAS)* and *RAF (B-RAF)* genes (Maik-Rachline et al., 2019; Sanchez-Vega et al., 2018). Driver mutations in MAPK/ERK related genes lead to hyperactivation of this survival signalling cascade and thus contribute to uncontrolled tumour proliferation and metastasis formation. More recently, its role in the regulation of lifespan in different organisms has been established, following the findings that reduced IIS extends lifespan across species. In this context, genetic modulations of the MAPK/ERK signalling pathway have been shown to extend lifespan of various species. In yeast, deletion of RAS2 resulted in doubled mean lifespan (Fabrizio et al., 2003). In worms, the knockout of *lin-45* (mammalian orthologue of *RAF1*) has been shown to shorten lifespan (Okuyama et al., 2010). In flies, expression of dominant negative *Ras* and overexpression of the *Nf1* gene, have also been shown to extend lifespan (Slack et al., 2015; Tong et al., 2007). Finally, in mice, deletion of *RasGrf1* was shown to extend lifespan and result in improved health parameters (Borrás et al., 2011). To decipher the role of the MAPK/ERK signalling pathway in human longevity, we generated flies and mice harbouring the human variants in the *RAF1* and *NF1* gene, to assess their effect on lifespan. When we introduced the $Nf1^{\mathrm{Phe1148Leu}}$ variant in the fruit fly, we could show that flies that carry the variant in a homozygous state have a reduced lifespan in both sexes, while heterozygous flies show no difference in comparison to wildtype flies. These findings suggest that this variant in the *Nf1* gene did likely not contribute to the longevity

phenotype of the long-lived individuals in which it was identified. As we could not generate flies harbouring the *Raf1*^{Asp633Tyr} variant, we opted for the generation of mice and have recently started a lifespan experiment that entails both sexes and heterozygous and homozygous animals. At this point, i.e. after one year of follow-up, neither detrimental nor beneficial phenotypes have been observed. Hence, we cannot yet draw any conclusions regarding the *in vivo* effects of this variant.

We also tested the effect of RGS treatment on life- and healthspan in the fly and could show that the drug extends lifespan in both sexes (mean lifespan extension of 6-12 %). These effects on lifespan are comparable to similar studies conducted in flies with the MEK kinase inhibitor trametinib and TOR inhibitor rapamycin, that yielded a medium lifespan extension of ~ 9-14.7 % (Bjedov et al., 2010; Slack et al., 2015). At high doses, trametinib treatment resulted in early-life mortality in flies (Slack et al., 2015). We observed no toxicity at high doses in RGS treated flies, speaking for a high safety profile of the drug, which has also been reported in early clinical trials of RGS treatment in humans (Silverman et al., 2015). Maintained gut homeostasis is known to be an important mediator of health, disease and lifespan (Fan et al., 2018). We showed that RGS treatment ameliorated age-related decline in gut homeostasis in female flies, i.e. reduced ISC over-proliferation, reduced dysplasia and elevated autophagy in the gut, which is likely to have contributed to the observed lifespan extension. These gut-related phenotypes are comparable to previous findings in rapamycin and trametinib treated flies. Rapamycin treatment has previously been shown to reduce gut dysplasia and to increase autophagy (Bjedov et al., 2010; Fan et al., 2015; Regan et al., 2018). Similarly, trametinib treatment in flies has been shown to repress intestinal stem cell division and maintain gut barrier function, contributing to overall sustained gut homeostasis (Regan et al., 2018). In contrast to female flies, male flies, are known to show no or only minor gut dysfunction with increasing age (Regan et al., 2016). The fact that we observed lifespan extension in both female and male RGS treated flies thus implies that the gut is not the sole mediator. Follow-up experiments, i.e. through genetic epistasis experiments using constitutively active and null mutants of downstream targets of the MAPK/ERK and PI3K/AKT/mTOR signalling pathways, such as FoxO, S6K and anterior open (AoP) are needed to determine the exact mechanism of action of RGS treatment in *D.melanogaster*. Tissue specific analysis will answer the question which other tissues might contribute to the lifespan extension.

5.2 The role of MAPK/ERK signalling pathway activity

Although the role of the MAPK/ERK signalling pathway has been well established in lifespan, the exact underlying mechanisms and the role of individual proteins, such as the ERK kinases, remains largely unclear and appears contradictory. The ratio of phosphorylated ERK over total ERK is commonly described in the literature as a read-out for MAPK/ERK signalling pathway activity (Martinez-Lopez et al., 2013; Masaki et al., 2003; Mitic et al., 2015). On the one hand, reduced ERK signalling activity by means of treatment with the MEK kinase inhibitor trametinib results in lifespan extension in flies (Slack et al., 2015). On the other hand, elevated ERK signalling activity has been reported in long-lived dwarf Snell mice and caloric restricted mice (Ikeyama et al., 2002; Madsen et al., 2004). In rats, both age-related decline (Hutter et al., 2000; Y. Liu et al., 1996; Zhen et al., 1999) and age-related incline in MAPK/ERK signalling pathway activity have been reported in *ad libitum* fed animals (H. J. Kim et al., 2002). Interestingly, this was not the case in caloric restricted rodents. In rapamycin treated mice, elevated ERK 1/2 transcripts have been found (Fok et al., 2014). Moreover, in human lung and dermal fibroblasts, elevated ERK signalling activity was shown to extend replicative lifespan (Tresini et al., 2007). In general, MAPK/ERK signalling kinetics have been reported to be distinct in longer- and shorter-lived mammals and birds, as shown by Elbourkadi et al. (2014) who found that fibroblasts of longer-lived species displayed delayed but prolonged ERK activation in response to oxidative stressors. Taken together, these findings suggest a variable regulation of the MAPK/ERK signalling pathway that is highly context and organism specific.

In this thesis, we used this read-out to assess functionality of the genetic variants observed in long-lived individuals **(Chapter 3)** and RGS treatment in cells and flies **(Chapter 4)**. Intriguingly, we found the *Raf*1$^{\mathrm{Asp633Tyr}}$ and *Nf1*$^{\mathrm{Phe1112Leu}}$ variants to alter MAPK/ERK signalling pathway activity in opposing directions in mESCs, while the *Rras*$^{\mathrm{Glu118Lys}}$ and *Rps6ka4*$^{\mathrm{Ala394Val}}$ variants had no effect. In MEFs, the *Raf1*$^{\mathrm{Asp633Tyr}}$ and *Nf1*$^{\mathrm{Phe1112Leu}}$ variants showed functional effects on pathway activity, but opposite to the results in mESCs. In mouse tissues of the *Raf*1$^{\mathrm{Asp633Tyr}}$ and *Nf1*$^{\mathrm{Phe1112Leu}}$ variants analysed in this study, we observed no effect on pathway activity at the age of three months. This could, however, be explained by tissue specificity and hence the wrong tissues analysed and functional effects *in vivo* only becoming prominent at a later point in life. We also did not observe functional effects on pathway activity in flies harbouring the *Nf1*$^{\mathrm{Phe1148Leu}}$ variant.

While RGS is described as a dual inhibitor of the MAPK/ERK and PI3K/AKT/mTOR signalling pathways, we could confirm MAPK/ERK signalling inhibition *in vitro* and found, somewhat surprisingly, elevated MAPK/ERK signalling activity *in vivo*. Although the exact mechanism of action of RGS remains elusive, modulations of the MAPK/ERK, and possibly also the PI3K/AKT/mTOR signalling pathway, appear to underlie the observed lifespan extension. Recently, a study of red ginseng treatment in fruit flies reported elevated levels of MAPK/ERK signalling pathway activity and downregulated AKT activity, resulting in increased lifespan (Hou et al., 2020), which at least partially supports our *in vivo* findings of RGS treatment. Our results in both chapters of this thesis are in line with previously reported findings in the literature regarding MAPK/ERK signalling pathway activity and its differential regulation *in vitro* and *in vivo*. Depending on the environment, i.e. cell type, tissue and organisms, it appears that MAPK/ERK signalling pathway activity is highly variable and can have both detrimental and beneficial effects on lifespan. Therefore, we conclude that MAPK/ERK signalling pathway activity alone cannot be used as a direct read-out for improved life- and healthspan, but rather as a first functionality assessment of genetic and pharmacological interventions.

5.2.1 MAPK/ERK signalling pathway activity in the Raf1^{Asp633Tyr} variant *in vitro*

RAF1, also called C-RAF, belongs to a family of serine/threonine-kinases, together with A-RAF and B-RAF. The well-conserved RAF kinase family is best known for its role in signal transduction in the MAPK/ERK signalling pathway. All three family members share a catalytic kinase domain at the C-terminus of the protein and a regulatory domain, consisting of a cysteine-rich and Ras-binding domain towards the N-terminus (Maurer et al., 2011). Raf kinases are activated by Ras proteins through homo- and heterodimerization (Garnett et al., 2005; Rushworth et al., 2006). RAF1 is known to do most crosstalk to neighbouring pathways, inhibiting apoptosis through negative regulation of ASK-1 and MST-2, and inhibiting proliferation and angiogenesis through Rb. RAF1 has also been described to activate the nuclear transcription factor-kB, an important regulator of survival, inflammatory- and immune-related functions. B-RAF is the main activator of downstream located MEK and ERK proteins (Reuter et al., 1995), which explains its prominent role in tumour formation. Strikingly, the introduction of oncogenic *B-RAF* mutations in *RAF1*, were unable to turn *RAF1* into an oncogene because of its low intrinsic kinase activity (Emuss et al., 2005). While mutations in RAF1 are rare, overexpression of the full-length protein has been shown to increase proliferation both *in vitro* and *in vivo* (Niault & Baccarini, 2010). Its overexpression has been

reported in a variety of human cancers, such as hepatocellular carcinoma (Rivaet al.,1995; Hwanget et al., 2004, Blasco et al., 2011) and is used as an early biomarker for lung adenocarcinoma (Cekanova et al., 2007). In contrast, another study reported *RAF1* as a tumour suppressor in hepatocarcinogenesis and showed that *RAF1* ablation resulted in increased proliferation in human cancer cells. Moreover, the researchers found reduced ERK activation in these cells.

Genetic studies in mice have further elucidated distinct roles of the three ubiquitously expressed Raf kinase family members: *A-Raf* knockout mice are born, but die within the first three weeks of life due to neurological and gastrointestinal defects. *B-Raf* and *Raf1* knockout mice die in utero between day 10.5 and 12.5 due to vascular and neuronal defects and liver apoptosis, highlighting their essential roles during development. ERK activation was found unimpaired in fibroblasts of these *Raf1* deficient mice, suggesting MEK independent and dependent activation of ERK through *Raf1* (Hüser et al., 2001; Mercer et al., 2005b; Mikula et al., 2001; Yamaguchi et al., 2004). This is also highlighted in a double knockout study of *Raf1* and *A-Raf*, in which no significant change in ERK activation was reported (Mercer et al., 2005). Taken together, these findings in *Raf1* mutant cells suggest underlying compensatory mechanisms by other members of the Raf kinase family and possibly other genes, given the crosstalk to other signalling branches.

The complexity of MAPK/ERK signalling activity is also illustrated in the human context, in which gain-of-function mutations in *RAF1* and resulting elevated pathway activity have been reported to cause LEOPARD and Noonan syndrome, severe genetic diseases that cause growth retardation and cardiological defects (Pandit et al., 2007; Razzaque et al., 2007). Recently, a novel *RAF1* loss-of-function mutation, resulting in reduced pathway activity, has been reported to lead to a lethal progeroid syndrome (Wong et al., 2022). In this study, we found MAPK/ERK signalling pathway activity significantly upregulated in *Raf1*$^{\mathrm{Asp633Tyr}}$ mESCs and downregulated in MEFs. Given the incoherence of previously reported MAPK/ERK signalling pathway activity and RAF1 gain- or loss-of-function mutations, our findings do not enable us to draw a definite conclusion regarding the effect of the *Raf1*$^{\mathrm{Asp633Tyr}}$ variant on the functioning, i.e. improved or decreased, of RAF1.

5.2.2 MAPK/ERK signalling pathway activity *in vitro* in the Nf1^{Phe1112Leu} variant *in vitro*

Neurofibromin, encoded by the NF1 gene, is a well-studied, large protein that consists of 2818 amino acids in humans. It is a GTPase-activating-protein (GAP) that hydrolyses Ras-bound guanosine triphosphate (GTP) to guanosine diphosphate (GDP), thereby negatively regulating the MAPK/ERK signalling pathway. Because of its inhibitory role, it is also called a tumour-suppressor: when its function is compromised, Ras becomes hyperactive. Structurally, it contains multiple functional domains, such as a cysteine-serine-rich domain, a tubulin-binding-domain (TBD) and a central GTPase-activating protein-related domain, highlighting its role in diverse cellular functions. The cellular functions of Nf1 include neuronal differentiation, membrane localization and cell signalling among others (Ratner & Miller, 2015). Both the NF1 *Ala1127Val* and *Phe1112Leu* variants are localised in the TBD, which consists of amphipathic alpha-helices, supporting neurofibromin dimerization. The role of this domain remains largely unknown, but it is assumed to bind tubulins and thus regulate cell division (Sherekar et al., 2020).

Knockout mouse models have demonstrated the indispensable role of NF1 *in vivo*, showing that these mice die in utero between day 11.5 and 13.5 due to cardiac defects and exencephaly (Brannan et al., 1994; Gutmann & Giovannini, 2002). NF1 deficient cells display elevated MAPK/ERK signalling pathway activity (Sanchez-Ortiz et al., 2014). Studies in the fruit fly have also revealed a role of Nf1 in social behaviour, learning and normal circadian functioning (Georganta et al., 2021; King et al., 2020; Moscato et al., 2020; Williams et al., 2001). Social behaviour deficits, as a result of elevated MAPK/ERK signalling pathway activity, could be recapitulated in a mouse model and ameliorated through pharmacological MEK inhibition (Borrie et al., 2021). However, another study that aimed to study human *NF1* missense mutations in a mouse model, failed to recapitulate any of the phenotypes observed in humans and reported that functional consequences of these missense mutations were highly dependent on the genetic background (Li et al., 2016). In humans, *NF1* is best known for its role in neurofibromatosis type 1, a genetic disorder that affects roughly 1 in 3000 persons worldwide. Neurofibromatosis type 1 is characterised by mostly benign peripheral nerve sheath tumours and optic pathway glioma. Depending on the severity of the disease, these tumours may degenerate and elevate the risk for development of brain, blood and skin cancers, which in turn reduces the life expectancy of these patients. NF1 and its role in RASopathies, diseases characterised by elevated MAPK/ERK signalling, has already been described as early as in 1882 by Frederick von Recklinghausen, which is why neurofibromatosis type 1 is also known

as ‘Von Recklinghausen’s disease’ (Crump, 1981; Wilkins & Brody, 1971). Since then, more than 1400 human mutations have been identified in this gene, most of which are loss-of-function germline mutations (Xu et al., 1992; Legius et al., 1993; Shannon et al., 1994) and thus result in increased MAPK/ERK signalling pathway activity. No gain-of-function mutations have been described in the literature. In this thesis, the observed reduction in MAPK/ERK signalling pathway activity in the $Nf1^{Phe1112Leu}$ variant, suggests improved functioning of NF1 in mESCs and stands in contrast to disease-associated loss-of-function mutations. In MEFs, elevated MAPK/ERK signalling pathway activity suggests reduced functioning of NF1 in these cells. We did not observe any effects on pathway activity in flies and mice harbouring this variant.

5.3 Proteomic and metabolic signatures of the Raf1^{Asp633Tyr} and Nf1^{Phe1112Leu} variants in the MAPK/ERK signalling pathway

Proteins and metabolites are highly adaptive and offer the closest currently possible snapshot of physiological functioning and are therefore used as biomarkers in a range of (age-related) pathologies. The role of proteomic and metabolomic homeostasis in physiological functioning and ageing is also reflected in the ‘hallmarks of ageing’, underlying loss of proteostasis, altered intercellular communication and deregulated nutrient sensing. Recent efforts have compiled the current understanding of the changing human proteome with age and pathway enrichment analysis brought IIS, EGF/EGFR and MAPK/ERK signalling forward among others (Johnson et al., 2020; Moaddel et al., 2021). It has also become evident that age-related changes of the proteome and metabolome and their effects on lifespan are highly variable, depending on the organism, tissue and organ. For instance, insulin-like growth factor 1 (IGF1), which activates both the PI3K/AKT/mTOR and MAPK/ERK branches of the IIS, has been consistently shown to extend lifespan when downregulated in different organisms (Wrigley et al., 2017). In humans, however, IGF1 levels correlate negatively with ageing and higher levels are associated with maintained muscular fitness and overall reduced mortality (Andreassen et al., 2009; Rahmani et al., 2022). In plasma and skeletal muscle of elderly, ERK1 and 2 levels have been found to be elevated (Moaddel et al., 2021). In this thesis, we reported significantly altered proteome structure in the $Raf1^{Asp633Tyr}$ and $Nf1^{Phe1112Leu}$ mESC lines. While both cell lines showed some overlapping proteomic changes, predominantly illustrated by elevated

expression of the upstream located EGFR receptor and Grb2 adaptor proteins, downstream DEPs differed between the two cell lines. Upregulated EGFR expression is generally described in the context of different cancers (Holdman et al., 2015; Zhang et al., 2013).It is well known that the MAPK/ERK pathway is subject to a range of negative feedback loops and to stand in close contact to other branches of the IIS network (Lake, Corrêa & Müller, 2016; Sturm et al., 2010; Mendoza, Er & Blenis, 2011; Fey et al., 2012). Generally, proteomic analysis revealed more downregulated proteins in the *Raf1*^{Asp633Tyr} variant (Raf1, Mek1, Mek2, Erk2, Rras) and more upregulated proteins in the *Nf1*^{Phe1112Leu} variant (Ras family, Mek1 and ribosomal S6 kinases). These results stand in contrast to the functional effects on MAPK/ERK signalling pathway activity in mESCs and thus strongly suggest negative feedback loops to explain these contradictory findings.

Pathway enrichment analysis revealed that oxidative phosphorylation and metabolic pathways including glycolysis are downregulated in the *Raf1*^{Asp633Tyr} variant. Cancerous cells are known to induce a metabolic shift, mostly characterized by the downregulation of oxidative phosphorylation and favouring of aerobic glycolysis (Frederick et al., 2020; Zheng, 2012), described by the 'Warburg effect' (Warburg, 1925). The 'Warburg effect' is characterised by cancer cells favouring the less-efficient glycolysis and decreasing oxidative phosphorylation to maintain proliferation despite normally functioning mitochondria and oxygen supply. The proteomic and metabolic signatures of the *Raf1*^{Asp633Tyr} and *Nf1*^{Phe1112Leu} variants in this study do not mirror such tumour-promoting metabolic changes and we did not observe abnormalities in proliferation of these cell lines, which is why we rule the possibility out that these variants are cancerous. Mild inhibition of oxidative phosphorylation is known to extend lifespan in worms, flies and mice (Copeland et al., 2009; Lapointe & Hekimi, 2008; S. J. Lee et al., 2010). Recently, it was shown in *C.elegans* that lifespan could be extended through simultaneous inhibition of mitochondrial respiration and phosphorylation dependent activation of AMPK, through overexpression of a highly conserved vaccinia virus-related kinase (Park et al., 2020). AMPK is a major nutrient sensor in the IIS network, known to do extensive crosstalk with the MAPK/ERK signalling pathway (Yuan et al., 2020). Given the functional similarity and close proximity of these kinases, these findings offer the possibility that our findings, i.e. elevated MAPK/ERK signalling pathway activity and downregulation of oxidative phosphorylation in the *Raf1*^{Asp633Tyr} variant, mirror these lifespan extending phenotypes in the worm. Following the discovery that DR extends lifespan in a robust and highly conserved manner, many follow-up studies aimed to determine the underlying mechanisms through micro- and macronutrient

diet composition analyses. Since then, it has become evident that protein restriction alone is sufficient to mediate many of the lifespan extending effects (Pamplona & Barja, 2006) and varying ratios of protein and carbohydrate restriction have resulted in different effects on lifespan and fecundity in fly and mouse models (Kwang et al., 2008; Mirzaei et al., 2016; Solon-Biet et al., 2014). Moreover, both restriction and supplementation of micronutrients, such as amino acids, have been shown to extend lifespan in different organisms. In *C.elegans,* the addition of all amino acids except phenylalanine and aspartate extended lifespan, while glucose supplementation diminished those effects, highlighting the importance of the metabolic context (Edwards et al., 2015). Contrarily, dietary restriction of the amino acid methionine has been shown to robustly extend lifespan in worms, flies, mice and rats (Cabreiro et al., 2013; Miller et al., 2005; Orentreich et al., 1993; Troen et al., 2007).

We performed targeted metabolomic analysis in mESCs and found a significantly different metabolomic profile of the *Raf1*$^{\text{Asp633Tyr}}$ and *Nf1*$^{\text{Phe1112Leu}}$ variant in comparison to wildtype cells. TCA cycle metabolites and amino acids were predominantly downregulated in both cell lines. In the *Raf1*$^{\text{Asp633Tyr}}$ cell line, we found SAH as the only significantly upregulated amino acid. Cystathionine was strongly reduced in the *Raf1*$^{\text{Asp633Tyr}}$ cell line and upregulated in the *Nf1*$^{\text{Phe1112Leu}}$ cell line. Both SAH and cystathionine belong to the transsulfuration pathway, which metabolises cysteine and homocysteine through the intermediate cystathionine (Steegborn et al., 1999). Recently, it has been shown that SAH supplementation in *C.elegans* extends lifespan through mimicking methionine restriction (Ogawa et al., 2022), highlighting a beneficial effect of elevated SAH levels. In cell and mouse models, cystathionine alone has been shown to alleviate damage caused by the ER stressor, tunicamycin (Maclean et al., 2012). The role of cystathionine metabolism and lifespan has also recently been highlighted in *D.melanogaster*, in which deletion of cystathionine-β-synthase decreased lifespan and deletion of cystathionine-γ-lyase increased lifespan (Shaposhnikov et al., 2022). Further analysis of the exact role of these metabolites is needed, but our results suggest metabolic rewiring of the transsulfuration pathway in both variants, which may at least partially explain the longevity phenotype observed in the long-lived individuals harbouring them.

5.4 Improved stress resistance in the Raf1^{Asp633Tyr} variant

Improved stress resistance has been linked to lifespan and longevity in different cell types and model organisms (Harshman et al., 1999; Johnson et al., 2002; Lithgow & Walker, 2002; Murakami, 2006). Stress resistance is known to decrease with age, likely because of an impaired ability to activate stress response pathways, including the MAPK/ERK signalling pathway (Bansal et al., 2015; Dues et al., 2016).

Extended lifespan as a result of metabolic rewiring in the transsulfuration pathway has been shown to be accompanied by increased oxidative stress resistance (Shaposhnikov et al., 2022). Also, in the long-lived Ames dwarf mice, enhanced functioning of the transsulfuration pathway with an increased flux of methionine has been reported as an underlying cause of their improved oxidative stress resistance (Brown-Borg et al., 2001; Brown-Borg et al., 2005; Uthus & Brown-Borg, 2006). Also in humans, a recent study reported a unique metabolic profile in long-lived individuals, characterised by upregulation of the methionine transsulfuration pathway and reduced content of specific amino acids (Mota-Martorell et al., 2021). Oxidative stress occurs due to an imbalance between the production and degradation of reactive oxygen species (ROS), which are generated as by-products of metabolic processes (Pizzino et al., 2017). The homeostasis of ROS generation and removal is also referred to as redox balance and disturbances in it are known to underlie many (age-related) pathologies (Franco & Vargas, 2018). Elevated ROS levels are also known to underlie different 'hallmarks of ageing', such as cellular senescence and mitochondrial dysfunction (van der Rijt et al., 2020). In the long-lived *Rasgrf1* knockout mice, the researchers reported reduced levels of oxidative stress and that their overall metabolic profile resembled that of dietary restricted mice (Borrás et al., 2011). In rat hepatocytes, it was shown that old cells responded worse to H2O2 induced oxidative stress than young cells and that pharmacological inhibition of the MAPK/ERK and PI3K/AKT/MTOR signalling pathways sensitised these cells to oxidant injury and cell death (Ikeyama et al., 2002). In this Thesis, we showed an elevated stress response in mESCs and MEFs harbouring the *Raf1*^{Asp633Tyr} variant. Our findings suggest that modulations in MAPK/ERK signalling pathway activity in mESCs and MEFs promote an elevated response to different stressors. Future work in *Raf1*^{Asp633Tyr} mice will reveal whether these mice exhibit reduced oxidative stress *in vivo*. Additionally, it would be interesting to compare their metabolic profiles to caloric restricted and rapamycin/trametinib treated mice.

5.5 Limitations

5.5.1 CRISPR/Cas9 in haploid AN3-12 mouse embryonic stem cells

The recent discovery that CRISPR/Cas9 technology can be individually tailored for a broad spectrum of applications in genome editing has revolutionised biomedical research. One main concern that hampers its translation into clinical use, is the occurrence of unwanted off-target effects. To promote high specificity of genomic editing and mitigating potential off-target effects, we made use of the double-nicking strategy using the D10A Cas9 mutant with paired guide RNAs (Ran et al., 2013). Previous studies have shown that the occurrence of off-target mutations is extremely low when using the D10A nickase because it drives the cellular repair mechanism into homology-directed repair rather than more error-prone non-homologous end joining (Chiang et al., 2016; Cho et al., 2014; Shen et al., 2014). *In silico* predictions of gRNA on- and off-target binding and cut-to-mutation distance were additional factors considered in this study to ensure maximum specificity. Through targeted sequencing of generated clones, we ensured that all cell lines only contained the desired point mutation and, when needed, silent mutations to disrupt the PAM sites and/or create a restriction site for screening of the colonies. However, we did not conduct whole genome sequencing of the generated cell lines, leaving the possibility that off-target effects occurred at different genomic locations. For the purpose of this study, it was essential to use a murine cell line that would allow for downstream *in vitro* applications. Moreover, it strengthened the likelihood that functional effects are conserved in the mouse, which we used to follow functional variants up in more detail. A limiting factor is that functional effects may differ between model organisms or cell lines used, as the results in this study suggest. We therefore propose that future studies investigate the heterozygous *Raf1*$^{\mathrm{Asp633Tyr}}$ variant in mouse and human cell lines. In this study, we opted for AN3-12 mESCs given that these cells have successfully been used for CRISPR/Cas9 genome engineering in previous studies (Allmeroth et al., 2021; Chylinski et al., 2019; Horn et al., 2018; Kroef et al., 2022). This AN3-12 cell line provides a valuable tool for functional genomics, as it allows for precise genomic editing in the haploid state and does not possess karyotypic abnormalities, such as polyploidy, that could affect the functional characterisation. Stem cells are a commonly used cell line in biomedical research because of their unlimited capacity to divide and differentiate into different cell types (Gonzalez & Bernad, 2012). These characteristics pose advantages for experimental purposes, but also set them apart from the majority of differentiated cells in the human body and other cell lines. In diploid or polyploid cells,

CRISPR/Cas9 genome editing often leads to one allele being successfully edited, while the other allele remains wildtype and thus, may mask functional effects of the introduced variant. By using a haploid cell line, we avoided this potential problem. A limitation of this approach is, however, that the variants found in long-lived individuals were identified in the heterozygous state. As we expected potential mild functional effects, we aimed to increase their functionality by first generating homozygous cell lines. Once successfully edited in the haploid state, these AN3-12 cells spontaneously become diploid (Elling et al., 2017). To mirror the cellular environment of most cells in flies, mice and humans as closely as possible, we used these cells in the diploid state for the functional characterisation of the variants.

5.5.2 Follow-up in humans

The nature of this study entails a power limitation, as we are investigating extremely rare and family-specific genetic variants. The number of carriers is therefore extremely low and possible functional effects need to be rather big to be able to observe them. For this reason, we first analysed the variants in the homozygous state and subsequently followed them up *in vivo*. Additionally, it would be highly interesting to follow our findings up in the humans that carry the variants under study. As most of the long-lived individuals are deceased at present, we aim to determine if their offspring carry these variants as well. If this is indeed the case, we can, for instance, study their proteomic and metabolomic profiles and perform a more targeted analysis of the transsulfuration pathway and stress resistance. This will help us answer the question of whether the observed functional effects are conserved. Moreover, the health and disease-related parameters observed in these individuals might further help in terms of a more targeted analysis of mouse tissues harbouring the *Raf1*^{Asp633Tyr} and *Nf1*^{Phe1112Leu} variants. Such information would for instance facilitate the search for the most likely tissue targets and additional functional read-outs.

5.5.3 RGS treatment

We could show promising geroprotective effects for RGS treatment in flies, but it would be key to recapitulate these health- and lifespan-extending effects in other model organisms, such as the mouse. RGS has been described as a dual inhibitor of the MAPK/ERK and PI3K/AKT/MTOR signalling pathways. However, we could only show effects on the MAPK/ERK signalling pathway and these effects appear to vary between cell types. The exact mechanism of action of RGS remains elusive and should be determined in future studies. Also, it is likely that RGS has more tissue targets than the gut, which should be assessed in a tissue-

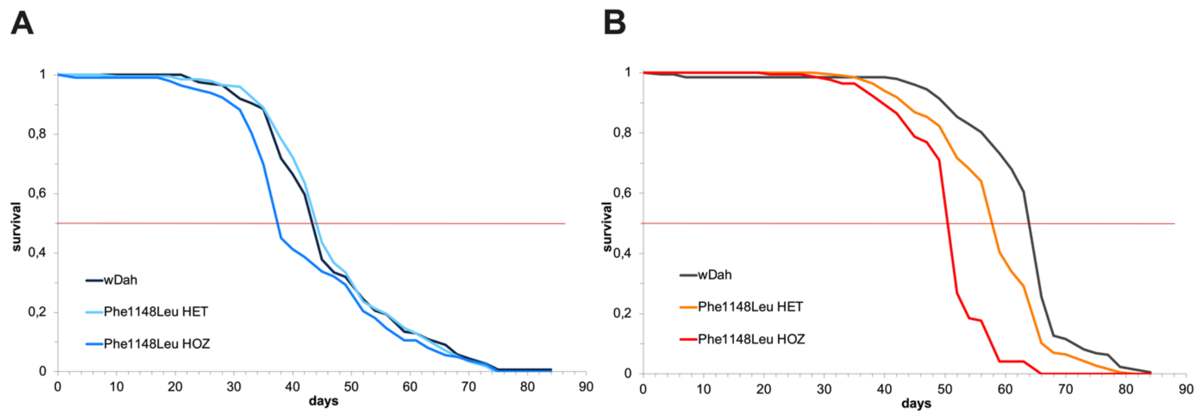
specific manner. In translational studies with the ultimate aim to bring promising geroprotective drug candidates into human use, it is critical to determine the latest possible onset of drug treatment to still elicit beneficial effects and whether short-term treatment would be sufficient. We therefore suggest to assess different treatment regimes, such as late- or midlife onset and intermittent RGS feeding, as it has been done in similar studies of other lifespan-extending drugs (Juricic et al., 2022).

5.6 Conclusion

Our findings in **Chapter 3** of this thesis suggest that functional effects on MAPK/ERK signalling pathway activity result in improved stress resistance, mediated through proteomic and metabolomic rewiring in the *Raf1*^{Asp633Tyr} variant. While the interpretation of these results remains speculative, proteomic and metabolomic rewiring in the *Raf1*^{Asp633Tyr} suggests differential regulation of the transsulfuration pathway. Follow-up experiments, determining the flux of associated metabolites, will shed light on their potential role in mediating health and lifespan promoting cellular effects. *In vivo* analysis of the heterozygous *Nf1*^{Phe1148Leu} variant in *D.melanogaster* revealed no effect on life- and healthspan, while the homozygous variant appears detrimental. The fact that we observed significant effects *in vitro*, i.e. reduced MAPK/ERK signalling pathway activity and proteomic and metabolomic rewiring, offers the possibility, however, that some functional effects are not conserved in the fly. Further functional analysis of the *Raf1*^{Asp633Tyr} and *Nf1*^{Phe1112Leu} variants in the mouse at older time points and different tissues will show whether the functional effects observed in mESCs and MEFs are conserved, and thus may have contributed to the longevity phenotype of the long-lived individuals belonging to the LLS.

Our findings in **Chapter 4** of this thesis reveal promising results in *D.melanogaster* to further study RGS as a potential geroprotective drug. We have presented that RGS extends lifespan in a similar manner than previously reported geroprotective drugs- such as rapamycin and trametinib- without, negatively affecting fecundity and inducing adverse side effects or toxicity. In contrast to previous studies reporting lifespan promoting effects through the pharmacological inhibition of the MAPK/ERK signalling pathway, we provide evidence that the mild elevation of such has beneficial effects on health and lifespan in flies. We demonstrate that the MAPK/ERK signalling pathway is highly adaptable to its environment and an important mediator of cellular responses, such as stress resistance and possibly longevity.

Supplementary Figures



Supplementary Figure 3.1 | The homozygous *Nf1*^{Phe1148Leu} variant reduces lifespan in *D.melanogaster.*

A+B repeated lifespan assay of wildtype (black), heterozygous and homozygous *Nf1*^{Phe1148Leu} flies of male **(A)** and female **(B)** flies. Lifespan of heterozygous, male animals did not differ significantly from wildtype flies, while homozygous animals showed significantly reduced lifespan. Lifespan of heterozygous, female animals appeared slightly reduced, while homozygous animals showed significantly reduced lifespan (n = 160 flies per condition, $p < .001$, log-rank test)

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List of Publications

Results in Chapter 3 have been published here:

Helena Hinterding, Maarouf Baghdadi, Thies Gehrmann, Pasquale Putter, Nico Lakenberg, Erik B. van den Akker, P. Eline Slagboom, Joris Deelen, Linda Partridge (2023). Functional characterisation of rare variants in genes encoding the MAPK/ERK signalling pathway identified in long-lived Leiden Longevity Study participants. *bioRxiv*. doi: https://doi.org/10.1101/2023.06.01.541708

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