Regulation of metabolism by DNA-binding ability and acetylation of FOXO in *Drosophila*

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ABBREVIATIONS

ΔV3	null mutants
AA	Amino acid
AGE	Advanced Glycation End-product
AMPK	AMP-activated protein kinase
APO	Apolipoprotein
Apoltp	Apolipoprotein lipid transfer particle
ATG	Autophagy related gene
ATGL	Adipose Triglyceride lipase
BCAA	Branched-chain amino acids
bgm	Bubblegum
bmm	Brummer
BP	Biological process
CBP	CREB-binding protein
CC	Cellular component
CDK1	Cyclin-dependent kinase 1
CK1	Casein kinase 1
CPT1	Carnitine palmitoyltransferase-1
DBD	DNA-binding domain
DBD2	DNA-binding mutants
DEP	Differentially expressed proteins
DILP	Drosophila ILP
DR	Dietary restriction
EAA	Essential amino acids
FOXO	Forkhead Box O
G6Pase	Glucose-6-phosphatase
gck	Glucose carboxykinase
GO	Gene ontology
GSK3	Glycogen synthase kinase 3
GST	Glutathione S-transferase
HDAC	Histone deacetylase
HNF4	Hepatocyte nuclear factor 4
IGF	Insulin-like growth factor
IIS	Insulin/IGF signaling
ILP	Insulin-like peptide
InR	Insulin(-like) receptor
IP	Immunoprecipitation
IPC	Insulin-producing cells
IRS	Insulin receptor substrate
Lip3	Lipase 3

Lip4	Lipase 4
Lipa	Lysosomal acid lipase
LPL	Lipoprotein lipase
MF	Molecular function
NEAA	Non-essential amino acid
NES	Nuclear exclusion signal
NLS	Nuclear localization signal
NSBP1	Nematode sterol binding protein 1
OXPHOS	Oxidative phosphorylation genes
PDK1	Phosphoinositide-dependent kinase 1
pepck	Phosphoenolpyruvate carboxykinase
PI3K	Phosphoinositide-3-kinase
PIP2	Phosphatidylinositol (4,5)-diphosphate
PIP3	Phosphatidylinositol (3,4,5)-trisphosphate
PKB	Protein kinase B
PML	Promyelocytic leukemia protein
PolyQ	Polyglutamine
PP2A	Phosphatase 2A
PTM	Post-translational modification
SGK1	Serum- and glucocorticoid-inducible kinase 1
SIRT	Sirtuin
SREBP1c	Sterol regulatory element-binding protein 1c
SYA	Sugar-yeast-agar food
TAD	Transactivation domain
TAG	Triacylglycerol
TOR	Target of Rapamycin
TORC1	TOR complex 1
TORC2	TOR complex 2
YAA	Defined diet
YP	Yolk protein

SUMMARY

Population ageing is a major social problem, and researchers are exploring ways to promote healthy ageing. One area of investigation is the evolutionarily conserved insulin-insulin like growth factor signaling pathway (IIS), the reduced activity of which has been largely associated with improved health and longevity in different species. FOXO transcription factors are downstream effectors of this pathway and have been identified as mediators of the healthspan and lifespan benefits. Besides regulating ageing, FOXOs play a crucial role in the regulation of energy homeostasis and metabolism. In order to coordinate all the different functions associated with the protein, their action is specifically regulated by post-translational modifications and interactions with binding partners. However, the molecular mechanisms behind FOXO regulation *in vivo* have not been fully elucidated.

In this PhD project, I used the model organism *Drosophila melanogaster* to investigate the specific contribution of DNA-binding ability and acetylation in the regulation of FOXO-related functions. In the first part of the study, I used previously generated mutant flies expressing constitutively acetylated or deacetylated dFOXO proteins to study the influence of this modification on lifespan and metabolism. We found that dFOXO acetylation plays a crucial role in the response to yeast starvation and, partially, cholesterol starvation. To have a deeper insight into the lysine residues whose acetylation regulates dFOXO function, I generated novel partial acetylation mutants and observed overlapping phenotypes with the complete acetylation mutants. Acetylation of FOXO also seemed to drive metabolism towards lipogenesis. Moreover, constitutively acetylated dFOXO protected flies from the detrimental effects of a high-sugar diet. Finally, we described a new role of dFOXO as potential mediator of methionine depletion.

In the second part of the project, I used dFOXO mutant flies in which the DNA-binding ability of the protein was abolished to identify functions that are independent of its direct transcriptional ability. Our results indicate that dFOXO-mediated response to sugar starvation is completely dependent on its DNA-binding ability, whereas it promotes lipid usage under starvation independently of its transcriptional ability.

Moreover, we observed that dFOXO-DBD mutants switch from carbohydrates to lipids as preferential energy source upon starvation.

In conclusion, this PhD study sheds light on the specific metabolic functions associated with dFOXO and on its regulatory mechanisms *in vivo*.

INTRODUCTION

1. Introduction

1.1 Ageing – What can we do about it?

Understanding how we grow old and finding ways to delay death has been a topic of human interest for centuries. Today, with people living much longer than in the past, our awareness of human aging has especially increased. As ageing represents the major risk factor for many human diseases (López-Otín et al., 2023), the greying population poses major social and economic challenges for our society. Fortunately, ageing has proved to be a malleable process that can be slowed through dietary, pharmacological and genetic interventions (Riera et al., 2016; Teumer et al., 2016; Fontana and Partridge, 2015). In this context, the study of the evolutionarily conserved insulin/insulin-like growth factor 1 signaling (IIS) pathway and its downstream components is of particular importance. Manipulation of this network has been shown to consistently extend lifespan in several model organisms, making the IIS pathway a prominent target of ageing research (Fontana and Partridge, 2015).

1.1.1 Demographic changes and challenge of population ageing

Many countries across the world are experiencing growth both in the number and proportion of elderly in the population, turning aging into a topic of global importance (Olshansky et al., 2019). As a result of medical and technological advances, healthier life styles, food availability and reduced child mortality, life expectancy has dramatically increased over the past two centuries (Oeppen et al., 2002). In the EU, life expectancy has been rising steadily for several decades, increasing from 69 to 84 in just 60 years (Ageing Europe, 2019). According to the World Health Organization, between 2015 and 2050 the proportion of the world's population over the age of 60 will almost double, while the number of persons aged 80 or older will triple (WHO), posing a serious economic challenge to our society. We are living longer, but not healthier. The drastic rise in overall life expectancy has not been matched by a similar increase in healthy life expectancy (Crimmins, 2015), resulting in a greater part of life spent in late-life illness (Jagger et al., 2008).

Ageing is the number one predisposing factor for the onset of many diseases (López-Otín et al., 2023), including metabolic disorders. One example of metabolic deregulation that occurs during ageing is a decline in glucose tolerance and insulin sensitivity, leading to type 2 diabetes (Fröjdö et al., 2009). Additionally, old age is associated with alterations in lipid metabolism, with an increased risk of developing cardiovascular disease (Cho et al., 2020). Age-related diseases both negatively impact on an individual's health and quality of life and represent a serious economic burden for our ageing society. Thus, understanding the underlying molecular mechanisms of ageing and developing effective interventions to extend healthspan, the disease-free period of life (Nikolich-Zugich et al., 2016), are of primary importance.

1.1.2 Ageing is a malleable process

A growing body of research has demonstrated that nutritional, pharmaceutical, and genetic interventions not only prolong life, but also improve age-related functional decline (Stein and Murphy, 2012). In an attempt to define common denominators of ageing in different organisms, López-Otin et al. had identified cellular and molecular hallmarks to describe ageing (López-Otin et al. 2013): DNA instability, telomere attrition, epigenetic alterations, loss of proteostasis, deregulated nutrient-sensing, mitochondrial dysfunction, cellular senescence, stem cell exhaustion, altered intercellular communication; more recently, disabled macroautophagy, chronic inflammation and dysbiosis have also been added to the list (López-Otin et al. 2023). Since ageing is a dynamic and plastic process, the use of interventions to directly target these mechanisms can help delay ageing and prevent age-related pathologies. For instance, mouse models of pulmonary fibrosis and aplastic anemia have revealed therapeutic benefits from telomerase activation using a gene therapy approach (Povedano et al., 2018; Bär et al., 2016). The use of senolytics to recapitulate the natural clearance of senescent cells has also proved efficient in the cure of pathologies associated with cellular senescence (Zhu et al., 2015). In mice, induced senolysis is able to extend longevity (Xu et al., 2018). Interventions on gut microbiota composition restore age-related neurological decline in middle-aged mice (Cryan et al., 2019). Furthermore, different strategies aimed at reducing the activity of nutrient sensing

pathways extend the lifespan in multiple model organisms, including primates (Colman et al., 2009; Mattison et al., 2012), indicating that some of the advantages of a lowered activity in nutrients sensing may also apply to humans.

1.2 The insulin nutrient-sensing network in ageing

Among the characterized hallmarks of ageing, “deregulated nutrient sensing” was the first identified to modulate the ageing process through the insulin/IGF-1 signaling (IIS) pathway (Kenyon, 1993). Manipulation of components of this pathway has been shown to extend lifespan in various model organisms (Fontana and Partridge, 2015).

1.2.1 The insulin/IGF signaling pathway

The IIS pathway is an evolutionarily conserved nutrient-sensing network that controls diverse cellular and organismal processes, such as growth, metabolism, stress, reproduction and longevity (Fontana et al., 2010). When insulin-like peptides (ILPs) bind to the insulin receptor, the insulin signaling is activated and integrates signals of growth, development, reproduction, stress resistance and lifespan (Giannakou and Partridge, 2007). In vertebrates, insulin secreted by pancreatic β-cells regulates glucose and lipid metabolism, insulin-like growth factors (IGFs) regulate growth and relaxins, produced by the ovaries, help regulate reproduction (Klein, 2016). In invertebrates, a variable number of ILPs function as both regulators of glucose and as growth factors. *C. elegans* genome codes for about 40 ILPs (Kaletsky and Murphy, 2010), while in *Drosophila* eight insulin-like peptides (DILPs) were described (Grönke et al., 2010). DILPs are secreted by insulin-producing cells (IPCs) in the brain and fat body with various spatiotemporal expression patterns and functions depending on the development stage (Brogiolo et al., 2001; Bai et al., 2012; Nässel et al., 2016; Post et al., 2018). Interestingly, manipulations leading to decreased dilp2 expression or the triple deletion mutation dilp2-3,5 were showed to increase longevity (Grönke et al., 2010). More recently, it was proposed that DILP2 signals influence lifespan through deactivation of glycogen phosphorylase, the key enzyme in the utilization of reserves

of glycogen (Post et al., 2018). Moreover, overexpression of DILP6 in the fat body also promoted lifespan by decreasing DILP2 peptide secretion (Bai et al., 2012).

The signal transduction starts when, in presence of nutrients, insulin/DILPs are secreted and bind to their receptors, activating a complex phosphorylation cascade (Figure 1.1). Unlike mammals, where insulin and IGF peptides signal through independent receptors, in *Drosophila* a single common receptor signals downstream of different ILPs (Grewal et al., 2009). In summary, the binding determines a conformational change that triggers activation of the receptors by autophosphorylation. This consequently leads to phosphorylation of an Insulin Receptor Substrate – IRS1-4 in mammals, *chico* in *Drosophila* – which further allows activation of the regulatory protein phosphoinositide-3-kinase (PI3K) (Barthel et al., 2005; Grewal et al., 2009). In addition to IRS, other substrates can be phosphorylated. For instance, activation of ShC activates the MEK1/2-MAPK cascade (Yenush et al., 1996), important mediator of the mitogenic and growth effects of DILPs (Orme et al., 2006).

When activated, PI3K converts membrane phosphatidylinositol (4,5)-diphosphate (PIP2) to phosphatidylinositol (3,4,5)-trisphosphate (PIP3). Accumulation of PIP3 at the membrane leads to phosphorylation and activation of the protein kinase dPDK1 (*Drosophila* phosphoinositide-dependent kinase-1), which in turns recruits and activates AKT (or protein kinase B) at the plasma membrane (Piper et al., 2005). AKT activity is further regulated by two additional pathways: inhibition by dephosphorylation via protein phosphatase 2A (PP2A) (Vereshchagina et al., 2008) and activation via the TOR complex 2 (TORC2) (Hietakangas and Cohen, 2007).

AKT phosphorylates several proteins involved in the regulation of metabolism. Among these, glycogen synthase kinase 3 (GSK3) and protein phosphatase 1 (PP1) are inactivated and activated, respectively, to promote glycogen synthesis (Newgard et al., 2000; Beurel et al., 2015). In both mammals and flies, FOXO transcription factors are a key downstream target of AKT and the central node of the pathway. Under nutrient availability, phosphorylation of highly conserved residues of FOXO by AKT leads to its nuclear exclusion and inactivation for interaction with the scaffold protein 14-3-3. In contrast, under nutrient depletion, reduced activity of the pathway results in release of

FOXO and expression of genes involved in stress resistance, ageing and metabolism (Jünger et al., 2003; Puig et al., 2003).

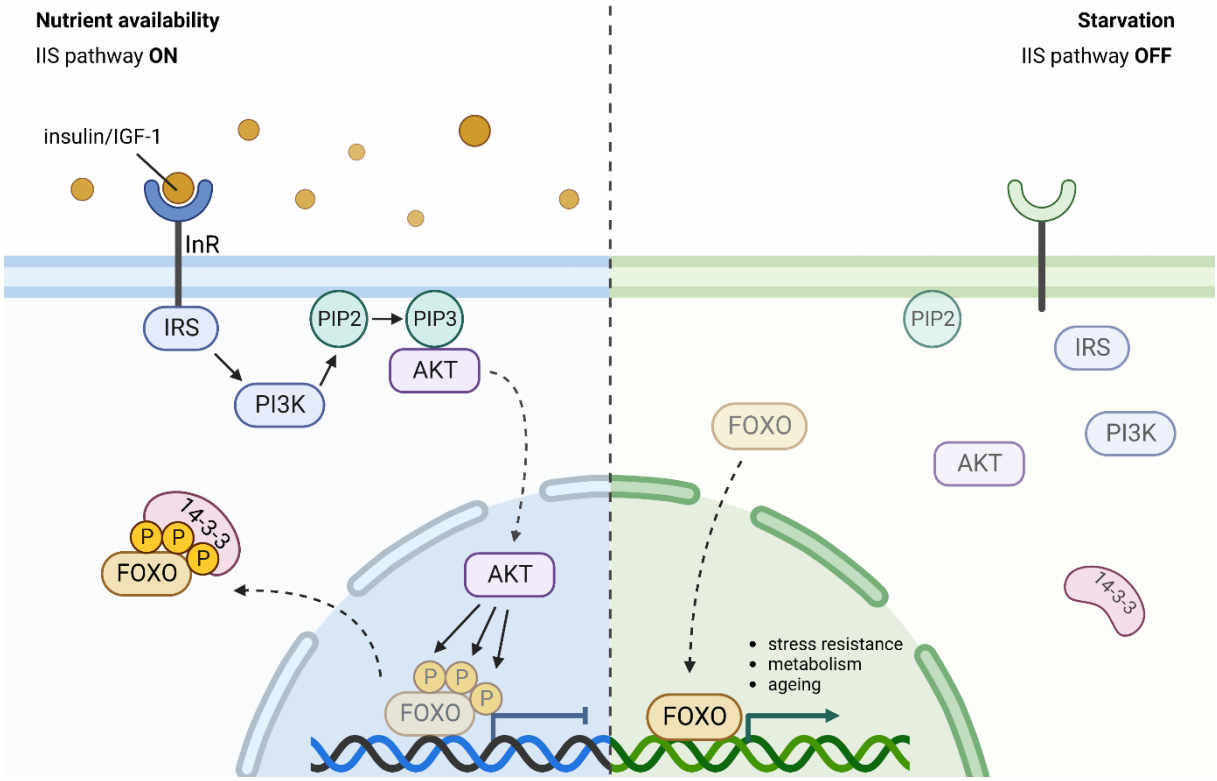

Figure 1.1: Schematic representation of the Insulin/Insulin-like growth factor signaling (IIS) pathway.
Under nutrient availability, activation of the IIS pathway leads to AKT-mediated phosphorylation of FOXO and inactivation by nuclear exclusion. In contrast, under starvation, reduced activity of this pathway results in release of FOXO and activation of target genes involved in a variety of homeostatic functions, including stress resistance, metabolism, and ageing. Created with Biorender.com.

1.2.2 Reduced IIS pathway extends lifespan

30 years ago, loss of the *daf-2* gene in the worm *C. elegans* was found to double longevity (Kenyon et al., 1993). Originally discovered for its role in controlling the dauer stage (Riddle et al., 1981), several years later *daf-2* was discovered to encode the insulin/insulin-like growth factor (IGF) receptor (Kimura et al., 1997). Similar to the worms, mutation of the insulin receptor (InR) in *Drosophila* was also found to increase lifespan (Tatar et al., 2001). Today, more and more studies have confirmed that lowered

activity of this pathway is associated with health and lifespan benefits in different organisms, including vertebrates (Fontana et al., 2010). In mice, mutation of the IGF-1 receptor (IGF-1R) increases lifespan and oxidative stress resistance in females (Holzenberger et al., 2003) and improves insulin sensitivity in male mice (Duran-Ortiz et al., 2021). Moreover, female (but not male) mice with long-term anti-IGF1R antibody treatment also exhibited longer lifespans (Zhang et al., 2020). Besides the insulin receptor, other mutations in components of the IIS pathway were identified to extend the lifespan in different species. For instance, mutation of *chico* (insulin-receptor substrate) in flies (Clancy et al. 2001) or IRS1 in mice (Selman et al., 2008) promote longevity. In addition, it was recently found that reducing insulin signaling by neuron-specific IRS-1 knock-out promotes health benefits in male mice (Baghdadi et al., 2023).

The high level of interspecies conservation of this pathway makes it very attractive to study ageing in *Drosophila*, because of all the advantages that comes with this model organism: relatively short lifespan, low maintenance costs and ease of genetic manipulation. In *Drosophila*, the simultaneous mutation of three insulin-like peptides (dilp2-3,5) is able to extend the lifespan by 30-50% (Grönke et al., 2010). In the same way, over-expression of an antagonist of PI3K (dPTEN) in the head fat body was shown to increase the lifespan by 50% (Hwangbo et al., 2004).

Notably, the longevity benefits related to manipulation of the IIS pathway seem to hinge on the presence of the downstream transcription factor FOXO. In both worms and flies, FOXO is required to provide the lifespan extension upon reduced insulin signaling (Kenyon et al., 1993; Slack et al., 2011; Yamamoto and Tatar, 2011). In addition, *foxo-null* mutants are short-lived in *C. elegans* and *Drosophila* (Lin et al., 2001; Slack et al., 2011), and over-expression of dFOXO in both muscle and fat body increases longevity in flies (Demontis and Perrimon, 2010; Giannakou et al., 2004; Hwangbo et al., 2004). In mice, the life-extending effect of lowered insulin signaling requires the presence of FOXO3 but not FOXO1 (Yamaza et al., 2010; Shimokawa et al., 2015). Interestingly, human genetic association studies have also implicated FOXO3 in human longevity (Broer et al., 2015; Martins et al., 2016; Willcox et al., 2016).

1.2.3 IIS and dietary restriction

The IIS nutrient-sensing network is highly conserved in evolution and easily manipulated by diet. As a result, dietary restriction (DR), described as the reduction of nutrient intake without causing malnutrition, has proven to be a solid lifespan and healthspan extending intervention in diverse species. DR was first shown to extend the lifespan of rats (McCay et al., 1935); later on, it was proved that this method can extend the lifespan also in yeast (Jiang et al., 2000; Lin et al., 2000), worms (Klass, 1977), flies (Chapman and Partridge, 1996), mice and primates (Colman et al., 2009; Mattison et al., 2017).

It has been reported that FOXOs have an important role in mediating the DR response (Jiang et al., 2019). In yeasts, DR was unable to prolong the chronological life of mutants with simultaneous loss of the FOXO protein orthologs FKH1 and FKH2 (Postnikoff et al., 2012). In *C. elegans*, *daf-16* regulates immunity and promotes longevity by suppressing feeding behavior, which mimics a DR-like condition (Wu et al., 2019). Intermittent fasting led to noticeably increased FOXO1 and FOXO4 gene expression in rat skeletal muscles (Furuyama et al., 2002). DR also increased the levels of *FOXO1* and *FOXO4* mRNA expression in murine liver, skeletal muscle and adipose tissue (Yamaza et al., 2010). Together with the direct increase in FOXO protein expression, DR also changes the expression of a number of FOXO target genes (Yamaza et al., 2010; Miyauchi et al., 2019; Kim et al., 2014). Moreover, both a heterozygous and a homozygous *foxo3*-knockout mice did not live longer on the DR regimen, indicating that FOXO3 is necessary to mediate the lifespan benefits (Shimokawa et al., 2015). In a *Drosophila* study by Giannakou et al. (2008), a distinction between a role for dFOXO in DR and a requirement for dFOXO in the extension of lifespan by DR was made. It was suggested that dFOXO is not essential for extending the lifespan of flies through dietary restriction, but that its activity can affect the response. In particular, when dFOXO was knocked out, dietary restriction still increased the lifespan of fruit flies. However, when dFOXO activity was reduced, the lifespan extension was also reduced, indicating that dFOXO is not necessary but modulates the response to dietary restriction (Giannakou et al., 2008).

1.2.4 Alternative approaches to DR and role of FOXOs

Restriction of specific macronutrients has also been shown to be effective in providing the same benefits as DR, with the benefit of lowering its side effects (Lee and Longo, 2016). Among the different types, reduced intake of proteins and amino acids is the most effective pro-longevity intervention in multiple model organisms, providing the same strong impact on lifespan as complete dietary restriction (Mirzaei et al., 2014). Changing the relative proportions of macronutrients in a diet can produce an increase in lifespan equivalent to that seen when access to all dietary nutrients is restricted (Mair et al., 2005; Skorupa et al., 2008). The use of fixed concentrations of sugar while reducing the yeast (protein source) significantly extended the lifespan of flies, whereas restriction of dietary sugar resulted in only a modest extension, suggesting an important role for protein restriction in lifespan extension (Mair et al., 2005). Notably, depletion of a single essential amino acid (methionine) in a normal diet can extend lifespan in both flies (Grandison et al., 2009) and mice (Miller et al., 2005). Removal of three branched-chain amino acids (BCAA) from the food increases metabolic health and longevity in flies and mice (Juričić et al., 2020; Richardson et al., 2021). Moreover, it was recently shown that, while methionine deprivation acts via an independent mechanism, the beneficial effects derived from restriction of all EAAs depend on the amino acid sensor GCN2 (Srivastava et al., 2022). This kinase was shown to phosphorylate the transcription factor FOXO to promote its activity (You et al., 2018), suggesting that FOXO might be involved in the response to amino acid starvation. FOXO proteins have already been shown to be activated during amino acid starvation and crucial for survival under nutrient restricted conditions (Kramer et al., 2008). Even though the TOR pathway has been identified as the predominant amino acid sensing network (Wullschleger et al., 2006), the exact downstream mechanisms activated by specific amino acid(s) restriction, their contribution to the lifespan benefits and the interplay with the IIS pathway, are still under investigation.

Different dietary and genetic lipid-related interventions have also been shown to extend lifespan in model organisms, often times in an IIS-dependent fashion. In worms, phosphatidylcholine feeding promotes the nuclear accumulation of DAF-16/FOXO and

increases lifespan (Kim et al., 2019). In *daf-2* mutants, a reduction in insulin signaling activity leads to longer lifespan and upregulation of the lysosomal lipase LIPL-4, which hydrolyzes fats such as cholesterol and triglycerides. Long-lived worms are lean and lipid hydrolysis is dependent on reduced insulin signaling (Wang et al., 2008). The longevity effect reported in response to ASM-3 (acid sphingomyelinase-3) knockdown depends on the functions of the insulin signaling genes *daf-16/foxo* and *daf-18/pten* (Kim and Sun, 2012). The compound α-lipoic acid, previously reported to prolong life in worms (Benedetti et al., 2008; Brown et al., 2006), also extends life in both female and male flies (Bauer et al., 2004). Moreover, full life extension via dietary restriction in *Drosophila* involves the expression of fatty acid β-oxidation-related genes, which also depends on the activation of dFOXO signal (Lee et al., 2012). Although the specific mechanisms by which lipid interventions affect lifespan are largely unknown, it is clear that different interventions exhibit overlap with the canonical IIS aging pathway, and that in many cases FOXO proteins constitute a central node of mediation.

In humans, clinical trials based on dietary restriction are complicated since continuous reduction of calorie or macronutrient intake is not easy to practice in everyday life. Therefore, much effort has been put into researching drugs that can be repurposed as anti-ageing agents. These potential geroprotectors could simulate beneficial effects of DR without limiting the food intake. For instance, the drug rapamycin was consistently shown to increase lifespan in mice (Harrison et al., 2009) or flies (Bjedov et al., 2010; Regan et al., 2022), also under short treatment regime (Juričić et al., 2022). Rapamycin acts on longevity-inhibiting TOR signaling and mimicking DR. TOR pathway has a connection to the IIS network through FOXO (Hay, 2011; Chen et al., 2010). In a *C. elegans* study, Robida-Stubbs et al. showed that rapamycin treatment increased lifespan in worms by inhibiting TORC1 and upregulating the transcription factors SKN-1/Nrf and DAF-16/FOXO. While only SKN-1/Nrf was required for the lifespan-extending effects of rapamycin, DAF-16/FOXO was important for maintaining proteostasis and resistance to oxidative stress. Inhibition of TORC1 might enhance longevity by decreasing protein synthesis, preserving protective gene translation, and increasing autophagy. In this context, the transcriptional responses mediated by SKN-1 and DAF-

16 are essential effectors of longevity resulting from genetic TORC1 inhibition (Robida-Stubbs et al., 2012).

In conclusion, the network of DR-related signaling pathways is highly interconnected and there is abundant evidence that FOXO factors play an important role in mediating their crosstalk. Even though many of the downstream gene targets of FOXO proteins have been identified in multiple organisms (Webb et al., 2016), further investigations are needed to unravel the exact mechanisms by which FOXO factors modulate lifespan and healthspan.

1.3 FOXO proteins

The first report of a FOX gene dates back to 1989, when Weigel et al. observed that the *Drosophila* gene *fkh* (*Forkhead)* encodes a nuclear protein; because of its localization, it was proposed that fkh acts as a transcription factor to regulate the expression of other genes (Weigel et al., 1989). To date, the number of known FOX proteins in humans amounts to 50 members, classified in 19 subgroups (FOXA-FOXS) (Golson & Kaestner, 2016; Jackson et al., 2010). Among these, FOXO proteins have been particularly well studied and characterized because of their implications in ageing (Broer et al., 2015; Martins et al., 2016; Willcox et al., 2016) and age-related diseases such as cancer (Hornsveld et al., 2018), cardiovascular disease (Yu et al., 2018), diabetes, (Pajvani & Accili, 2015), and neurodegeneration (Hu et al., 2019). However, the precise mechanism by which FOXO factors influence human longevity and health is not yet fully understood. In this context, the use of simplified animal models to study the molecular function of FOXO proteins in ageing represents a powerful resource.

1.3.1 Forkhead family of transcription factors, Subgroup O

FOXO proteins belong to the Forkhead family of transcription factors (subgroup O), characterized by the presence of an evolutionary conserved DNA-binding domain – the Forkhead box or FOX (Figure 1.2C) (Martins et al., 2016). The sequence similarity between Forkhead domains has been used as a criterion for the classification of FOX proteins into subfamilies (Lam et al., 2013).

The DNA-binding domain (DBD) of FOXO, also known as '*winged helix'*, consists of about 100 amino acids and folds into three alpha helices (H1-H3), three beta sheets (S1-S3) and two wing-like loops (W1, W2). While the third helix H3 represents the main DNA recognition element and makes sequence-specific contacts with the major groove of the DNA, the wings often interact with the minor groove or DNA backbone (Figure 1.2B) (Gajiwala & Burley, 2000). All FOXO-DBDs bind to DNA duplexes as monomers and recognize the consensus sequence 5'-(G/C)(T/A)AA(C/T)AA-3', identified as the FOXO-Recognized Element (FRE) (Obsil & Obsilova, 2011). Crystal

structure analysis of the FOXO1 DBD-DNA complex revealed that binding occurs through two specific amino acid residues that make direct contact with the DNA (Brent et al., 2007).

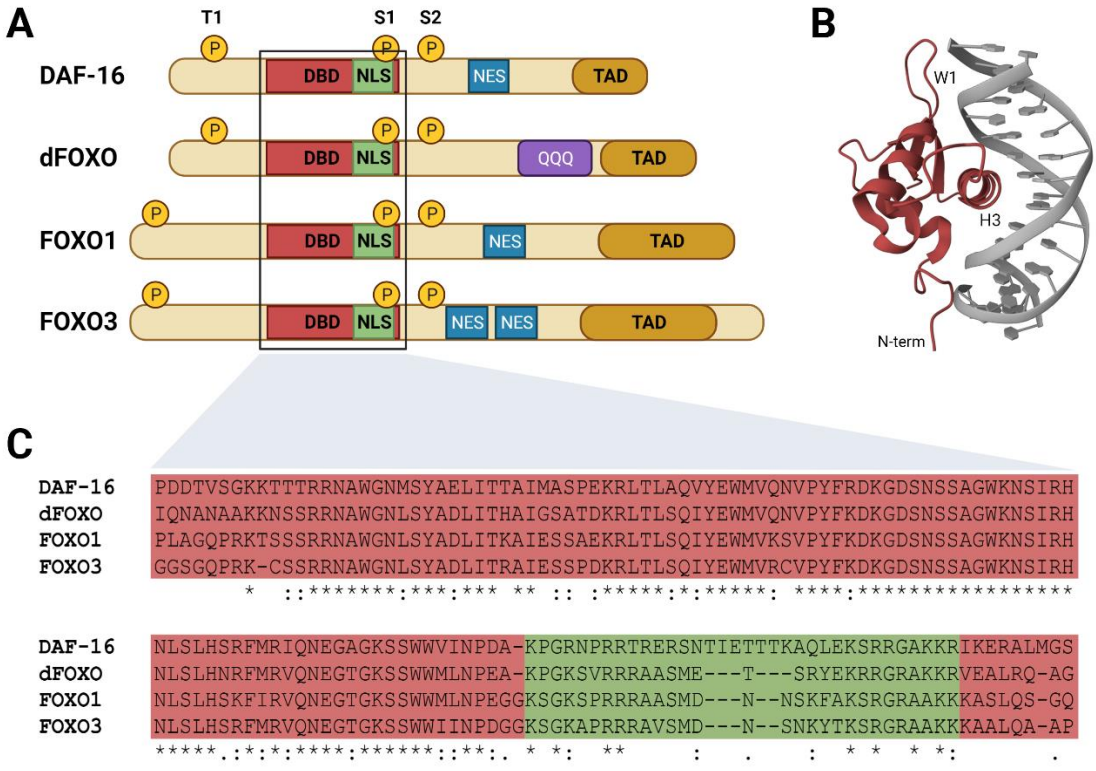

Figure 1.2: The FOXO DNA-binding domain (DBD) and NLS are conserved during evolution. A) Structural domains of FOXO proteins in *C. elegans* (DAF-16), *Drosophila* (dFOXO) and mouse (FOXO1, FOXO3). FOXOs share a highly conserved forkhead DNA-binding domain (DBD), a nuclear localization signal (NLS) and a transactivation domain (TAD); only mammalian FOXO proteins exhibit one or two nuclear export signal (NES). Three conserved AKT phosphorylation sites (one N-terminal threonine – T1 – and two C-terminal serines – S1 and S2) are indicated by circles. B) Crystal structure of FOXO1-DBD in contact with DNA. W1= wing 1; H3= helix 3. Obtained from PDB (code 3CO7). C) Amino acid alignment of the DBD of FOXO proteins in worm (DAF-16), fly (dFOXO) and mouse (FOXO1, FOXO3) using Clustal Omega. Residues in red represent the DBD, while green residues highlight the NLS. Consensus symbols indicate the degree of conservation: identical (*), strongly similar (:) or weakly similar (.).

In addition to the DBD, FOXO proteins also carry a nuclear localization signal (NLS), a nuclear export sequence (NES) downstream of the DBD and a C-terminal transactivation domain (TAD) (Figure 1.2A). Among these, the NLS is especially important for the modulation of FOXO function.

The NLS is rich in arginine and lysine residues and located at the C-terminal end of their DNA-binding domain (Brownawell et al., 2001; Zhang et al., 2002). Since this region overlaps with an AKT phosphorylation motif, phosphorylation of a serine residue in the consensus site modulates the function of the NLS and, consequently, of FOXO protein. Of note, in mammals the NLS harbors evolutionary conserved lysine residues that can be acetylated, with a key role in modulating dFOXO function (Wang et al., 2011).

Interestingly, mammalian FOXO1, -3 and -4 also have a leucine rich nuclear export sequence (NES) that functions co-operatively with the nearby NLS to promote nuclear exclusion. AKT-mediated phosphorylation induces a conformational change that exposes the NES and promotes export from the nucleus (Brownawell et al., 2001; Brunet et al., 2002). This motif was not found in the fly or in the worm, suggesting that FOXO shuttling outside the nucleus is only regulated by post-translational modification on the NLS.

Only in *Drosophila,* FOXO is characterized by the presence of a C-terminal polyglutamine stretch (polyQ). These repeats attach to other polyQ regions, modulating the interaction between transcription factors (Orr and Zoghbi, 2007; Atanesyan et al., 2012).

In mammals, FOXO proteins are encoded by four different genes (FOXO-1, -3, -4 and -6) and exert important cellular functions such as cell cycle, oxidative stress response, DNA repair and apoptosis (Webb and Brunet, 2014). They are expressed nearly everywhere in the body; however, each of the FOXO proteins is expressed differently in various tissues. For instance, FOXO1 and FOXO4 are mainly found in adipose tissue and skeletal muscle, respectively, while FOXO3 is present in several tissues, including the brain, heart, and kidneys. FOXO6, on the other hand, is mainly expressed during development and in nervous tissue (Furuyama et al., 2000; Fu et al., 2008). The pattern of expression determines specialized functions of these proteins, which include glucose and lipid metabolism, cardiac hypertrophy, myogenesis, neural survival and

synaptic plasticity (Kodani et al., 2020). However, despite their specific patterns of expression and functions, FOXO proteins have a high degree of conservation in their DBDs, which means they can bind to the same gene targets and perform at least similar functions in different tissues (Bullock, 2016). This core set of genes regulated by FOXO across tissues are involved in stress resistance, proteostasis, metabolism and growth (Webb et al., 2016). As FOXO proteins are conserved through evolution, this also applies in other species, suggesting that the primary function of FOXO factors as modulators of longevity is closely linked to these fundamental processes.

Invertebrates like *Drosophila* only contain one homolog, dFOXO (Jünger et al., 2003; Puig et al., 2003), making the fly an ideal model system to study its function independent of redundancy between FOXO paralogues. On the other hand, dFOXO is expressed in 4 different isoforms which differ in their 5'UTR region (Villa-Cuesta et al., 2010). However, there is no knowledge about potential different functions or tissue-specific expression.

1.3.2 Mechanisms of regulation of FOXO proteins

In the cell, FOXO acts as a molecular hub to integrate and convert the external stimuli of insulin, growth factors, nutrients and oxidative stress into specific biological responses. In order to be able to exert fine control over different functions and also among different tissues, FOXO factors are tightly regulated by multiple post-translational modifications (PTMs) and protein-protein interactions. It was proposed that different PTMs on FOXO act in concert as a molecular code read by selective FOXO-binding partners to rapidly adapt gene expression to the needs of the cell (Calnan and Brunet, 2008; Hill et al., 2014). PTMs of FOXO include phosphorylation, methylation, glycosylation, ubiquitination, acetylation, O-glycosylation and poly-ADPribosylation (Wang et al., 2016). These modifications control FOXO functions through adjusting their half-life, changing their subcellular localization, altering their DNA-binding affinity, controlling their transcriptional activity and regulating their interaction with co-factors (van der Vos and Coffer, 2011).

Phosphorylation of FOXO by AKT is one of the key regulatory mechanisms of these transcription factors, and the most characterized PTM. Genetic studies on the nematode *C. elegans* provided the first mechanistic proof that FOXO proteins are regulated by AKT-mediated phosphorylation (Lin et al., 1997), later confirmed in mammalian systems (Brunet et al., 1999). FOXOs contain four consensus motifs for AKT phosphorylation (RXRXXS/T); three of these phosphorylatable residues within the motifs (one N-terminal threonine – T1 – and two C-terminal serines – S1 and S2) are conserved across species (Greer and Brunet, 2008) (Figure 1.2).

Regulation of FOXOs via phosphorylation is quite intricate and involves other sites on these proteins. For instance, the serum and glucocorticoid-induced kinase (SGK) is also able to inhibit FOXO3a by phosphorylation, as this kinase is activated upon insulin signaling. Since the efficiency of phosphorylation by SGK and AKT on FOXO differs between the two kinases, it is likely that they work together to coordinately regulate FOXO transcription factors (Brunet et al., 2001). In addition, AKT phosphorylation creates recognition motifs for the casein kinase 1 (CK1) (Rena et al., 2002). In contrast, phosphorylation by AMPK, which happens on sites that don't overlap with the AKT-consensus motifs, is essential to activate FOXO upon low energy levels (Greer et al., 2007). In the same way, CDK1 (cyclin-dependent kinase 1) phosphorylation induces FOXO1 transcriptional activity (Yuan et al., 2008).

In several studies, acetylation was also suggested to have a key role in controlling dFOXO activity, especially in relation to metabolism. For the purpose of this thesis, acetylation and the effects on FOXO function will be described in more detail.

1.3.2.1 FOXO Acetylation

Acetylation involves the addition or removal of acetyl groups onto specific lysine residues through the opposite action of protein acetylases and protein deacetylases. In mammals, these include CBP (CREB-binding protein)/p300 and members of the Sir2/Sirt family of deacetylases, respectively (Matsuzaki et al., 2005). In both mice and flies, there is evidence for HDACs having a role in mediating deacetylation upon

nutrient starvation (Mihaylova et al., 2011; Wang et al., 2011); however, the exact molecular process leading to acetylation as well as the identification of potential specific, stress-dependent acetylation/deacetylation partners in *Drosophila* awaits further investigation.

Acetylation on FOXO can activate (Perrot and Rechler, 2005) or repress (Mihaylova et al., 2011; Wang et al., 2011) its function. In particular, repressive acetylation was observed to reduce FOXO binding to the DNA and increase its sensitivity to AKT-dependent phosphorylation, leading to downregulation in its transcriptional activity (Brent et al., 2008; Brunet et al., 2004; Matsuzaki et al., 2005; Qiang et al., 2010). In most cases, acetylation of transcription factors leads to an increase in their transactivation functions, mainly by enhancing their DNA-binding ability (Chen et al., 2001). Therefore, acetylation of FOXO was initially predicted to be an activatory modification. After extensive analysis, a "hit and run" model was proposed to explain why acetylation of FOXO attenuates its transcriptional activity. In the nucleus, FOXO1 binds to the target gene promoter and CBP is recruited to form the CBP-FOXO1 complex. The CBP-FOXO1 complex activates transcription of target genes via histone acetylation and recruits a preinitiation complex to the gene promoter. Subsequently, CBP induces acetylation at the DNA-binding domain of FOXO1 and attenuates its DNA-binding ability (Daitoku et al., 2004; Matsuzaki et al., 2005). This model of interaction between FOXO and CBP allows for the sophisticated control of target gene expression.

In a study by Wang et al. (2012) it was demonstrated that deacetylation of FOXO3 by sirtuins 1 and 2 (SIRT-1, -2) promotes FOXO3 ubiquitination and degradation via Skp2, implying that acetylation of FOXO increases its stability (Wang et al., 2012).

Previous observations have suggested that acetylation regulates multiple metabolic and physiological functions of FOXO proteins in mammals and flies. A mouse knock-in study revealed a fundamental role of FOXO1 acetylation in the regulation of glucose and lipid metabolism (Banks et al., 2011). In a study by Sundaresan et al. (2009), it was shown that SIRT3 stress-dependent activation and deacetylation of FOXO3 is determinant for FOXO translocation inside the nucleus and activation of antioxidant genes (Sundaresan et al., 2009). A study in hepatocytes showed that FOXO1–SIRT1 interaction in the nucleus overrides the AKT-mediated phosphorylation and increases

expression of target genes for gluconeogenesis (Frescas et al., 2005). On the other hand, acetylation of this transcription factor in the fly was shown to be highly dependent on the nutritional state, with dFOXO being deacetylated under starvation and acetylated after refeeding (Banks et al., 2011; Wang et al., 2011). Deacetylation of FOXO proteins by Sir2/Sirt deacetylases was found to also be important to regulate food intake in *Drosophila melanogaster* and mammals (Hong et al., 2012).

Repressive acetylation of mammalian FOXO proteins takes place at lysine residues located within the NLS (Daitoku et al., 2004; Jing et al., 2007). Some of these residues are evolutionarily conserved across species (Figure 1.3), suggesting that this kind of acetylation also happens in *Drosophila* (Wang et al., 2011), although direct evidence is still lacking.

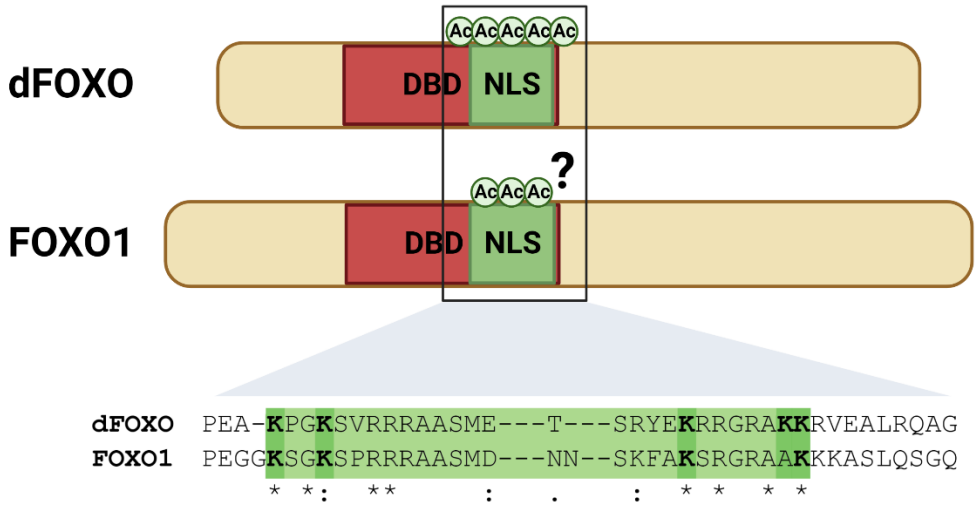

Figure 1.3: Inhibitory acetylation of FOXO NLS in mammals and (possibly) *Drosophila*.
In mammals, lysine residues within the NLS can be acetylated, with a inhibitory effect on FOXO. Some of these residues are evolutionarily conserved, thus it is possible that this regulatory mechanism also applies to *Drosophila*. DBD: DNA-binding domain; NLS: Nuclear localization signal. Amino acid alignment of FOXO NLS in fly (dFOXO) and mouse (FOXO1) was done using Clustal Omega. Consensus symbols indicate the degree of conservation: identical (*), strongly similar (:) or weakly similar (.).

1.3.2.2 FOXO DNA binding and subcellular localization

The main functional consequences of FOXO inhibition through phosphorylation or acetylation include 1) decreased ability to bind DNA and 2) translocation of FOXO proteins from the nucleus to the cytoplasm. Nuclear localization of FOXO has directly been linked to lifespan extension in worms (Oh et al., 2005; Lehtinen et al., 2006).

The phosphorylation of FOXOs has been proposed to directly inhibit DNA binding (Zhang et al., 2002). This may suggest that AKT signaling is primarily required for the release of FOXOs from the DNA, and that the cytoplasmic retention is a consequence of the masking of the NLS. More in detail, AKT-mediated phosphorylation within the NLS mediates the interaction with the 14-3-3 protein. These scaffold proteins bind to phosphorylated FOXOs in the nucleus and mediate their relocation into the cytoplasm. Masking of the NLS results in cytoplasmic accumulation and inactivation of FOXO (Brunet et al., 2002; Zhao et al., 2004). The ability of 14-3-3 to induce conformational changes in the NLS of FOXO has also been demonstrated by crystallographic structural studies (Obsilova et al., 2005). Once in the cytoplasm, phosphorylated FOXO can undergo ubiquitination and, eventually, degradation (Aoki et al., 2004). Opposite to AKT action, dephosphorylation of the NLS by PP2A mediates the dissociation of the FOXO/14-3-3 complex and promotes FOXO activity inside the nucleus (Singh et al., 2010; Yan et al., 2008).

One example of positive regulation by phosphorylation is AMPK. This kinase does not affect FOXO3 subcellular localization, but it phosphorylates nuclear FOXO3 and activates transcription of energy-producing genes, while inactivating energy-consuming pathways (Greer et al., 2007). This underlies the tight connection between FOXO PTMs and regulation of metabolism.

In *Drosophila*, phosphorylated dFOXO interacts with another scaffold protein, Melted, which recruits FOXO to the plasma membrane in close proximity to AKT. This might be needed to completely inactivate FOXO, as well as to position FOXO in close proximity with other signaling pathways, such as the TOR pathway (Teleman et al., 2005).

FOXO acetylation in response to stress stimuli also affects its DNA-binding capacity and subcellular localization. Structural studies of the complex FOXO3-DBD/DNA showed that Lys245 interacts directly with the phosphate group of DNA, suggesting that acetylation of this residue may prevent this interaction (Tsai et al., 2007). Moreover, both cell culture and *in vivo* studies have shown that acetylated FOXO1 remains cytoplasmatic under serum starvation, preventing its transcriptional activity in the nucleus (Banks et al., 2011; Qiang et al., 2010). In pancreatic β-cells, FOXO1 acetylation induces the interaction between FOXO1 and PML (promyelocytic leukemia protein) and the relocalization of FOXO1 to the nuclear bodies (Kitamura et al., 2005).

It was proposed that acetylation and phosphorylation cooperate to synergistically regulate FOXO in response to different stimuli, rather than individually affecting its function (Wang et al., 2017). Acetylation of FOXO3a by CBP/p300 has been suggested to promote AKT-mediated phosphorylation at Ser253 in the NLS motif (Senf et al., 2011; Matsuzaki et al., 2005). It is possible that acetylation of FOXO in lysine residues in the NLS induces a conformational change of the protein, which results in lower affinity for the DNA and increased susceptibility to phosphorylation by AKT. AKT phosphorylation in the NLS increases the binding of FOXO to 14-3-3 proteins and decreases the interaction with CBP/p300, consequently losing the ability for chromatin remodeling (Matsuzaki et al., 2005).

In the same way, the interplay between FOXO acetylation and monoubiquitination determines the localization of the protein, since the two PTMs compete for the same lysine residues. The deubiquitination of FoxO4 by the deubiquitinase USP7/HAUSP influences the status of FOXO acetylation and localization (van der Horst et al., 2006).

The primary function of FOXO proteins is in the nucleus; however, recent studies have demonstrated that FOXO exerts a transcriptional function also inside mitochondria, where it binds to mitochondrial DNA (Fasano et al., 2019; Lettieri-Barbato et al., 2019). Upon glucose restriction, activation of AMPK/MEK/ERK signaling results in FOXO3a translocation inside the mitochondria and allows the transcription of oxidative phosphorylation (OXPHOS) genes, restoring cellular ATP levels (Fasano et al., 2019).

Worth noting, the mito-to-nucleus shuttling of FOXO1 seems to depend on the dephosphorylation of the protein upon starvation (Lettieri-Barbato et al., 2019).

1.3.3 Roles of FOXO independent of DNA binding

1.3.3.1 Co-binding with other transcription factors: FOXO partners in crime

As described, FOXO ability to bind DNA is regulated by PTMs and directly affects its capacity to activate gene transcription. However, FOXO can regulate transcriptional responses in a number of ways that do not always require direct binding to the DNA; FOXO interacts with a variety of transcription factors that can help specify target-gene expression.

These mechanisms include:

1. co-binding to the DNA dependent on the interaction with another protein, that itself binds a nearby DNA sequence (requires FOXO binding to the DNA) (Yamamura et al., 2006; Seoane et al., 2004). The initial interaction between FOXO and its binding partner can determine changes in the proteins, altering the DNA-binding specificity of the transcription factors involved (Schmitt-Ney et al., 2020).

2. binding to another DNA-binding protein (does not require FOXO binding to the DNA) (Cook et al., 2015; Czymai et al., 2010; Ramaswamy et al., 2002).

This type of interaction seems to have a particularly important role in regulating hepatic glucose and lipid metabolism (Cook et al., 2015). Both mammalian cell culture and *in vivo* studies have suggested that FOXO1 controls lipid metabolism independent of DNA-binding ability (Cook et al, 2015; Matsumoto et al., 2006), and have indicated that it might rather act as a co-activator of another transcription factor (Cook et al., 2015).

1.3.3.2 Non transcriptional functions of FOXO

Even though FOXO proteins are mostly known for their role inside the nucleus as transcription factors, several studies have described functions that are independent of their ability to bind DNA. For instance, FOXO1 has also been found to act in the cytoplasm, where it regulates autophagy (Zhao et al., 2010) and ERK activity (Pan et al., 2017). Of note, the interaction between FOXO1 and the autophagy related 7 (ATG7)

protein is mediated by acetylated residues on FOXO (Zhao et al., 2010). The DNA-binding-independent role of FOXO1 in autophagy was confirmed by a recent study, where FOXO1-DBD mutants showed a decrease in activity and DNA binding, but preserved the interaction with cytoplasmatic ATG7 protein (Sablon et al., 2022). In mammals, over-expression of FOXO1 carrying a mutation that impairs its ability to bind DNA was found to block the cell cycle but not to affect apoptosis, suggesting that this function doesn't depend on direct transcriptional capacity of FOXO (Ramaswamy et al., 2002). In a mammalian cell culture and *C. elegans* study, mutated DAF-16/FOXO was found able to contribute to tolerance of UV-induced DNA damage independently of its transcriptional activity (Daitoku et al., 2016).

These findings suggest that FOXO proteins have additional roles outside DNA binding, including those of transcriptional co-regulators or cytoplasmic roles unrelated to transcription factors (Figure 1.4). This would add another level of complexity to the protein's regulatory actions. It is unclear, however, whether these DNA-binding independent functions are evolutionarily conserved also in other organisms like *Drosophila*, and whether they also affect other physiological processes *in vivo*.

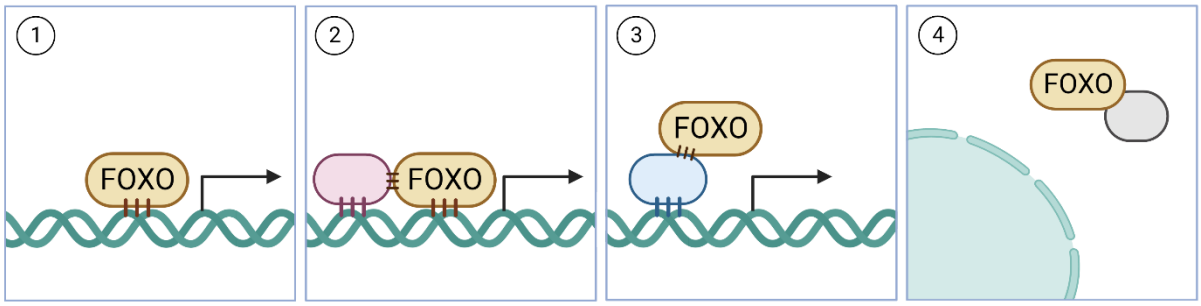

Figure 1.4: Role of FOXO dependent and independent of DNA-binding.
Schematic representation of the main different modes of action of FOXO proteins. Transcriptional functions: 1) Direct binding to the DNA; 2) Indirect binding, through another protein making DNA-contact; 3) Pairwise binding, with both proteins making DNA-contact. Non transcriptional functions: 4) Cytoplasmatic interaction with other proteins (e.g., ATG7).
Adapted from: Schmitt-Ney et al., 2020; Zhao et al., 2010.

1.4 FOXO & metabolism

Many studies have shown a pivotal role of FOXO proteins in regulating metabolism and energy homeostasis in response to nutrient stress. In general, activation of the IIS pathway upon nutrient availability leads to AKT-dependent phosphorylation of FOXO and its inactivation in the cytoplasm; upon starvation, FOXOs are released and activate the transcription of energy metabolism and stress resistance genes (Gross et al., 2008; Jünger et al., 2003; Puig et al., 2003). Activatory phosphorylation of FOXO by AMPK was proven crucial to activate the transcription of specific FOXO target genes to adapt to changes in energy levels (Greer et al., 2007). By modulating the expression of genes involved in glucose uptake and utilization, as well as lipid metabolism and storage, FOXO can affect various aspects of these metabolic functions (Kousteni et al., 2012). Of note, FOXO acetylation was implied in the regulation of many metabolic processes, such as glucose (Langlet et al., 2017; Frescas et al., 2005; Hirota et al., 2003) and lipid (Banks et al., 2011; Mori et al., 2014; Daemen et al., 2013; Chakrabarti et al., 2011) metabolism. Dysregulation of FOXO has also been linked to a variety of metabolic disorders, such as diabetes and obesity (DeFronzo et al., 2015), highlighting the importance of understanding its role in metabolic homeostasis.

1.4.1 FOXO in glucose metabolism

The role of FOXO in gluconeogenesis and glycolysis has been particularly well studied in mouse models (Cheng et al., 2011), where FOXO1 has been designated as the general regulator of energy metabolism (Kousteni et al., 2001). This protein is mainly expressed in tissues involved in glucose homeostasis, like fat tissue, pancreas and liver (Matsumoto et al., 2007). In mice, homozygous null mutation of FOXO1 is embryonically lethal (Furuyama et al., 2004; Hosaka et al., 2004), confirming the non-replaceable role of this factor.

In vitro studies in mammalian cells confirmed that FOXO1 induces transcription of genes for enzymes involved in glucose metabolism, such as the glucose 6-phosphatase (G6Pase) (Nakae et al., 2001; Schmoll et al., 2000) and the phosphoenolpyruvate carboxykinase (PEPCK) (Yeagley et al., 2001). Activation of FOXO1 target genes in hepatocytes results in enhanced gluconeogenesis and

increased release of glucose from hepatocytes (Gross et al., 2008; Puigserver et al., 2003; Frescas et al., 2005). Moreover, this mechanism is influenced by the acetylation state of FOXO, where Sirt-dependent deacetylation of FOXO1 triggers the expression of gluconeogenic genes (Frescas et al., 2005).

From a molecular perspective, the tissue-specific metabolic functions of FOXO are fine-tuned by the interaction with other binding partners. For example, in liver, intestine, kidney and pancreas, FOXO1 interacts with the steroid nuclear receptor HNF-4 (hepatocyte nuclear factor) (Hirota et al., 2003), a transcription factor that promotes glucose utilization (Hayhurst et al., 2001; Drewes et al., 1996). FOXO1 can repress the activity of HNF-4 by binding to its DNA-binding domain and inhibiting its transactivation. Activation of the IIS pathway and phosphorylation of FOXO1 by AKT decreases its binding affinity to HNF-4, leading to the induction of HNF-4 transactivation and the expression of glycolytic genes like *gck* (glucokinase). Interestingly, acetylated FOXO1 cannot bind to HNF-4, while Sirt-1 mediated deacetylation of FOXO1 enables its binding to the *gck* promoter and disruption of HNF-4 binding (Hirota et al., 2003). In a recent study, the interaction of FOXO1 with a co-repressor, SIN3A, was implicated in the selective expression of the pro-glycolitic gene *gck* in the liver (Langlet et al., 2017).

1.4.2 FOXO in lipid metabolism

Several studies have implicated FOXO1 in lipid metabolism and transport, as well as in the development of lipid-related diseases (Chu et al., 2011; Lettieri-Barbato et al., 2014; Wang et al., 2014; Yang et al., 2014). FOXO1 also coordinates the expression of genes related to adipocyte differentiation (Munekata et al., 2014) and white-to-brown fat conversion (Ortega-Molina et al., 2012).

In liver and adipose tissue, FOXO1 controls the expression of the adipose triacylglycerol lipase (ATGL) (Chakrabarti et al., 2009; Lettieri-Barbato et al., 2014), a key limiting enzyme in lipolysis whose expression stimulates the usage of triacylglycerols (TAGs) via the “canonical pathway” (Zimmermann et al., 2004). It is also involved in the expression of lipoprotein lipase (LPL) and carnitine

palmitoyltransferase-1 (CPT1) (Kamei et al., 2003; Zhang et al., 2006) and drives the secretion of triglycerides into the plasma by enhancing the expression of apolipoprotein ApoC-III (Almonte et al., 2004; Gross et al., 2008). On the other hand, FOXO1 was shown to induce the expression of lysosomal acid lipase (Lipa) (Lettieri-Barbato et al., 2013) and autophagy-related genes (Xiong et al., 2012) in the mouse adipose tissue and liver, both involved in the lipophagy-mediated utilization of TAGs (Dong et al., 2017).

Several studies have highlighted the function of FOXO in cholesterol metabolism. In a human cell culture study, FOXO was shown to regulate expression of ABCA6, an intracellular ATP-binding-cassette transporter responsive to cholesterol (Gai et al., 2013). HNF-4, a co-repressor of FOXO1, regulates lipid and cholesterol metabolism (Hirota et al.,2003; Hayhurst et al., 2001). In mice, FOXO3 and Sirt6 have been implied in cholesterol biosynthesis, as hepatic deficiency of Sirt6 leads to elevated cholesterol levels whereas Sirt6 or FOXO3 overexpression improves hypercholesterolemia in obese mice (Tao et al., 2013). In particular, FOXO3 was found to suppress expression of SREBP-2, key regulator of cholesterol biosynthesis (Tao et al., 2013).

As FOXO1 controls both glucose and lipid metabolism, the expression of specific gene programs relies on specific FOXO modifications and/or interactions with different binding partners to induce a carbo-lipid switch. In a cell culture study, the interaction of FOXO1 with a co-repressor, SIN3A, was found to regulate hepatic lipid and glucose metabolism (Langlet et al., 2017). In addition, a mice study using constitutively acetylated ($FOXO1^{KQ/KQ}$) and constitutively deacetylated ($FOXO1^{KR/KR}$) FOXO1 clearly demonstrated the significance of FOXO acetylation *in vivo* in the switch between lipid and glucose metabolism (Banks et al., 2011). In contrast to $FOXO1^{KQ/KQ}$ animals, which were embryonic lethal, $Foxo1^{KR/KR}$ mice were characterized by hyperglycemia and insulin resistance, which implies a defective insulin action on hepatic glucose metabolism. As a result of the increased gluconeogenesis and decreased glycolysis, these mice displayed excessive hepatic glucose output; on the other hand, they had low levels of free fatty acids and triglycerides, suggesting that constitutively deacetylated FOXO1 mice preferentially rely on lipids as source of energy (Banks et al., 2011). The results indicate that during periods of nutrient depletion, the deacetylation

of Foxo1 facilitates the production of glucose and, as the fasting continues, the metabolic response shifts from glucose production to lipolysis. Therefore, the acetylation of FOXO1 may act as a safeguard to prevent excessive catabolic activity of FOXO (Banks et al., 2011). Further studies have provided evidence for a role of FOXO acetylation in mediating lipid metabolism, with lipolysis being enhanced upon SIRT1-mediated deacetylation (Mori et al., 2014; Daemen et al., 2013; Chakrabarti et al., 2011).

Interestingly, it was proposed that FOXO1-mediated lipid synthesis modulation doesn't depend on its direct biding to the DNA, but rather on binding to a transcriptional co-regulator (Cooks et al., 2015; Matsumoto et al., 2006). However, the molecular mechanisms behind this regulation are still unclear.

1.4.3 *Drosophila* FOXO in energy metabolism

Studies in *Drosophila* have confirmed that dFOXO protein is involved in the regulation of energy metabolism, driving the expression of the gluconeogenic gene *pepck* and of the lipolytic gene *brummer* (*bmm*) (Wang et al., 2011) under starvation. The fly fat body is the functional analogous of the mammalian tissues for energy storage and homeostasis (Hwangbo et al., 2004). Indeed, as in mammalian tissues, lipid droplets are also found in fly tissues and can be hydrolyzed by lipases (Baker and Thummel, 2007). One mechanism to access the lipid stores is via the autophagic machinery of the cell, in a process defined "lipophagy" (Lettieri-Barbato et al., 2013). Like mammalian FOXO, dFOXO protein has also been implicated in the expression of autophagic proteins (Bai et al., 2013) and *lipase 4* (*lip4*), which participates in lipid catabolism during fasting (Vihervaara and Puig, 2008). This evidence suggests a potential role of dFOXO in lipophagy (Webb and Brunet, 2014).

The link between FOXO and cholesterol regulation seems to be evolutionary conserved in invertebrates. In worms, the binding between FOXO/DAF-16 and the nematode sterol binding protein 1 (NSBP1) mediates the effects of cholesterol on insulin/IGF-1 signaling (Cheong et al., 2013). Also the vitamin-D receptor NHR-8 interacts with daf-16/FOXO to regulate steady-state cholesterol levels in *C. elegans* (Magner et al., 2013).

More recently, cholesterol has been shown to regulate FOXO/DAF-16 localization in the nucleus and to mediate fasting-induced longevity (Ihara et al., 2017). In *Drosophila*, the nuclear receptor DHR96, a direct gene target of dFOXO, acts as a nutrient sensor for low cholesterol levels (Bujold et al., 2010). However, whether regulation of cholesterol metabolism relies on its DNA-binding ability or specific PTMs has not yet been investigated.

A side effect of high fat or sugar consumption is hypertriglyceridemia, which is often correlated with metabolic disorders such as insulin resistance and obesity (DeFronzo et al., 2015). *Drosophila* has already been used as a model to study complications derived from an excess of dietary sugar (van Dam et al., 2020; Lennicke et al., 2020; Morris et al., 2012; Musselman et al., 2011; Rovenko et al., 2015) and represents a valuable tool for the understanding of metabolic conditions that mirror human pathologies. Preliminary studies have already implicated dFOXO in survival upon sugar-induced lifespan dysregulation (van Dam et al., 2020), but further experimental testing is needed for a deeper mechanistic understanding of the role of FOXO in this context.

1.5 Aims of the thesis

FOXO transcription factors constitute the central node of the IIS pathway, with a pivotal role in modulating ageing and metabolism. As these proteins respond to a variety of stimuli upon different metabolic and stress conditions, the specificity of their action is regulated by post-translational modifications and interactions with binding partners. In particular, acetylation is an evolutionarily conserved mechanism that regulates FOXO function, influencing its cellular localization and its ability to bind to DNA or other effector proteins. Moreover, besides its known function as a transcription factor, in recent years more and more studies have suggested that FOXO has regulatory roles independent of DNA binding. However, the precise role of FOXO acetylation on longevity and metabolism, as well as its DNA-binding independent functions, are not fully investigated. In mammals, FOXO proteins are encoded by four different genes, whereas *Drosophila* genome only harbors one *dfoxo* gene, constituting a powerful genomic tool to investigate FOXO function independent of the redundancy of its mammalian paralogs.

With this in mind, making use of previously generated dFOXO mutant lines to either mimic dFOXO acetylation or abolish its binding to DNA, I aim to shed light on the specific contribution of dFOXO acetylation and DNA-binding ability in regulating physiological functions *in vivo*.

Section I: Role of dFOXO acetylation

Previous studies have suggested that FOXO acetylation may mediate the response to starvation and regulate metabolism, but how acetylation regulates these functions *in vivo* is not fully understood. Thus, I aim to:

- assess the role of dFOXO acetylation in the response to specific metabolic stress, such as starvation or excess of nutrients;

- investigate the acetylatable lysine residues that are important in mediating dFOXO function, by generating novel partial acetylation mutant flies;

- analyze the proteomic profile of dFOXO mutant flies subjected to specific nutrient starvation.

Section II: Role of dFOXO DNA-binding ability

By using dFOXO DNA-binding domain mutant flies, I will investigate the metabolic functions that are dependent and independent of FOXO capacity to bind DNA. In particular, I will research the role of FOXO DNA binding in regulating sugar and lipid metabolism.

MATERIALS & METHODS

2. Materials & Methods

2.1 Fly work

2.1.1 Fly maintenance

Fly stocks were maintained and experiments were conducted at 25ºC on a 12h light and 12h dark cycle, at 65% humidity. Experimental flies were reared at controlled densities into fly stock bottles containing 1SYA (sugar-yeast-agar) food (see Table 2.1 below). Newly eclosed adult flies were allowed to mate for 48h, then sorted according to gender and transferred to the experimental food.

2.1.2 Fly lines used in this study

dFOXO mutant flies used in this study were generated by Dr. Victor Bustos. Briefly, he generated knock-out parental lines in which a region of the dFOXO gene was replaced by an attP site using ends-out homologous recombination (Huang et al., 2009). pGEattBGMR constructs containing modified portions of the d*foxo* gene were injected into embryos of the KO-parental lines, allowing reintroduction by site-directed integration.

All mutant flies used for experiments were backcrossed for at least 6 generations into the wild-type white Dahomey (wDahT) strain, which did not contain the endosymbiotic bacterium *Wolbachia pipientis* (Grönke et al., 2010).

Table 2.1: ***Drosophila stocks used in this study.***

Fly line	Chromosome	Obtained from	Function
w[DhaT]		(Grönke et al., 2010)	Experiments
w;;dfoxoΔV3	3	Victor Bustos	Experiments
w;;dfoxo-V3-3xFLAG	3	Victor Bustos	Experiments
w;;dfoxo-V3-mCherry	3	Victor Bustos	Experiments
w;;dfoxo-V3-DBD2-3xFLAG	3	Victor Bustos	Experiments
w;;dfoxo-V3-5KR-3xFLAG	3	Victor Bustos	Experiments
w;;dfoxo-V3-5KQ-3xFLAG	3	Victor Bustos	Experiments

w,vas-int;dfoxoΔV3w[-]	3	Victor Bustos	Microinjection
w;;dfoxo-V3-2KQ-3xFLAG	3	Bruna Di Giacomo	This study
w;;dfoxo-V3-3KQ-3xFLAG	3	Bruna Di Giacomo	This study
w;;dfoxo-V3-3KQII-3xFLAG	3	Bruna Di Giacomo	This study
w;;dfoxo-V3-2KQ-mCherry	3	Bruna Di Giacomo	This study
w;;dfoxo-V3-3KQII-mCherry	3	Bruna Di Giacomo	This study
w;;dfoxo-V3-5KQ-mCherry	3	Bruna Di Giacomo	This study
w;;dfoxo-V3-5KR-mCherry	3	Bruna Di Giacomo	This study

2.1.3 Preparation of fly media

2.1.3.1 SYA media

For fly stock maintenance and yeast/sugar starvation experiments, SYA media was used and starvation food was prepared omitting the respective component during normal 1SYA preparation. For high-sugar diet experiments, sugar was increased 2-fold (S10%) or 4-fold (S20%) in the final concentration.

Table 2.2: ***SYA media used for fly maintenance and generation of experimental flies.***

	1SYA (standard)	**SYA -Yeast**	**SYA -Sugar**	**S10%**	**S20%**
Sugar	50g	50g	-	100g	200g
Yeast	100g	-	100g	100g	100g
Agar	15g				
Nipagin (10% in EtOH)	30ml				
Propionic acid	3ml				
H_2O	Up to 1L				

2.1.3.2 Defined diet

Holidic media were prepared according to (Piper et al., 2013), adjusting the amino acid content to an exome-matched AA ratio (Piper et al., 2017) (Table 2.3). Briefly, sucrose, agar, amino acids with low solubility (L-isoleucine, L-leucine and L-tyrosine), metal ions and cholesterol were combined with milliQ water and autoclaved at 120 °C under constant stirring for 15 min, using Mediaclave10 media preparator (Integra Biosciences). After autoclaving, filter-sterilized stock solutions of acetate buffer, amino acids, vitamins, nucleosides, choline, inositol and preservatives were added. Specific nutrients starvation food (YAA - cholesterol, - vitamins, - nucleic acids, - trace elements, - lipids) was prepared omitting the respective component. The final volume was adjusted adding the same volume of ethanol or water present in the standard defined diet. Diets with modified amino acid content were prepared similar to holidic media by only reducing or completely removing the specific amino acid in the EAAs stock solution (Table 2.4). To perform the sugar starvation assay in the defined diet, sucrose was omitted from the preparation. To test the effect of other nutrients deprivation on the dFOXO mutant flies, a food including only sugar and amino acids was prepared. In the table, components omitted in the respective starvation diets are highlighted in bold.

Table 2.3: ***Holidic media recipes, adapted to an exome-matched amino acid ratio. Amino acids or other nutrients modified in experimental diets are highlighted in bold.***

** in YAA - nucleic acids, YAA - lipids or YAA - cholesterol/lipids, stocks of nucleic acids and lipids were prepared separately.

Holidic media recipes	**YAA (standard)**	**YAA -Sugar**	**Sugar + AAs**	**YAA - specific ingredient**
Total volume (L)	1	1	1	
mM sucrose	50	-	50	
mM biologically available N	200	200	200	
Agar (g) ***Sucrose** (g)* ***Cholesterol** (ml)*	20 17.12 15	20 - 15	20 17.12 -	

Isoleucine* *(g)*	1.12	1.12	1.12	omitted in YAA - cholesterol
Leucine* *(g)*	2.03	2.03	2.03	
Tyrosine* *(g)*	0.93	0.93	0.93	
Acetate Buffer (ml)	100	100	-	
CaCl2 *(1000x, ml)*	1	1	-	omitted in YAA - trace elements
MgSO4 *(1000x, ml)*	1	1	-	
CuSO4 *(1000x, ml)*	1	1	-	
FeSO4 *(1000x, ml)*	1	1	-	
MnCl2 *(1000x, ml)*	1	1	-	
ZnSO4 *(1000x, ml)*	1	1	-	
MilliQ water (ml)	805.09	805.09	805.09	
Nucleic acids/lipid solution *(ml)*	8	8	-	omitted in YAA - nucleic acids or YAA - lipids **
EAA* *(ml)*	60.51	60.51	60.51	
NEAA* *(w/o cys, ml)*	60.51	60.51	60.51	
Glutamate* *(ml)*	15.79	15.79	15.79	
Cysteine* *(ml)*	7.10	7.10	7.10	
Vitamins *(ml)*	21	21	-	omitted in YAA - vitamins
Folic acid *(ml)*	1	1	-	
Propionic acid (ml)	6	6	-	
Nipagin (ml)	15	15	-	

Table 2.4: ***Amino acid content in stock solutions of standard and modified YAA media.*** **** In YAA -AAs, all the EAAs and NEAAs were completely removed.***

	***AAs amount in YAA**	***AAs in YAA-AAs (all)**	***AAs in YAA-R**	***AAs in YAA-M / M30%**
EAA stock solution	(g/200 ml)	(g/200 ml)	(g/200 ml)	(g/200 ml)
F (L-phenylalanine) H (L-histidine) K (L-lysine) T (L-threonine) V (L-valine) W (L-tryptophan) **R** (L-arginine) **M** (L-methionine)	3.47 2.25 4.69 3.81 4.13 1.10 5.61 2.07	- - - - - - - -	3.47 2.25 4.69 3.81 4.13 1.10 **-** in YAA-R 2.07	3.47 2.25 4.69 3.81 4.13 1.10 5.61 **-** in YAA-M **0.62** in YAA M30%
EAAs added as solid	(g/l of medium)	(g/l of medium)	(g/l of medium)	(g/l of medium)
I (L-isoleucine) L (L-leucine) Y (L-tyrosine)	1.12 2.03 0.93	- - -	1.12 2.03 0.93	1.12 2.03 0.93
NEAA stock solution	(g/200 ml)	(g/200 ml)	(g/200 ml)	(g/200 ml)
A (L-alanine) D (L-aspartic acid) G (L-glycine) N (L-asparagine) P (L-proline) Q (L-glutamine) S (L-serine)	3.79 4.03 2.64 3.54 3.36 3.85 4.74	- - - - - - -	3.79 4.03 2.64 3.54 3.36 3.85 4.74	3.79 4.03 2.64 3.54 3.36 3.85 4.74
Other AA stock Solution	(ml/l of medium)	(ml/l of medium)	(ml/l of medium)	(ml/l of medium)
C (L-Cysteine) (50 mg/ml stock) E (L-Glutamate) (100 mg/ml stock)	7.10 15.79	- -	7.10 15.79	7.10 15.79

2.1.4 Lifespan and stress assays

For lifespan assays, 150 once mated female flies per genotype and diet were transferred into vials containing different experimental diets (SYA- or YAA-based), at a density of 15 flies per vial. Flies were transferred into new vials every 2-3 days, and the number of dead flies was scored on the day of the transfer. The sorting day was classified as day 0 of the lifespan experiment.

For full starvation or yeast starvation assays, flies were sorted by sex at 15-20 flies per wide plastic vial, 8-10 vials per genotype. Flies were first kept 7 days on SYA food and then transferred to the starvation medium (1% w/v agarose, for full starvation, or SYA -yeast, for yeast starvation). Dead flies were scored 3 times per day.

2.1.5 Fecundity assay

For fecundity assays, eggs were collected over 16-20 h periods at several timepoints during the first 1-2 weeks of lifespan experiments. The number of eggs laid per vial at each time point was scored using a hand counter. The values are expressed as the mean number of eggs laid per female fly per 24 h ± SD or as cumulative eggs laid per female fly.

2.1.6 Developmental assay and body weight

For development timing, flies were allowed to lay eggs for 5h on grape juice plates. 200 eggs per genotype and diet were collected and transferred to vials at a density of 20 per tube. Pupae formation and, eventually, adult flies eclosion were recorded 4 times a day at 3-hours intervals.

For body weight measurement, batches of 2 flies were briefly anesthetized under CO_2 and weighted on a ME235S analysis balance (Sartorius Mechatronics). A total of 20 flies per genotype was measured.

2.2 Molecular biology methods

2.2.1 Cloning of injection plasmids

Donor constructs containing mutations of the V3 region of dFOXO were obtained by custom gene synthesis (Genscript) and subsequently subcloned into the pGEattBGMR vector. All restriction digest reactions were performed with enzymes provided by NEB according to their user's manual. Antarctic phosphatase (NEB #M0289) was used for dephosphorylation of 5'-ends of the pGEattBGMR vector and T4 DNA Ligase (NEB) was used for ligation reactions. Chemically competent OneShot TOP10 *Escherichia coli* (LifeTechnologies) were used for transformation of ligation reactions or plasmids following the manufacturer's instructions. For positive selection of transformants, 100 µg/mL ampicillin in LB plates was used. To purify plasmids out of bacteria, the QIAprep Miniprep or Midiprep Kits (Qiagen) were used. Cloning success was verified by Sanger sequencing at Eurofins Genomics. Vectors and method used are listed below (Table 2.5).

Table 2.5: ***Vectors used for cloning.***

Name	Vector backbone	Primer name	Method
pUC19-V3 2KQ	pUC19	Expression vector	Restriction digest via NheI/SpeI
pUC19-V3 3KQ	pUC19		
pUC19-V3 3KQII	pUC19		
pGEattBGMR-V3-2KQ-FLAG	pGE attB GMR-FLAG	Injection vector	
pGEattBGMR-V3-3KQ-FLAG	pGE attB GMR-FLAG		
pGEattBGMR-V3-3KQII-FLAG	pGE attB GMR-FLAG		
pGEattBGMR-V3-2KQ-mCherry	pGE attB GMR-mCherry		
pGEattBGMR-V3-3KQ-mCherry	pGE attB GMR-mCherry		

pGEattBGMR-V3-3KQII-mCherry	pGE attB GMR-mCherry		
pGEattBGMR-V3-5KQ-mCherry	pGE attB GMR-mCherry		
pGEattBGMR-V3-5KR-mCherry	pGE attB GMR-mCherry		

2.2.2 Generation of new transgenic fly lines

pGEattBGMR gene replacement constructs were injected into embryos of the dFOXO KO-parental line ΔV3 w[-], previously generated by Dr. Victor Bustos. Microinjections were performed with ~200 ng/µL DNA by Jacqueline Eßer at the Max Planck Institute for Biology of Ageing, or sent out to the FlyORF Injection Service. All generated fly lines were homozygous viable, except for the line w;;dfoxo-V3-3KQ-mCherry, that was only viable in the heterozygous state.

Table 2.6: ***New transgenic fly lines generated in this study.***

Transgenic flies	Vector used	Injected into
w;;dfoxo-V3-2KQ-3xFLAG	pGEattBGMR-V3-2KQ-FLAG	w,vas-int;dfoxoΔV3w[-]
w;;dfoxo-V3-3KQ-3xFLAG	pGEattBGMR-V3-3KQ-FLAG	w,vas-int;dfoxoΔV3w[-]
w;;dfoxo-V3-3KQII-3xFLAG	pGEattBGMR-V3-3KQII-FLAG	w,vas-int;dfoxoΔV3w[-]
w;;dfoxo-V3-2KQ-mCherry	pGEattBGMR-V3-2KQ-mCherry	w,vas-int;dfoxoΔV3w[-]
w;;dfoxo-V3-3KQ-mCherry/ Tm3Sb	pGEattBGMR-V3-3KQ-mCherry	w,vas-int;dfoxoΔV3w[-]
w;;dfoxo-V3-3KQII-mCherry	pGEattBGMR-V3-3KQII-mCherry	w,vas-int;dfoxoΔV3w[-]
w;;dfoxo-V3-5KQ-mCherry	pGEattBGMR-V3-5KQ-mCherry	w,vas-int;dfoxoΔV3w[-]
w;;dfoxo-V3-5KR-mCherry	pGEattBGMR-V3-5KR-mCherry	w,vas-int;dfoxoΔV3w[-]

2.2.3 Preparation of genomic DNA and Polymerase chain reaction (PCR)

To genotype flies by PCR, genomic DNA (gDNA) was isolated from individual flies. Therefore, single flies were homogenized in a tube containing 50µL Squishing buffer (10mM Tris, 25mM NaCl, 1mM EDTA) with freshly added Proteinase K (final concentration, 200 µg/mL) and incubated for 30min at 37ºC. After inactivation of the Proteinase K at 95 °C for 3 min, the fly homogenate was centrifuged for 15 min at 16,000 x g, and the supernatant containing gDNA was either immediately used to perform PCR or stored at 4 °C until further usage. Standard genotyping PCRs were performed with HotStarTaq Plus MasterMix (Qiagen), following the manufacturer's instructions and using 1µl of DNA template.

Table 2.7: ***Primers used for genotyping.***

Name	Target	Fwd/Rev	Sequence
SOL669	dFOXO-V3 region	fwd	TGCACAACCGCTTTATGAGGGT
SOL670	dFOXO-V3 region	rev	CAGTTGATAGTTACCTGTGGAG
SOL728	V3 Tags	fwd	TCACAGCCCAGCGTGGTGACCTCGCCACCATCCT
SOL729	V3 Tags	rev	AGTTATGGTACCGGCGCGCCAATTAAAGTGATATTA

2.2.4 Agarose gel electrophoresis

PCR products were analyzed on TAE buffered 0.5-2% agarose gels, depending on the expected band size. DNA was stained by 10 µL GelRed (Thermo Fisher Scientific) per 100mL agarose gel and electrophoresis was performed on horizontal electrophoresis cells (BioRad) for 30-40 minutes at 130 V. Hyperladder 50bp or 1 kb (Bioline) was used as size marker. For cloning, DNA fragments were eluted from the gel using the QIAquick Gel Elution Kit.

2.3 Biochemistry

2.3.1 Protein extraction and immunoblotting

15-20 fly tissues per sample were homogenized in ~300µl RIPA buffer (Pierce, Thermo Fisher Scientific), freshly supplemented with Protease inhibitors (Pierce, Thermo Fisher Scientific), using a hand homogenizer. After centrifugation, samples were boiled for 5 min and 5% β-mercaptoethanol was added in a 1:4 ratio. Equal amounts of proteins were loaded and separated on pre-stained 7.5% / any kDa SDS-PAGE gels (Bio-Rad). Proteins were wet-transferred onto an unsupported nitrocellulose membrane (GE Healthcare) at 100V for 40 min and, subsequently, blocked in 5% non-fat dry milk powder / TBST at room temperature. Primary antibody incubation was done overnight at 4ºC; the following day, membranes were incubated with HRP-coupled secondary antibodies for 1h at RT. Signal was recorded using ECL Western Blotting Detection Reagents (GE Healthcare) and the ChemiDocImager (BioRad).

For protein glycation assay, levels of glycation of whole flies (n=15 flies per samples) were assessed by western blotting using anti-AGE (Advanced Glycation End-product) antibody (1:2000, Millipore). The quantified lanes were normalized against Stainfree.

Table 2.8: ***Antibodies used for western blots.***

Antibody	Dilution	Source
dFOXO	1:5000	Dr. Nazif Alic (Giannakou et al., 2007)
FLAG (M2)	1:5000	Sigma (F1804)
mCherry	1:1000	Abcam (AB_2571870)
Ac-Lysine	1:1000	Santa Cruz Biotechnology (AKL5C1)
AGE (Advanced Glycation End-product)	1:2000	Millipore (AB9890)
Tubulin	1:10000	Sigma (T9026)
Actin	1:10000	Abcam (8224)

2.3.2 Immunoprecipitation

For IP/western blotting experiments to detect FOXO acetylation, 30 flies per sample were homogenized using a hand homogenizer in 600 µl lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 % Triton X-100), freshly supplemented with protease inhibitors (Pierce, Thermo Fisher Scientific) and deacetylase inhibitors (Trichostatin A, 500 nM; Sodium butyrate, 10 mM). The lysates were then centrifuged at full speed for 15 min at 4°C to remove debris; a portion of the lysate was retained as input and the rest was incubated (o/n, 4°C) with 40 µl of pre-equilibrated anti-FLAG M2 agarose beads (Sigma). The resin was centrifuged at 5000 g for 1 minute to discard the supernatant (unbound fraction) and then washed x3 with 500 µL TBS. Bound FLAG-tagged proteins were eluted by boiling the beads in loading buffer (50mM Tris pH 6.8, 2%SDS, 10%glycerol, 1% -mercaptoethanol, 12.5mM EDTA, 0.02% bromophenol blue) for 5 minutes. IP-samples were run on SDS-PAGE gels (BioRad) for western blot analysis.

2.3.3 Malpighian tubules dissection and imaging

Dissection of Malpighian tubules was performed as previously described in van Dam et al. (2020). Briefly, mated female flies kept on a 4XSugar 1XSYA diet (S20%) for 28 days were anesthetized on ice and dissected in ice-cold PBS. Malpighian tubules from 15 flies per genotype and condition were transferred onto poly-L-lysine coated slides, kept at 4°C over-night and imaged the next day on a Zeiss Axioscan 7 Microscope Slide scanner in both bright and polarized light. To quantify the tubule stone phenotype, Malpighian tubules per fly per fly were scored as described in Figure S9 and assigned to categories (0-4) based on the severity of the phenotype.

2.3.4 Larval fat body dissection and imaging

Fat body from mCherry-tagged dFOXO mutant larvae was dissected in cold 1xPBS under a stereomicroscope and fresh tissues were transferred on poly-L-lysine coated slides. For detection of the endogenous mCherry fluorescence and imaging, a Leica SP8X confocal microscope was used (FACS & Imaging Core Facility).

2.3.5 Whole body lipid assay

Lipid assays were performed as previously described in Grönke et al. (2003). 5 female flies per sample were homogenized in 100 µL PBS+0.05%Tween-20 using Fastprep-24 system (MP Biomedicals™). After inactivation at 70°C for 5 minutes, homogenates were clarified with centrifugation and incubated (50 µL) with 200 µL pre-warmed assay buffer (Infinity™ Triglycerides reagent, Thermo Scientific). Values were determined based on a standard curve using Triglyceride standards (Cayman Chemical) and normalized to protein content measured by BCA assay (Pierce).

2.3.6 Uric acid assay

Total uric acid content of female flies kept for 28 days on a standard (ctrl) or 4Xsugar diet (S20%) was performed using the QuantiChrom™ Uric Acid Assay Kit (DIUA-250, BioAssay Systems) as previously described (van Dam et al., 2020). 5 flies per sample were homogenized in 100 µL PBS+0.05%Tween-20 using Fastprep-24 system (MP Biomedicals™). Homogenates and standards were loaded in a 96-well plate, incubated with assay buffer for 30 minutes at 30°C and quantified spectrophotometrically measuring absorbance at 590nm in a plate reader (Tecan Life Sciences).

2.3.7 Mass spectrometry

Samples were prepared according to the protocol for LC MS/MS from the Proteomics Facility of the Max Planck Institute for Biology of Ageing (version April 2020). 4 biological replicates were used per condition.

Whole frozen bodies (15 per sample) were first disrupted using TissueLyser II (QIAGEN), and the pellet resuspended in 150 µL of freshly prepared lysis buffer (6M Guanidinium chloride, 2.5mM TCEP, 10mM CAA, 100mM Tris-HCl). Samples were heated at 95°C for 20 minutes and then sonicated with the Bioruptor (30 sec sonication, 30 sec break, 10 cycles, high performance). After centrifugation at 20000g for 20 minutes, supernatant was collected into new tubes and diluted 10 times with 20mM Tris to measure concentration by Nanodrop. 300 µg of protein, diluted 10-fold

in 20mM Tris, was digested o/n at 37°C with Trypsin (Promega, Mass Spectrometry grade) in a ratio trypsin:protein of 1:200 (w/w). The next day, digestion was stopped by addition of 100% formic acid (FA) to 1% (final concentration). Peptide cleaning was performed with 30µg C18 StageTips (Thermo Fisher Scientific), previously wetted with 60% Acetronitrile (ACN)/0.1% FA and then equilibrated with 0.1% FA. Sample elution was done in 80 µl 40%ACN/0.1% FA by centrifuging (500g x 10min). Peptides were consequently dried using SpeedVac Vacuum Concentrator and the pellet resuspended in 20 µl of 0.1% FA. Peptides concentration was assessed by NanoDrop and 4 µg of peptide for each sample were again dried in a SpeecVac and used for mass spectrometry (Orbitrap Fusion). Quantification of peptides and proteomic analysis was done by Dr. Ilian Atanassov (Proteomics core facility).

2.3.8 Proteomic analysis and GO terms enrichment

Differentially expressed proteins (DEPs) were visualized with Venn Diagrams for up- and down-regulated proteins created using the open source FLASKI version 3.14.10 (Iqbal A. et al., 2021; https://flaski.age.mpg.de/). GO analysis was performed through the DAVID app of FLASKI (Iqbal A et al., 2021) selecting the terms BP, MF and KEGG. The web server Revigo (Supek F. et al., 2011; http://revigo.irb.hr/) was used to summarize GO terms by removing redundant terms. Significantly enriched GO terms were sorted by adjusted p-value (Bonferroni $p<0.05$) and presented as barplot or dotplot. Barplot representation was created with GraphPad prism and top 10 significant terms for each category were shown. Dotplot representation was done by Dennis Gadalla. 40 unique top GO terms were selected sorting them by Bonferroni-adjusted p-value within each group, then in sequence selecting the respective top term in each group until 40 terms were reached. Dotplots were clustered by the adjusted p-value using the clustermap algorithm (method: “average”, metric: “euclidean”) of the python seaborn package (v0.11.1).

For visualization of DEPs with volcano plots, the web app VolcaNoseR (Goedhart J. and Luijsterburg M.S., 2020; https://huygens.science.uva.nl/VolcaNoseR2/) was used. Significance (adjusted p-value) and fold-change were converted to −Log10(adj. p-

value) and Log2(fold-change), respectively. The cut-off of for significance was set to 0.05, while no threshold was set for the fold-change.

2.4 Statistical analysis

Statistical analysis for lifespan and stress assays was performed in Excel (Microsoft) using log-rank test. All other statistical analysis were performed in GraphPad Prism. Individual statistical tests are mentioned in the respective figure legends. All data are presented as average ± standard deviation (SD). Significance was determined according to the p-value: *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

SECTION I: dFOXO ACETYLATION

3. Section I: dFOXO acetylation

3.1 Introduction

FOXO transcription factors regulate a variety of biological processes, including cell growth, metabolism, and survival. Therefore, the function of FOXO is tightly regulated by a variety of post-translational modifications, including acetylation (Daitoku et al., 2011; Wang et al., 2016). In mammals, acetylation of FOXO that occurs on lysine residues within the NLS has been shown to decrease its transcriptional activity, by reducing DNA binding and promoting nuclear exclusion (Matsuzaki et al., 2005; Qiang et al., 2010). Since some of these residues are evolutionarily conserved, it has been proposed that this kind of acetylation also happens in *Drosophila* (Wang et al., 2011), although direct evidence is still lacking.

Previous observations have already suggested that acetylation regulates multiple metabolic functions of FOXO proteins in mammals and flies. A knock-in study in mice has revealed a fundamental role for the acetylation of FOXO1 in the regulation of glucose and lipid metabolism (Banks et al., 2011). On the other hand, deacetylation of lysine residues in the NLS was found to be necessary to activate the starvation response in *Drosophila* (Wang et al., 2011).

However, the exact role of FOXO regulation by acetylation, and its effect on lifespan and metabolism, has not fully been explored in the fly. Moreover, it is still not clear which lysine residues are responsible for modulating dFOXO function.

3.2 Flies used in this study

To better understand the role of dFOXO acetylation, in this study the following fly lines were used:

- As a wild-type-like positive control, a fly line in which the endogenous dFOXO protein was tagged with a FLAG tag, termed FLAG.

- A dfoxo-null mutant, termed *dfoxo-null* ΔV3, was used as a control for complete loss of dFoxo function.

- Fly lines in which 5 conserved Lys residues in the NLS were changed to glutamine (5KQ, acetyl-mimetic mutants) or arginine (5KR, acetyl-null mutants) were used to study the influence of the acetylation state of the dFOXO protein.

- A fly line carrying two-point mutations (H150A and N146A) in amino acid residues essential for DNA binding, the DBD2 mutant, was used to address the importance of the DNA-binding ability of dFOXO as opposed to its acetylation state.

All fly lines listed here were previously generated by Dr. Victor Bustos by replacing the endogenous *dfoxo* gene using ends-out homologous recombination (Huang et al., 2009) (Figure 3.1).

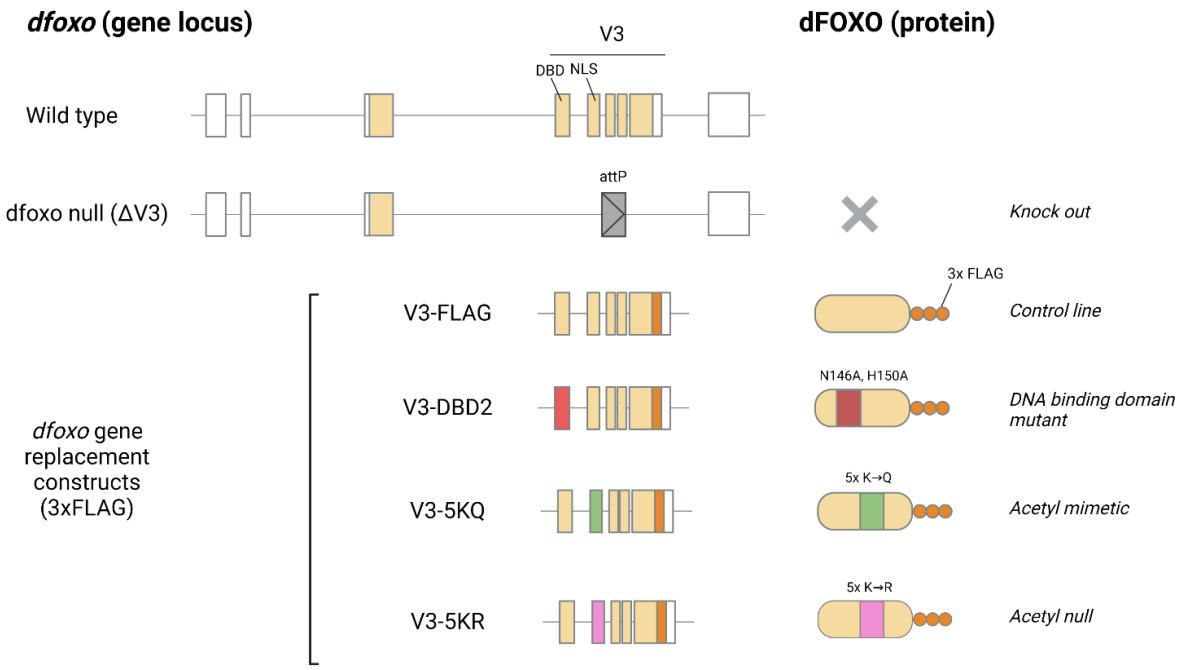

Figure 3.1: dFOXO gene locus.
Representation of *dfoxo* gene locus, FLAG-tagged gene replacement constructs and resultant dFOXO modified protein in the dFOXO mutant fly lines. In *dfoxo-null* flies, the region arbitrarily denoted as V3 was substituted with an attP site that allowed subsequent integration of modified DNA constructs into the gene locus. White boxes represent UTRs and yellow boxes represent CDS. Created with Biorender.com.

This first part of the results focuses on the analysis of the phenotypes related to the acetylation of dFOXO, while the second part discusses in more detail the results obtained for the DBD mutants.

3.3 Results

3.3.1 Role of dFOXO acetylation in mediating the response to specific nutrient starvation

3.3.1.1 Acetylation does not interfere with normal dFOXO function in vivo

In the fly, dFOXO controls biological processes such as lifespan, body size and fecundity (Jünger et al., 2003; Slack et al., 2011). To assess whether physiological fly phenotypes were altered by the modified FOXO alleles, several basic phenotyping assays of the FOXO acetylation mutants were performed. First, we evaluated the lifespan of these flies under continuous feeding or starvation. *foxo-null* ΔV3 mutants were shorter-lived (Figure 3.2A) and more sensitive to starvation (Figure 3.2B) compared to dFOXO-FLAG control flies and acetylation mutants (5KQ, 5KR). Moreover, both body weight and development were not altered by introduction of acetyl-mimicking or acetyl-null mutations, whereas ΔV3 mutants were smaller and had lower pre-adult viability (Figure 3.2C-E). This is consistent with previous observations (Slack et al., 2011) and suggests that the role of dFOXO as a transcription factor, but not its acetylation state, is important in the regulation of these physiological functions *in vivo*.

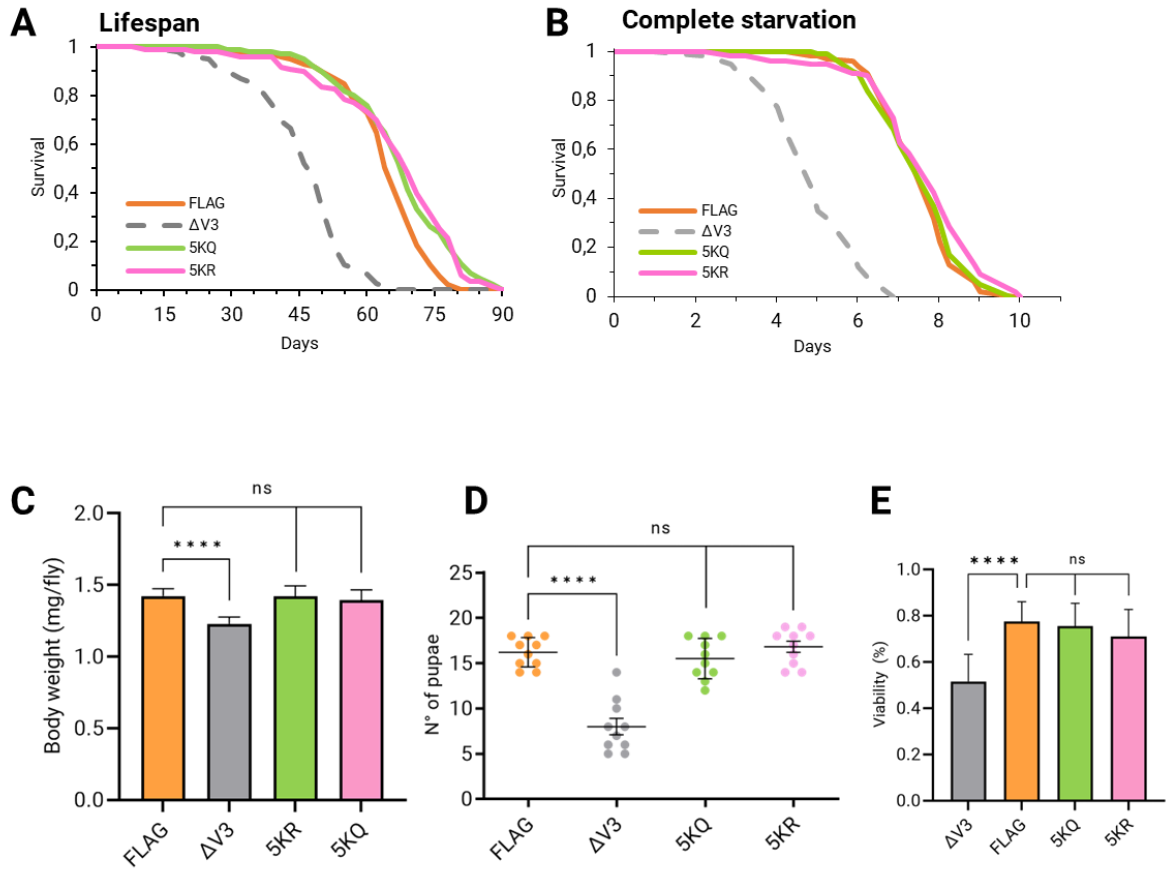

Figure 3.2: FOXO acetylation does not affect normal functions *in vivo*.
A) Female lifespan assay showed that, while ΔV3 (*dfoxo-null*) flies were shorter lived than FLAG control flies, acetyl-lysine mutants lived as long as control flies on a standard SYA diet. ***p<0.001 log-rank test, ΔV3 vs FLAG, 5KQ and 5KR flies. N=150/genotype. Performed by Victor Bustos. B) On a starvation medium, only ΔV3 flies were shorter-lived when compared to controls. ***p<0.001 log-rank test, ΔV3 vs FLAG flies. N=150/genotype. C) Female *dfoxo-null* flies had reduced body weight compared to control (FLAG) flies, while acetylation mutants (5KQ, 5KR) had similar body weight. ns p>0.05, ****p<0.0001, one-way ANOVA followed by Dunnett's multiple comparisons test vs control (FLAG). N=20/genotype. D-E) Development assay showed both a reduced number of pupae (D) and adults (E) in *dfoxo-null* ΔV3 flies with respect to FLAG control line, whereas 5KR and 5KQ flies showed similar development as control. ns p>0.05, ****p<0.0001, one-way ANOVA followed by Bonferroni's multiple comparisons test with control (FLAG). N=10 replicates/genotype, 20 eggs each.

3.3.1.2 dFOXO acetylation mediates the response to yeast starvation

Previous studies have suggested the existence of a strong connection between FOXO acetylation and response to nutrient availability, with acetylation decreasing during starvation and increasing again after refeeding (Banks et al., 2011; Wang et al., 2011). Based on this, and given our finding that acetylation mutants do not show sensitivity to complete starvation, we next examined the effect of specific yeast depletion both on a molecular (Figure 3.3A-C) and phenotypical (Figure 3.3D-F) level. By western blotting analysis, we measured protein expression and acetylation levels of FOXO in control (FLAG) flies that were continuously fed, kept on a yeast-deprived diet for 3 days or re-fed at different time points. We found that FOXO expression significantly increased upon yeast starvation and decreased again 30 minutes and 1 hour after refeeding. Interestingly, 2 hours re-fed flies showed a dramatic increase in FOXO expression (Figure 3B), comparable to the yeast-starved condition. In parallel with its stimulatory effects on protein expression, yeast starvation also decreased FOXO acetylation in flies, while refeeding at early time points enhanced it. At the latest time point, acetylation of FOXO was found to be reduced (Figure 3.3C), which goes hand in hand with FOXO increased protein levels. These observations suggest that acetylation may be involved in regulating the response to yeast starvation. Moreover, the increase of FOXO and paired decrease in its acetylation state possibly hint towards the existence of a compensatory mechanism to restore normal levels of acetylation and protein expression once the metabolic stress is over.

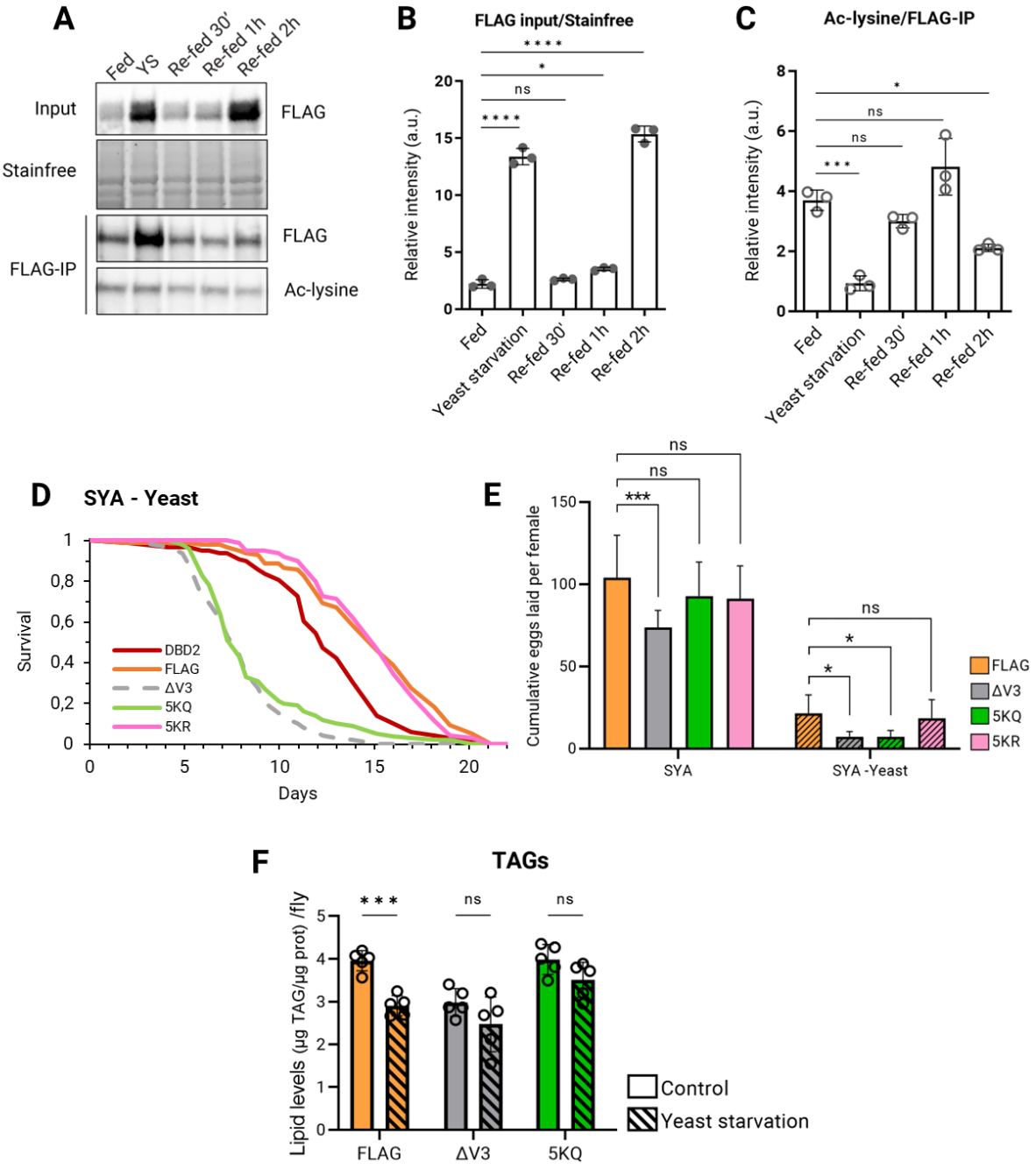

Figure 3.3: Acetylation of dFOXO modulates the response to yeast starvation.
A) Immunoblot showing FOXO expression level and acetylation during yeast starvation (YS) and time course refeeding (re-fed 30', 1h, 2h) in FLAG control flies. B) Quantification of (dFOXO-)FLAG band obtained from total protein extracts (input) showed that dFOXO expression increases during yeast starvation (YS), decreases upon 30'-1h refeeding and goes up again 2h after refeeding. C) Acetylation of FOXO, quantified as ac-lysine/FLAG in the immunoprecipitated samples (FLAG-IP), significantly decreases upon yeast deprivation and increases in the early stage of refeeding. ns $p>0.05$, *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$, one-way ANOVA followed by Tukey's multiple comparisons test. N=3 biological replicates/condition, 30 flies each. D) On a no-yeast diet (SYA -Yeast), ΔV3 and acetyl-mimetic

(5KQ) flies were sensitive to the absence of yeast. In contrast, acetyl-null (5KR) mutants were unaffected by this diet when compared to control flies (FLAG). ***p<0.001 log-rank test, ΔV3 and 5KQ vs FLAG. N=150/genotype. E) Fecundity assay on a yeast deficient diet shows that both *dfoxo-null* (ΔV3) and acetyl-mimetic (5KQ) female flies have reduced cumulative egg production compared to FLAG control and acetyl-null 5KR flies. ns p>0.05, *p<0.05, ***p<0.001, one-way ANOVA vs FLAG. N= 10 biological replicates/genotype, 5 flies each. F) TAG content of female flies kept for 3 days on control (SYA) or yeast deficient food showed that both *dfoxo-null* and 5KQ flies had no significant reduction in lipid levels after starvation. Diet: ****p<0.0001; genotype: ****p<0.0001; interaction: *p<0.05, two-way ANOVA. Bonferroni multiple comparison test: ns p>0.05, ***p<0.001. N=5 biological replicates per assay, each with 5 flies/genotype.

Next, we tested whether mutations that alter the acetylation state of FOXO would affect the survival of flies on a yeast deprived diet. In addition to control (FLAG), *foxo-null* (ΔV3) and acetylation (5KR and 5KQ) mutant flies, DNA-binding domain mutants (DBD2) flies were also included in the starvation assay to verify whether the response to this metabolic stress also depends on FOXO transcriptional ability. Flies carrying the acetylation mimicking mutations (dFOXO-5KQ) were short-lived as much as *foxo-null* flies under yeast starvation, whereas acetyl-null flies (5KR) as well as DBD2 mutants showed no difference compared to control (Figure 3.3D). These findings suggest that acetylation of FOXO mediates survival upon yeast starvation *in vivo* and that this is independent from the ability of FOXO to bind DNA. Fecundity of 5KQ mutant flies, comparable to controls on a standard diet, was also found to be reduced in the absence of yeast (Figure 3.3E), suggesting that acetylation might influence phenotypes other than survival under these nutritional conditions.

Acetylation of FOXO has been reported to modulate lipid metabolism (Banks et al., 2011) and to decrease lipolysis (Chakrabarti et al., 2011). Hence, we analyzed the lipid content of flies subjected for 7 days to a normal or yeast deficient diet. We observed a significant difference in the response to the diet in both *dfoxo-null* and 5KQ flies, which displayed an accumulation of lipid content post-starvation (Figure 3.3F). This suggest that lipid metabolism is influenced not only by the presence of dFOXO, but also by its acetylation state.

3.3.1.3 Complete amino acids deprivation does not recapitulate the yeast starvation phenotype of dFOXO acetylation mutants

Since yeast has a protein content of about 45-50% (Jach et al., 2022), in flies, dietary yeast starvation is often seen as equal to protein restriction. Moreover, FOXO transcription factors have been shown to be involved in amino acid metabolism (Bhardwaj et al., 2022), especially in the context of starvation (Slade et al., 2016; Kramer et al., 2008).

With this in mind, we switched from a standard SYA food to a chemically defined diet or flYAA (Piper et al., 2014; Piper et al., 2017) – in this thesis abbreviated as YAA (Figure 3.4A). After verifying that these mutants survived on the YAA diet similarly to SYA (Figure S1A), we tested the acetylation mutants on a medium specifically lacking all amino acids. Contrary to expectations, the lifespan of acetyl-mimic flies was similar to control flies on the YAA -AAs starvation medium (Figure 3.4B). This suggests that the sensitivity of 5KQ mutants on the yeast starvation food cannot simply be explained by protein restriction, but that the lack of other nutritional components (e.g., cholesterol, vitamins, lipids, nucleic acids, trace elements) might be responsible for the observed phenotype. To test this hypothesis, a food deprived of all additional components but sugar and amino acids was used to perform another starvation assay of the FOXO mutants. As shown by the survival plot in Figure 3.4C, acetylation mutants were sensitive to lack of nutrients other than sugar and amino acids, with a percentage change in median lifespan relative to standard YAA diet similar to *dfoxo-null* flies (Figure 3.3D).

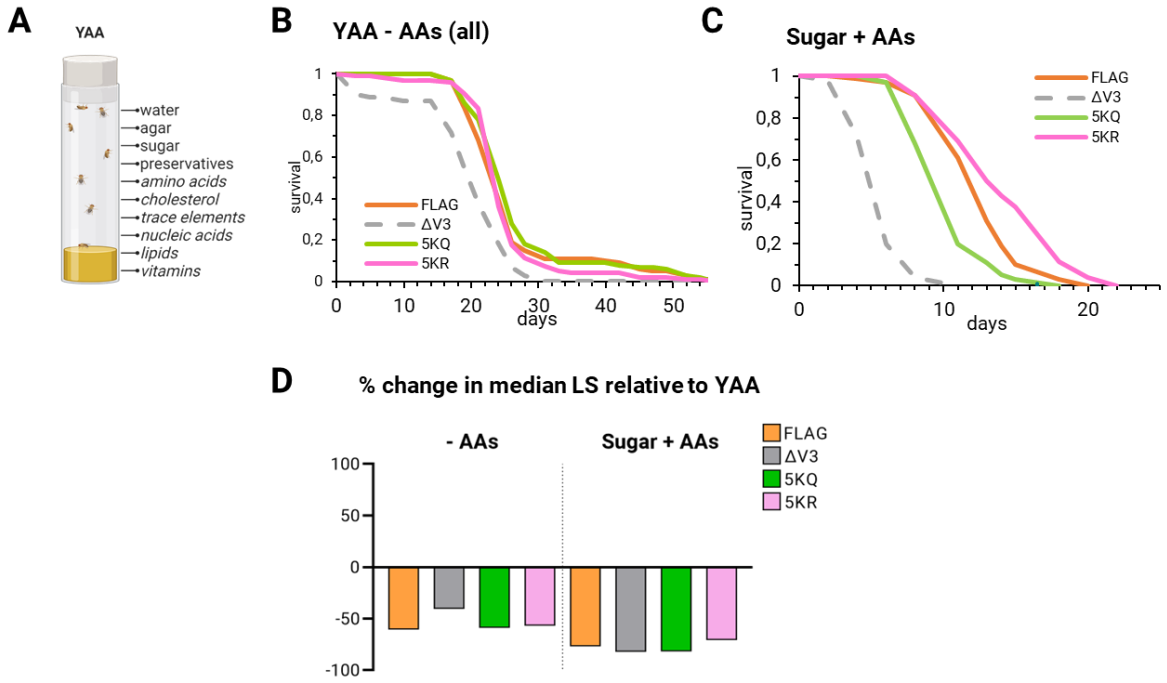

Figure 3.4: Amino acids starvation does not recapitulate the shorter-lifespan phenotype of dFOXO 5KQ mutants observed upon yeast starvation.
A) Schematic representation showing the purified ingredients that compose the defined diet. Created with Biorender.com. B) On a defined medium lacking all essential amino acids (EAAs), 5KQ flies had similar lifespan to FLAG control flies. ns, log-rank test, 5KQ vs FLAG flies. N=150/genotype. C) On a diet in which all nutrients but sugar and amino acids were removed, acetyl-mimetic flies were shorter-lived compared to control flies (FLAG, 5KR). 5KQ vs FLAG and 5KR, ***p<0.001 log-rank test. N=150/genotype. D) Summary of starvation stress presented as percentage change in median lifespan relative to standard YAA diet. 5KQ flies and *dfoxo-null* ΔV3 flies were more affected than others to nutrients starvation.

3.3.1.4 dFOXO acetylation might be involved in the response to cholesterol starvation

In the light of the above results, we next tried to identify the nutrient(s) that underlie the yeast-starvation sensitivity of the acetyl-mimetic flies and tested their resistance on diets systematically lacking all ingredients (Figure 3.5A and Figure S1B-G). While ΔV3 flies were consistently shorter-lived in all starvation media compared to control flies, 5KQ flies were only affected by deprivation of cholesterol, alone (Figure 3.5B) or in combination with lipid starvation (Figure S1F). On the other hand, combined cholesterol/vitamin deprivation did not reduce the lifespan of 5KQ flies compared to controls (Figure S1G), suggesting that the effect of vitamin starvation overrides the effect of cholesterol deprivation.

Despite shortening fly lifespan of about 50% in all genotypes, cholesterol starvation had a bigger impact on the acetylation mutants compared to control and *foxo-null* flies (Figure 3.5D). Moreover, reintroduction of cholesterol in a medium with only sugar and amino acids partially increased the lifespan of 5KQ flies compared to *foxo-null* flies (Figure 3.5C). However, acetyl-mimicking flies were still shorter-lived than controls on this diet and, comparing the percentage change in median lifespan of the cholesterol starvation diet and the sugar + amino acids food (Figure 3.5D), we noticed that cholesterol starvation did not fully recapitulate the phenotype observed in the previous assay (Figure 3.4D), implying that acetylation of dFOXO may be involved in mediating the response to starvation of multiple nutrients, including cholesterol.

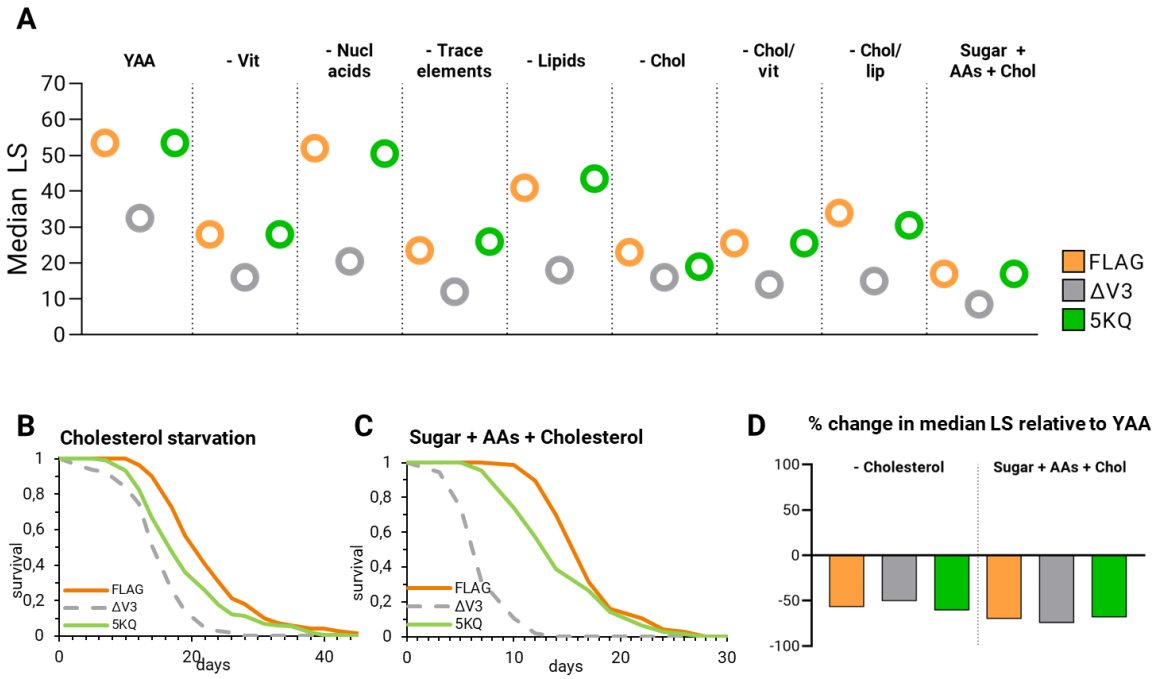

Figure 3.5: dFOXO acetylation might be required to mediate survival upon cholesterol starvation.
A) Median lifespans of female control (FLAG), dFOXO acetylation (5KQ) and dfoxo-null (ΔV3) flies subjected to different starvation mediums. (Lifespan experiments for YAA -Chol and for Sugar+AAs+Cholesterol are shown in (B) and (C), respectively. Lifespan experiments for all other diets are shown in FigureS1). ΔV3 mutant flies were consistently shorter-lived in all diets compared to FLAG control flies. ***p<0.001 log-rank test, ΔV3 vs FLAG. B) On a defined medium lacking cholesterol, both female 5KQ and ΔV3 flies showed a reduced lifespan when compared to FLAG control flies. ***p<0.001 log-rank test, 5KQ and ΔV3 vs FLAG. C) Reintroduction of cholesterol in a diet only composed of sugar and amino acids partially

rescued the lifespan of dFOXO 5KQ flies. ***p<0.001 log-rank test, 5KQ vs ΔV3. N=150/genotype in each assay. D) Summary of starvation assays presented as percentage change in median lifespan relative to standard YAA diet. Upon cholesterol deprivation, 5KQ flies had a higher reduction in median lifespan than FLAG and ΔV3 flies. On a diet with sugar, amino acids and cholesterol, 5KQ flies had a smaller change in median lifespan than FLAG and ΔV3 flies.

Prior evidence had already shown that FOXO proteins are indeed involved in cholesterol metabolism (Afschar et al., 2016; Cheong et al., 2013;), also in the context of acetylation (Tao et al., 2013). In the fly, cholesterol is required for the synthesis of steroid hormones that regulate development and fecundity (Sieber et al., 2015; Bownes, 1994). Based on this, we next wondered whether FOXO acetylation had a role in mediating phenotypes other than survival under cholesterol deprivation and checked effects on fecundity and development. While larval survival was completely abolished on the starvation diet in all genotypes (Figure S1H), fecundity seemed to be reduced depending on the mutation, the major effect being seen in the acetylation mutants (Figure 3.6A and 3.6C). Compared to FLAG control flies, already after 4 days 5KQ acetyl-mimetic flies showed reduced fecundity on a food without cholesterol (Figure 3.6B).

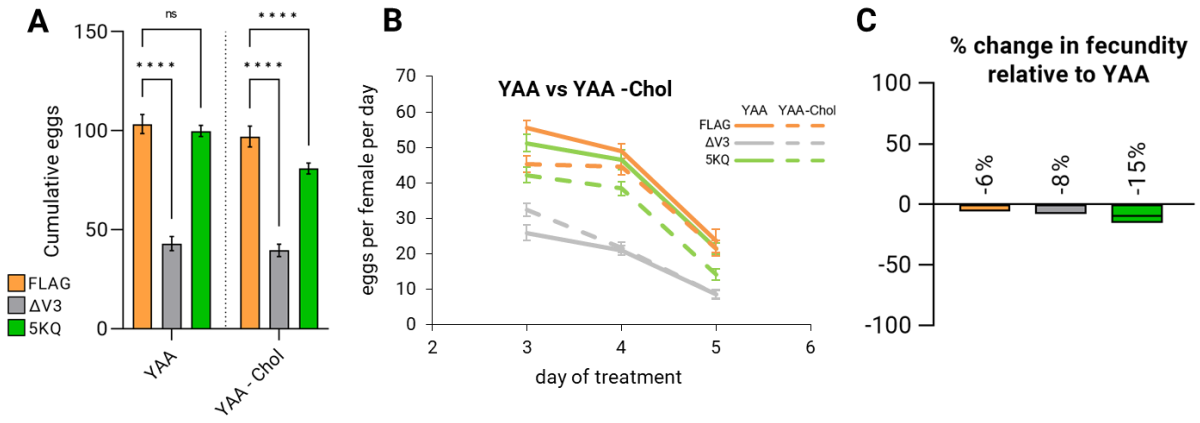

Figure 3.6: Cholesterol deprivation decreases fecundity of dFOXO acetylation mutants.
A) Comparison of cumulative egg production of control (FLAG), *dfoxo-null* (ΔV3) and acetyl-mimetic (5KQ) female flies on YAA control and cholesterol deficient diets. ns p>0.05, ****p<0.0001, one-way ANOVA. B) Complete deprivation of cholesterol reduced fecundity of control and dFOXO mutant flies already after 3 days. The difference is statistically significant

comparing ΔV3 or 5KQ with control flies. **p<0.01 Student's t-test, 5KQ vs FLAG. N= 10 biological replicates/genotype, 5 flies each. C) Summary of the effect of starvation stress (YAA -Cholesterol) presented as percentage change in fecundity relative to YAA standard food. 5KQ flies had the highest reduction in egg laying (-15%).

In conclusion, these results suggest that dFOXO acetylation plays a role in mediating survival, fertility and lipid utilization in response to yeast starvation. The observed phenotype may also, but not exclusively, be due to cholesterol starvation.

Future studies should attempt to identify the nutrients that, when depleted together with cholesterol, would induce a phenotypic response as great as that observed in yeast starvation, and further experiments should be performed to determine the molecular mechanisms behind FOXO acetylation in response to the availability of specific nutrients.

3.3.2 Effect of single EAAs drop out on dFOXO acetylation mutant flies

Previous studies suggest an important role of amino acids in modulating longevity in different species (Fontana and Partridge, 2015), but to date the underlying molecular mechanisms still remain poorly understood. In a recent publication, the role of the kinase GCN2 as an amino acid starvation sensor was investigated in detail. GCN2 function was found to be essential for ensuring fly survival under deprivation of specific EAAs, with the strongest effect seen upon arginine deprivation, whereas GCN2 mutant flies appear to be longer-lived compared to wild types upon complete methionine starvation. This suggests that methionine may be the only EAA whose deprivation is sensed by a GCN2-independent mechanism (Srivastava et al., 2022). Moreover, a study highlighted the existence of a link between FOXO and GCN2, since this kinase was shown to phosphorylate FOXO to promote its activity (You et al., 2018). With the idea to test whether dFOXO could be involved in the regulation of specific amino acid-mediated longevity, and whether this is influenced by its acetylation state, we checked the effect of arginine (YAA-R) and methionine (YAA-M) deprivation on lifespan, development and fecundity of FOXO acetyl-mimicking mutants.

As shown in Figure 3.7A-C, both R and M starvation reduced lifespan of flies, with the largest effect seen on the YAA-M diet. ΔV3 showed the greatest change in lifespan upon arginine deprivation, however, no striking difference was seen between the acetyl-mimetic mutants and control flies when observing the percentage change in median lifespan of flies on the EAA-deprived diet relative to the standard diet (Figure 3.7C). In terms of larval development, as expected the complete absence of arginine or methionine is lethal for all dFOXO mutants tested (Figure 3.7D). On the other hand, fecundity on the YAA-R diet was almost completely abolished (~99% reduction) for all FOXO mutants, while on the YAA-M food, although still dramatically reduced (~90%), analysis on this phenotype allowed a higher degree of differentiation between genotypes (Figure 3.7E-F). In particular, the acetyl-mimetic flies showed a significant reduction in egg laying compared to control flies (Figure 3.7E-F), visible already after 4 days (Figure 3.7G). However, egg-laying stopped almost completely in all genotypes by day 6.

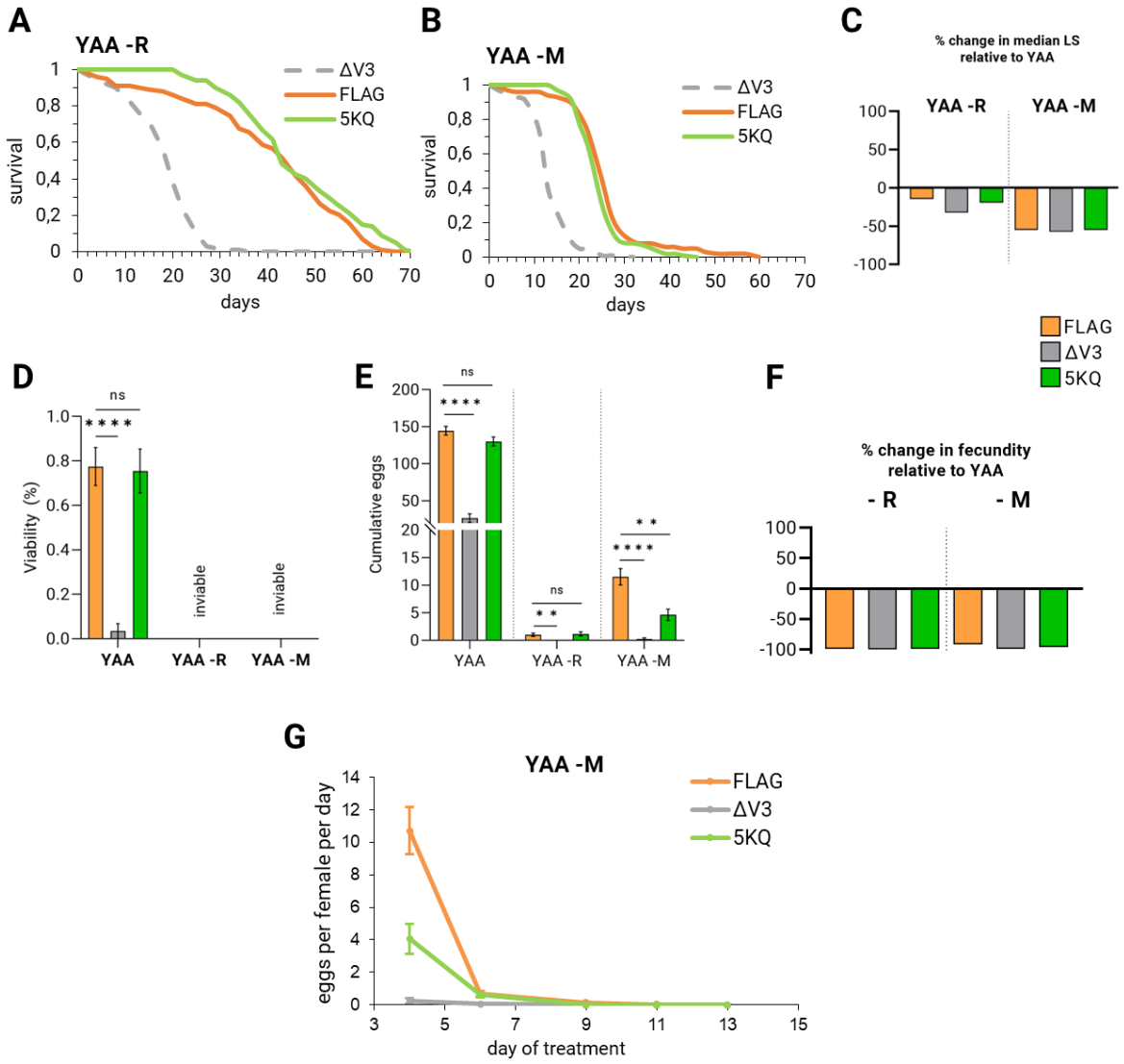

Figure 3.7: Effect of R and M deprivation on lifespan, development and fecundity of dFOXO acetylation mutants.

A-B) Lifespan curves of female dFOXO mutant flies subjected to Arginine (A) and Methionine (B) starvation food. N=150/genotype in each assay. C) Summary of starvation assays presented as percentage change in median lifespan relative to standard YAA diet. ΔV3 flies had the largest reduction upon arginine deprivation. Methionine starvation had a biggest impact on lifespan of tested flies, but no difference between genotypes. D) On a control YAA diet, only *dfoxo-null* (ΔV3) flies had a significantly reduced lifespan compared to FLAG flies. Deprivation of both R or M was developmentally lethal for all tested genotypes. ****p<0.0001, Student's t-test. N=10 replicates/genotype, 20 eggs each. E) Comparison of cumulative egg production of control (FLAG) and dFOXO mutants (ΔV3, 5KQ) on R and M deficient diets. Fecundity of ΔV3 flies was reduced compared to control FLAG both on YAA -R (**p<0.01, one-way ANOVA) and YAA -M (****p<0.0001, one-way ANOVA). With respect to FLAG flies, 5KQ mutants showed no difference in egg laying on a R deprived food but had significantly reduced cumulative eggs upon M deprivation (****p<0.0001, one-way ANOVA). N= 10 biological replicates/genotype, 5 flies each. F) Summary of the effect of starvation stress (YAA -R and -

M) presented as percentage change in fecundity relative to YAA standard food. Fecundity on the YAA-R diet is reduced by 99% in all genotypes. On the YAA -M diet, 5KQ flies had reduced egg laying compared to FLAG controls. G) Complete deprivation of methionine significantly reduced fecundity of control and 5KQ acetyl-mimetic mutant flies already after 4 days. **p<0.01 Student's t-test, 5KQ vs FLAG in YAA-M.

Based on these results, to test whether titration of this essential amino acid would allow larval development and/or emphasize the difference in fecundity of dFOXO mutants, a diet in which methionine levels were dropped down to 30% (YAA M30%) was included in the analysis. Although the general development time was slowed down in all genotypes (Figure 3.8B and S2B), reduction of methionine to 30% resulted in almost unchanged viability of FLAG control and acetyl-mimetic flies compared to their viability on a YAA standard diet (Figure 3.8A). On the other hand, viability (Figure 3.8A) as well as total number of pupae (Figure S2A) were significantly reduced in *dfoxo-null* flies, suggesting that dFOXO function might be important to mediate larval survival under deprivation of methionine. Interestingly, the larval development time (=time from egg to appearance of first pupa) of 5KQ acetyl-mimetic flies on the YAA M30% diet was significantly increased when compared to the same genotype on the standard YAA food (Figure 3.8C), hinting that development timing under methionine starvation might be partially affected by dFOXO acetylation.

Concerning fecundity, 5KQ acetyl-mimetic flies also showed a clear reduction in egg-laying upon partial (YAA M30%) methionine depletion (Figure 3.8D-F), highlighting an even stronger difference with respect to FLAG control flies at earlier time points.

In summary, acetylation of dFOXO appears to be involved in mediating fecundity and, in part, development in methionine-restricted diets. In the future, it would be interesting to further investigate the mechanism by which methionine deprivation regulates dFOXO deacetylation and the subsequent metabolic response induced by this modification. In addition, we could test these mutants on diets lacking single or combinations of other specific amino acids to explore potential roles of dFOXO as an amino acid starvation sensor.

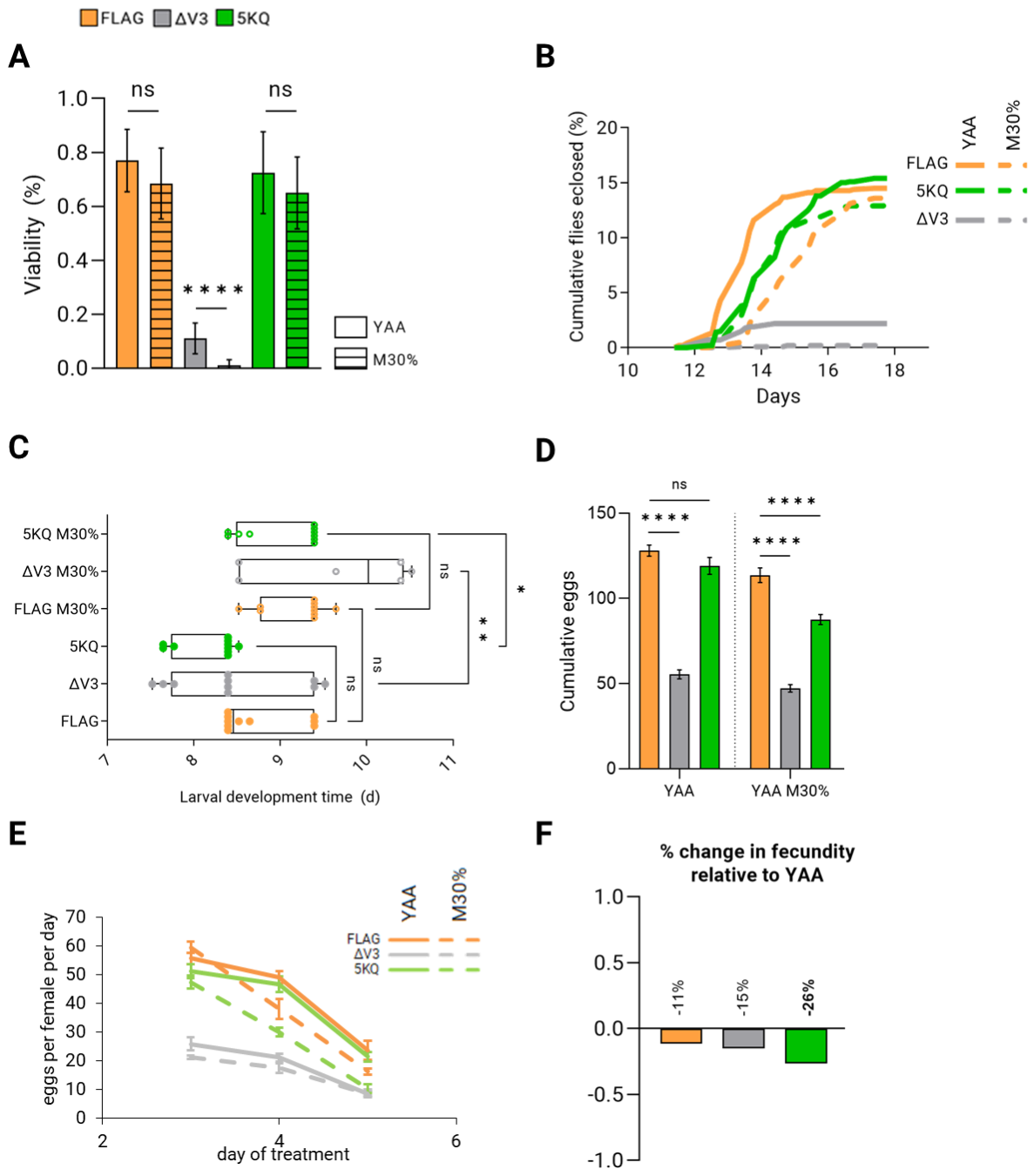

Figure 3.8: dFOXO acetylation might be required to mediate fecundity and (partially) development upon Methionine starvation.

A-C) Effect of methionine reduction (YAA M30%) on viability (A) and development of FLAG, ΔV3 and 5KQ mutant flies, expressed as eclosion rate (B) and larval development time (C). *dfoxo-null* ΔV3 flies showed significantly reduced eclosion compared to control flies (****p<0.0001, Student's t-test), while 5KQ flies subjected to the starvation diet showed an increased development time compared to the same genotype on a standard YAA diet (5KQ vs 5KQ M30%: *p<0.05). N=10 replicates/genotype, 20 eggs each. D-F) Effect of methionine reduction (YAA M30%) on fecundity of FLAG, ΔV3 and 5KQ mutant flies, expressed as cumulative eggs (D), eggs per female per day (E) and % change in fecundity relative to a standard YAA diet (F). 5KQ flies showed a consistent and significant decrease in the starvation

diet. Diet: ****p<0.0001; genotype: ****p<0.0001; interaction: ****p<0.0001, two-way ANOVA. Bonferroni multiple comparison test with FLAG: ns p>0.05, ****p<0.0001. N=5 biological replicates per assay, each with 5 flies/genotype. N= 10 biological replicates/genotype, 5 flies each.

3.3.3 Partial acetylation mutants

A protein alignment between human, mouse, worm and fly FOXO proteins showed that only three of the five lysine residues within the nuclear localization domain were evolutionarily conserved between all species (Figure 3.9A). Moreover, lysine residues that are acetylated are more likely to be conserved through evolution (Weinert et al., 2011). Thus, we were curios to verify whether mutation of only these three lysine residues was sufficient to cause the same phenotypes as observed in the dFOXO 5KQ mutant flies. Therefore, we designed new dFOXO mutant constructs in which only two (2KQ) or three (3KQ, 3KQII) of the corresponding lysine residues were mutated (Figure 3.9B). Furthermore, we also generated flies in which the partially acetylated dFOXO proteins were tagged with mCherry, which allows direct visualization of dFOXO subcellular localization *in vivo*.

3.3.3.1 Generation and validation of dFOXO partial acetylation mutants in vivo

Taking advantage of the genomic engineering tool previously established in our lab by Victor Bustos, new endogenous dFOXO mutants were generated by site-directed integration using the φC31/attP/attB system (Bischof et al., 2007). Briefly, donor constructs harboring mutations in the V3 region of *dfoxo*, which includes the DBD and the NLS, were subcloned into the pGEattBGMR vector tagged with either FLAG or mCherry tags. All constructs were verified by Sanger sequencing and microinjected into embryos of the previously generated dFOXO KO-parental line ΔV3 w[-]. Injected flies were crossed with wDahT wild type flies, and mutants were identified based on their red eye color and subsequently single-crossed again with wild type flies for amplification. The new mutant stocks were made homozygous before performing genotyping and subsequent sequencing (Figure 3.9C).

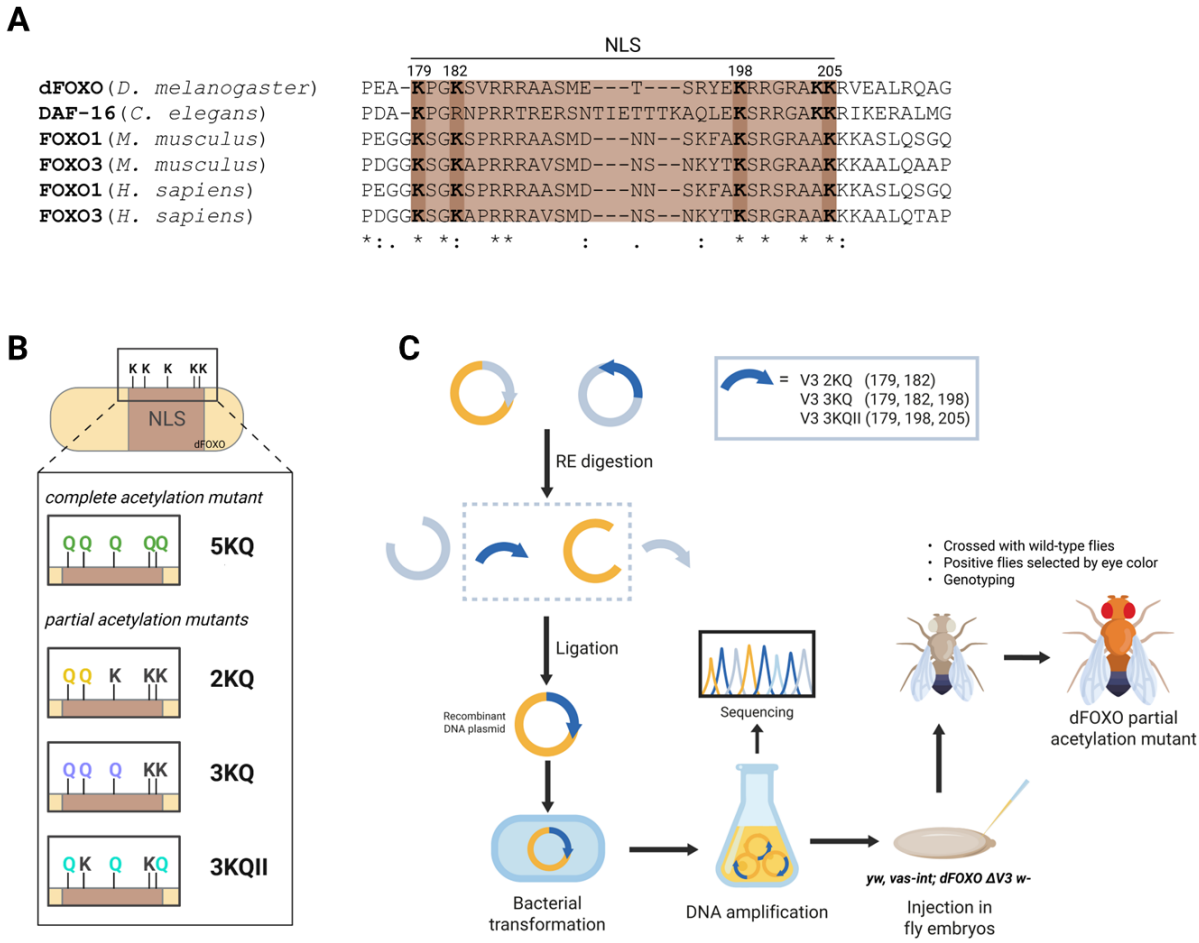

Figure 3.9: Generation of dFOXO partial acetylation mutant flies by cloning and microinjection.

A) Protein alignment of the FOXO NLS between human/mouse (FOXO1, FOXO3), worm (DAF16) and fly (dFOXO) show a high degree of conservation. Brown background highlights the NLS. Protein alignment was performed using Clustal Omega. Identical (*), strongly similar (:) or weakly similar (.) residues between sequences. Lysine residues highlighted in bold are conserved in *Drosophila*. B) Schematic representation of the NLS region in wild type dFOXO protein (top) and in acetyl-mimicking dFOXO (bottom). While in complete acetylation mutants all 5 lysines in the NLS were mutated to glutamine (5KQ), in the newly generated partial acetylation mutants only the evolutionary conserved residues were mutated (2KQ, 3KQ, 3KQII). Created with Biorender.com. C) Graphic representation of the cloning procedure used to generate the new dFOXO mutant constructs for injection and generation of new mutated fly lines. After microinjection, positive mutant flies were selected based on the eye color marker and subjected to multiple crossings to finally obtain a homozygous stock.

In both FLAG and mCherry tagged lines, PCR analysis confirmed the correct reinsertion of mutated V3 gene replacement constructs (Figure 3.10A and Figure S3A) and dFOXO protein expression was assessed by western blotting analysis (Figure 3.10B and Figure S3B).

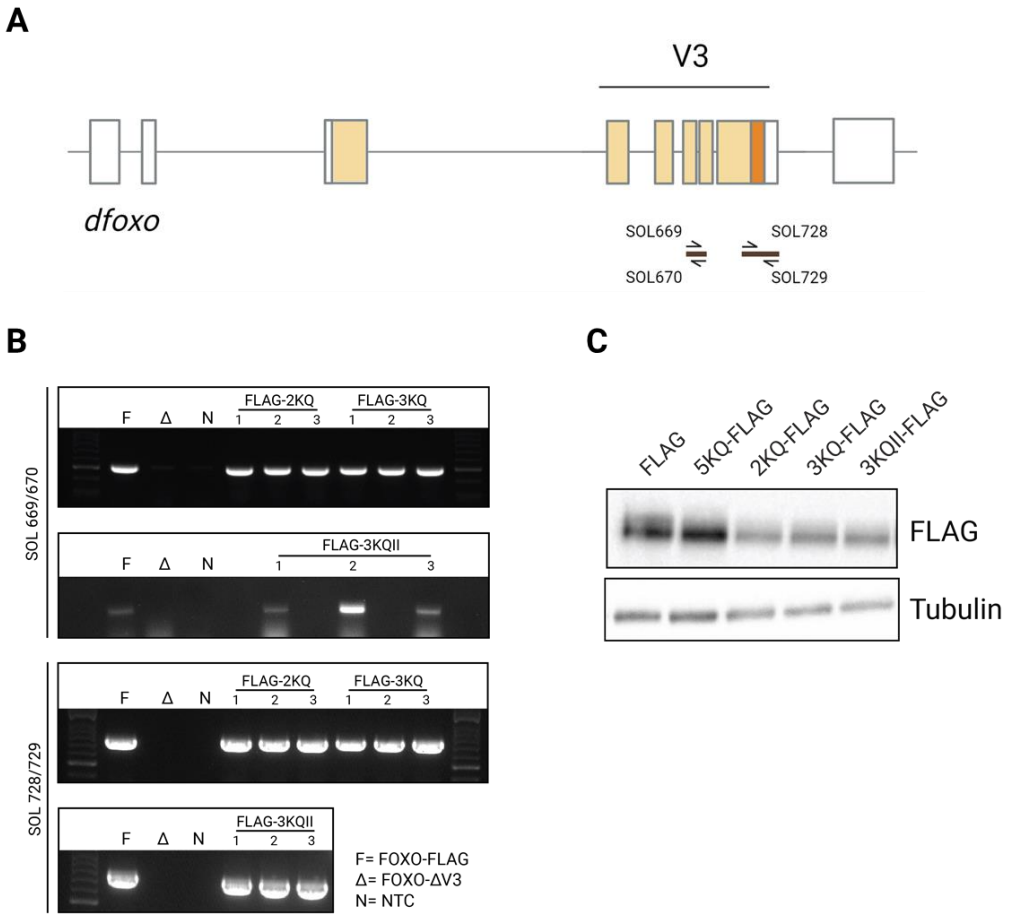

Figure 3.10: PCR verification and molecular validation of novel FLAG-tagged FOXO acetylation mutants.
A) Schematic of the *dfoxo* gene locus showing primer combinations used to validate correct integration of the mutated V3 replaced constructs. White boxes represent UTRs and yellow boxes represent CDS. The orange box represents the C-terminal FLAG tag. The V3 region was amplified using primers SOL669/670 and the TAG sequence was amplified using primers SOL728/729. Created with Biorender.com. B) PCR analysis confirmed the correct reinsertion of FLAG-tagged V3 gene replacement constructs. Numbers 1 to 3 indicate independent candidate fly lines tested. C) Immunoblotting on whole-body protein extracts from female flies confirmed dFOXO expression in the newly generated FLAG-tagged partial acetylation mutants (2KQ, 3KQ, 3KQII).

To verify whether the newly generated mutants had physiological phenotypes similar to control and complete acetyl-mimicking flies, FLAG-tagged partial acetylation flies were tested for lifespan, development and body weight on a standard diet. Consistent with the data obtained from 5KQ complete acetylation mutant flies, all new lines showed no change in any of the tested parameters (Figure 3.11A-C), suggesting that partial acetylation does not interfere with normal functions of the transcription factor *in vivo*. This was further confirmed by phenotyping of the mCherry-tagged partial acetylation lines (Figure S3C-D), also indicating that this tag does not interfere with the normal function of the protein.

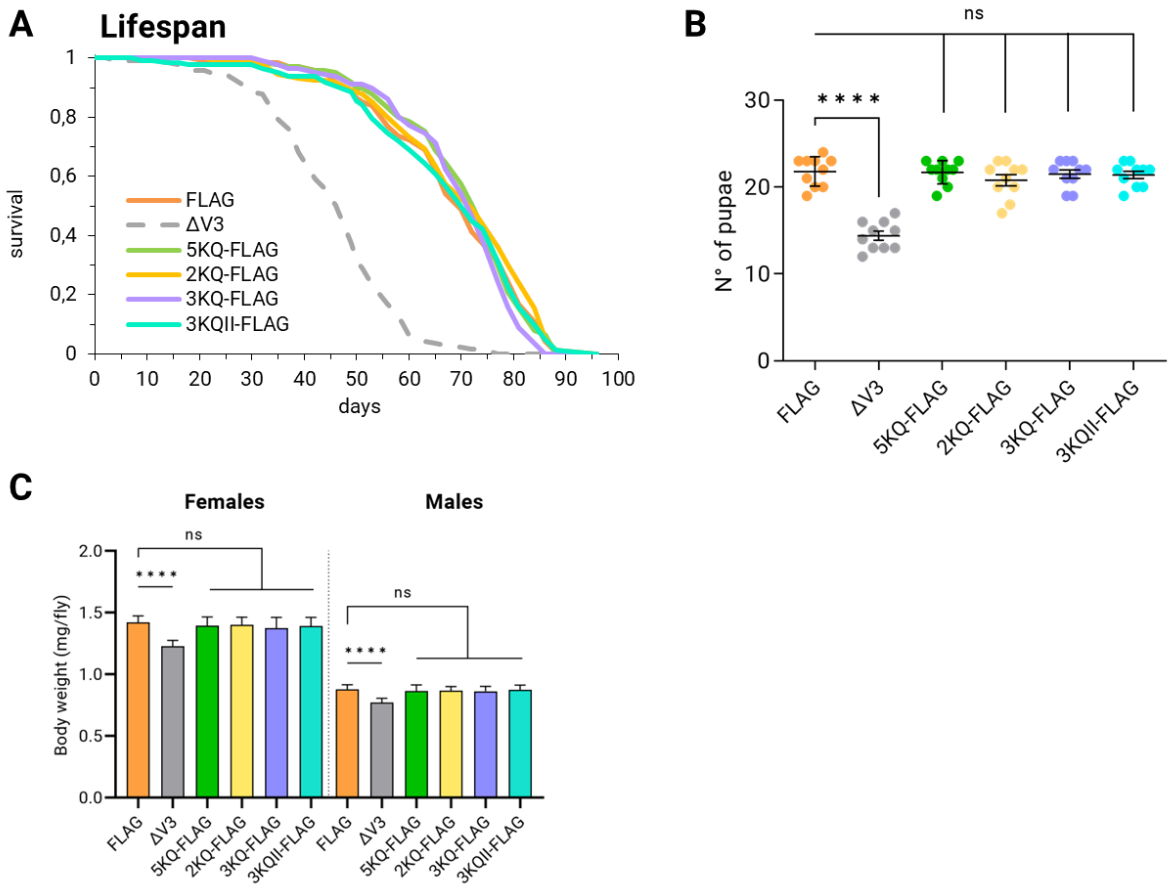

Figure 3.11: Partial acetylation mutants show normal lifespan, development and body weight. A) On a standard SYA diet, partial acetyl-lysine mutants (2KQ-, 3KQ-, 3KQII-FLAG) showed similar lifespan to FLAG control and complete acetylation mutant (5KQ) flies. ns p>0.05 log-rank test, 2KQ 3KQ, 3KQII vs FLAG and 5KQ. N= 150/genotype. B) With respect to FLAG control line, development assay showed a reduced number of pupae in *dfoxo-null* ΔV3 flies only. All partial and complete dFOXO acetylation mutants showed similar development as control. ns

p>0.05, ****p<0.0001, one-way ANOVA followed by Bonferroni's multiple comparisons test vs control (FLAG). N=10 replicates/genotype, 20 eggs each. C) In both female and male flies, partial and complete acetyl-lysine mutants had a similar body weight as FLAG control flies. ns p>0.05, ****p<0.0001, one-way ANOVA followed by Bonferroni's multiple comparisons test vs control (FLAG). N=20/genotype.

In addition, imaging of mCherry-tagged dFOXO in a partial acetylation line further confirmed the expression of the tagged protein *in vivo*. Interestingly, the pilot experiment also revealed that partial acetylation of FOXO prevented normal nuclear localization of the protein upon starvation (Figure 3.12). This is consistent with what has already been observed in mammalian (Banks et al., 2011; Qiang et al., 2010) and fly (Dr. Victor Bustos, unpublished data) cell culture with fully acetylated FOXO-NLS.

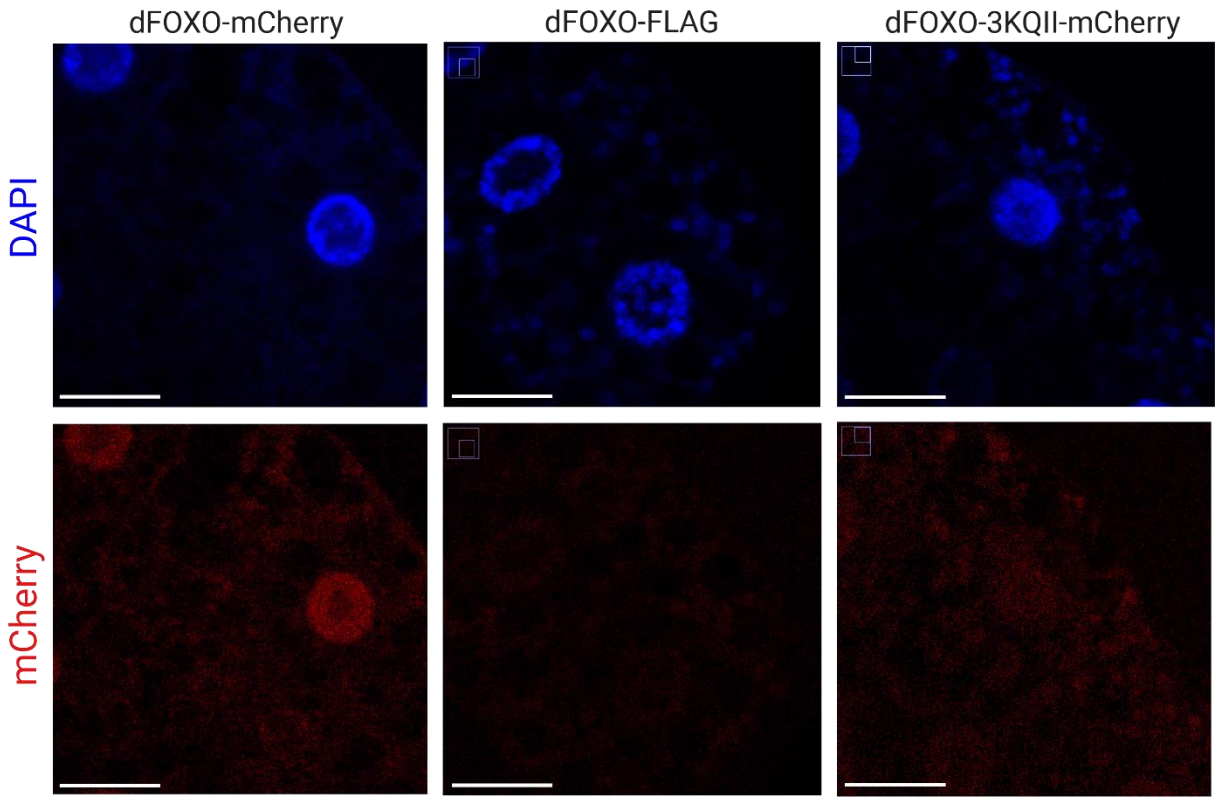

Figure 3.12: Acetylation affects cytoplasmic-nuclear shuttling of dFOXO *in vivo*.
Representative confocal images of larval fat body showed that, unlike dFOXO-mCherry, which localized mostly in the nucleus, partially acetylated dFOXO (dFOXO-3KQ-mCherry) was mostly cytoplasmatic under starvation. dFOXO-FLAG was included as negative control. Scale bar= 50 µm.

3.3.3.2 Partial acetylation of dFOXO is sufficient to induce yeast starvation sensitivity

Next, we tested whether only partial acetylation of the NLS would be sufficient to recapitulate the same yeast sensitivity phenotype observed in the dFOXO 5KQ mutant flies. On a SYA -Yeast diet, all partial acetylation mutants were shorter-lived as much as the complete acetylation mutant line and *foxo-null* (Figure 3.13A), suggesting that acetylation of only two lysine site is sufficient to regulate dFOXO function under yeast starvation. Consistent with this observation, partial acetylation was also found to reduce fecundity in absence of yeast (Figure 3.13B).

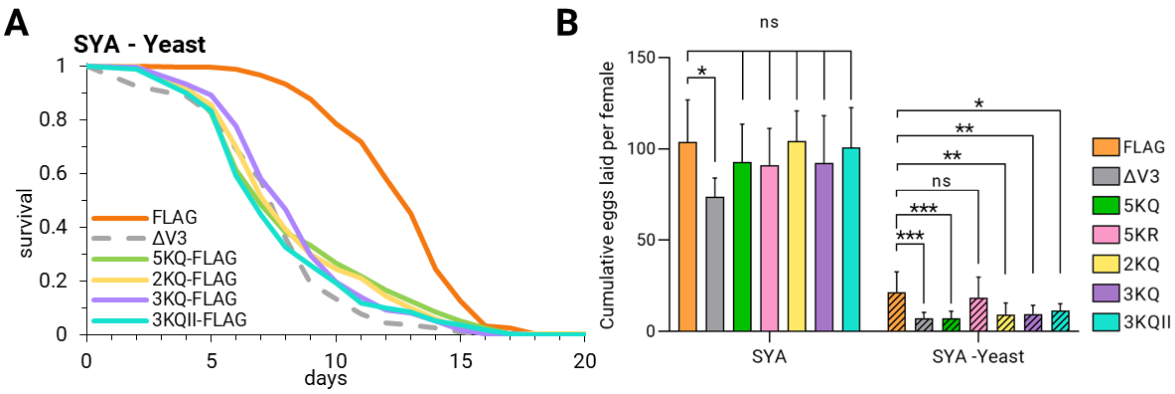

Figure 3.13: Mutation of 2-3 Lys residues is sufficient to recapitulate the yeast starvation phenotype of complete acetylation mutants.
A) Yeast starvation assay showed that both dfoxo-null flies (ΔV3) and complete/partial acetyl-lysine mutants (5KQ, 2KQ, 3KQ, 3KQII) were shorter-lived than FLAG control flies. ***p<0.001 log-rank test. N= 150/genotype. B) Fecundity assay showed that all acetylation mutants had similar egg laying with respect to FLAG control line. On a yeast deficient diet, both *dfoxo-null* (ΔV3) and complete acetylation mutant (5KQ) female flies had reduced cumulative egg production compared to FLAG control. Partial acetylation mutants also showed decreased egg laying. Fecundity of acetyl-null 5KR flies was not significantly different from FLAG control flies. ns p>0.05, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 one-way ANOVA vs FLAG. N= 10 biological replicates/genotype, 5 flies each.

In summary, partial acetylation of lysines in the NLS of dFOXO did not interfere with normal functions of the protein *in vivo* and recapitulated the phenotype observed in complete acetylation mutants upon yeast starvation. Additionally, partial acetylation of FOXO resulted in nuclear exclusion. Further studies are needed to confirm this

finding; in the future, the mCherry-tagged partial/complete acetylation mutant lines could be useful to investigate which acetylated Lys are specifically responsible for influencing the cytoplasmic-nuclear shuttling of FOXO. Moreover, it would be interesting to test whether wild type FOXO responds to different nutritional cues by changing its localization, and whether acetylation might have a different influence depending on the given starvation stress.

3.3.4 Proteome profiling of FOXO acetylation mutants upon yeast deprivation

In the light of the results obtained from the starvation assays, with the maximum effect and most consistent results observed with yeast starvation, we decided to use this paradigm to further investigate the molecular changes associated with yeast deprivation in *Drosophila* and the role of dFOXO acetylation in this context. Thus, we performed mass spectrometry and total proteome analysis on control (FLAG) and dFOXO complete acetylation (5KQ) female flies that were continuously fed or starved for 3 days on a yeast deprived food. Protein extraction from whole flies led to the detection of ~4500 proteins per sample (Figure S4A) and their principal component analysis showed consistent clustering based on both genotype and diet (one sample that was an outlier in the PCA was removed from subsequential analysis) (Figure S4B-C).

Comparing the yeast-starved to the fed condition, we observed that three days of yeast deprivation were sufficient to induce marked differential protein expression changes in both genotypes, with 1478 (416/1062) and 2268 (779/1489) proteins significantly up- and down-regulated under starvation in control and 5KQ, respectively. In particular, the majority of DEPs were observed in the acetylation mutants, suggesting that these mutants undergo a greater proteome change in response to the starvation diet (Figure 3.14B). 358 of the upregulated proteins and 836 of the downregulated proteins were shared between control and 5KQ flies (Figure 3.14A), suggesting that these proteins are involved in processes that need to be adapted in response to the lack of key nutrients contained in the yeast, and that these remain conserved in the context of the mutation. In fact, GO term analysis of these shared proteins revealed that, in the absence of yeast, processes related to catabolism, generation of energy and chromatin

condensation were increased, while gene expression and translation were drastically downregulated (Figure S5A-B).

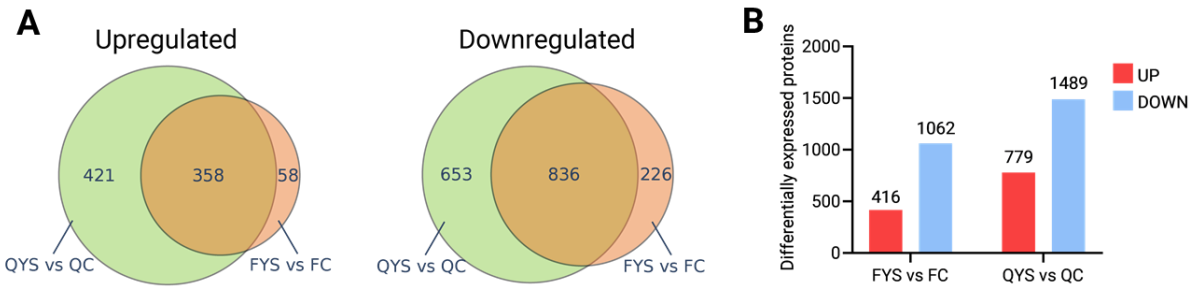

Figure 3.14: Up- and down-regulated proteins in dFOXO-5KQ vs FLAG flies.
A) Venn diagram of upregulated (left) and downregulated (right) differentially expressed proteins (DEPs) in the comparison QYS vs QC (green) and FYS vs FC (orange). B) Barplot representation of DEPs in the comparisons FYS vs FC and QYS vs QC. The exact number of DEPs is indicated above the bar. | Abbreviations: QC= dFOXO-5KQ flies, control diet; FC= dFOXO-FLAG flies, control diet; QYS= dFOXO-5KQ flies, yeast starvation; FYS= dFOXO-FLAG flies, yeast starvation. N=4 biological replicates.

3.3.4.1 FOXO acetylation mutants have downregulated transmembrane transport in normal feeding conditions

In order to identify processes that are regulated in 5KQ flies dependent on their mutation and independently of the starvation stress, we analyzed the difference of control and acetylation mutant flies under fed conditions. Interestingly, GO terms enrichment for this comparison showed that the mutants have a downregulation in processes involved in ion transport and transmembrane import (Figure 3.15A-B, BP= ion transport, import into cell, import across plasma membrane), as well as a downregulation of cellular component or molecular function terms related to vacuole transport (e.g., CC= endoplasmic reticulum membrane, ATPase-dependent transmembrane transport complex, SNARE complex; MF= active transmembrane transporter activity, SNAP receptor activity), suggesting that the constitutively acetylated state of FOXO may interfere with normal cellular trafficking.

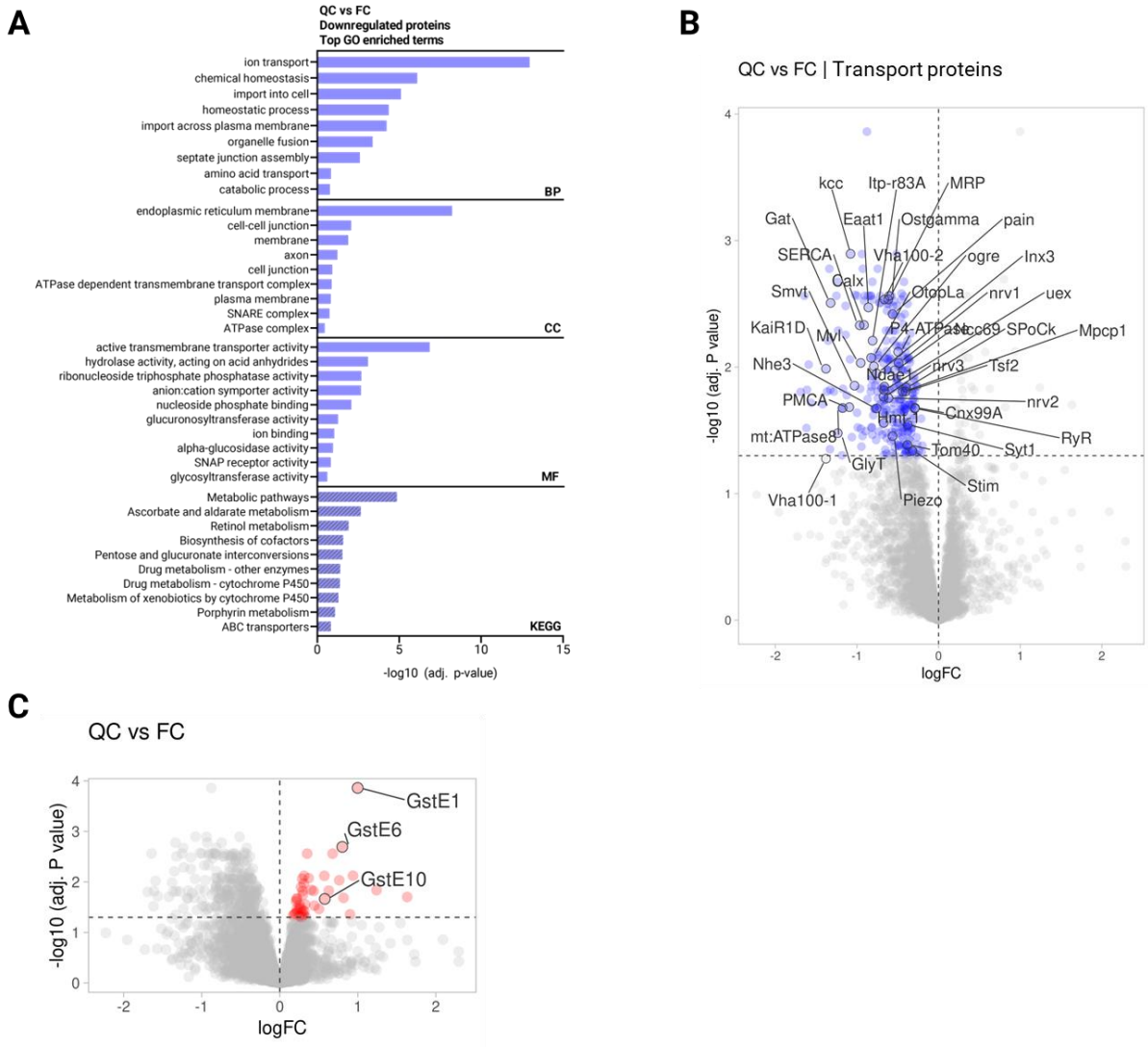

Figure 3.15: In fed conditions, FOXO acetylation mutants have decreased transmembrane transport and increased basal glutathione metabolism.

A) Barplot representation of significantly enriched top GO terms of downregulated proteins in the comparison QC vs FC. BP= biological process; MF= molecular function; CC= cellular component; KEGG= KEGG pathway. GO terms sorted by adjusted p-value. B) Volcano plot of DEPs in QC vs FC highlighting downregulation of proteins involved in transport across the plasma membrane. C) Volcano plot of DEPs in QC vs FC showing upregulation of GstE proteins, involved in glutathione metabolism. Significance (adjusted p-value) and fold-change are converted to −Log10(adj. p-value) and Log2(fold-change), respectively. Cut-off of p-value=0.05. Abbreviations: QC= dFOXO-5KQ flies, control diet; FC= dFOXO-FLAG flies, control diet.

3.3.4.2 Upon yeast starvation, FOXO acetylation mutants reduce levels of glutathione metabolism and lipid biosynthesis

Next, by analyzing the direct comparison of control vs acetylation mutants in the starvation condition, we identified proteomic profiles specific to each genotype and gained an overview of the processes affected by the mutation under metabolic stress. GO terms enrichment for this comparison pointed out that proteins associated with lipid biosynthesis were upregulated in the acetylation mutants compared to controls (Figure 3.16A and C, BP= lipid biosynthetic process, fatty acid biosynthetic process; MF= fatty acid ligase activity; KEGG= fatty acid biosynthesis). The high levels of fatty acid biosynthetic processes might explain the previous observation of stable TAGs content of 5KQ flies upon yeast restriction. Moreover, glutathione metabolism was also upregulated in 5KQ flies (Figure 3.16A, BP= sulfur compound metabolic process; MF= glutathione transferase activity; KEGG= glutathione metabolism) and in particular proteins from the GstE family (Figure 3.16B). Interestingly, 3 proteins involved in glutathione metabolism (GstE1, GstE6 and GstE10) were already found to be upregulated in the acetylation mutants under normal feeding conditions (Figure 3.15C), suggesting that the altered acetylation state of FOXO might boost glutathione metabolism, both under normal feeding and, even more, under yeast starvation.

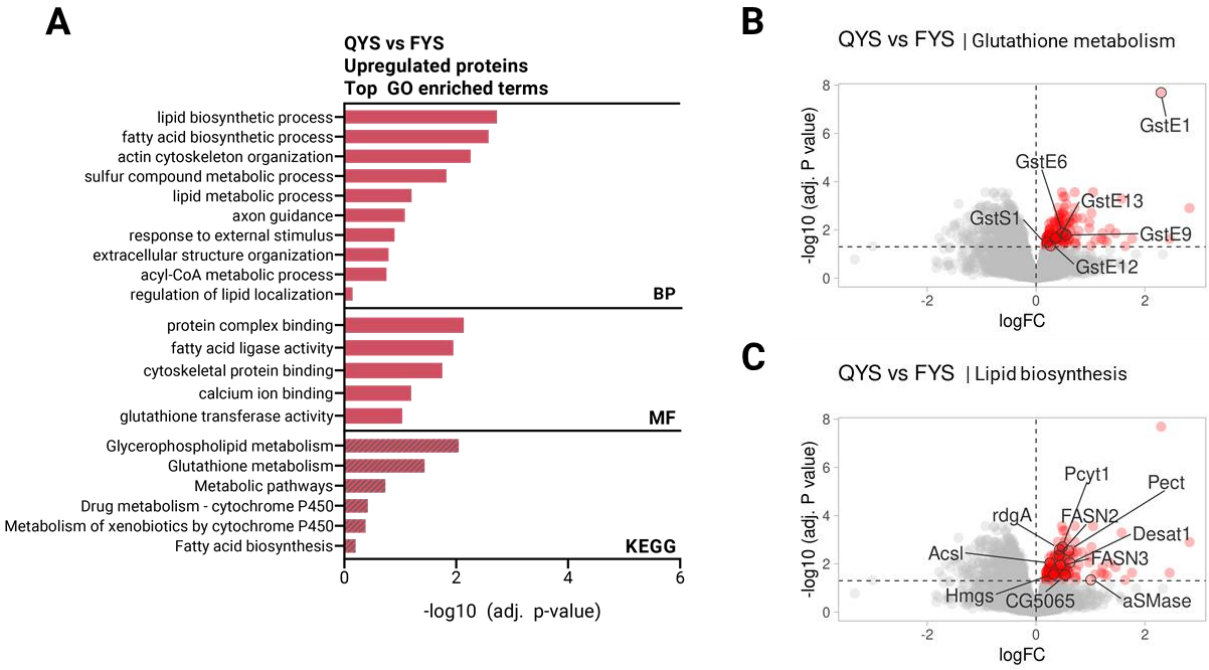

Figure 3.16: Upon yeast starvation, FOXO acetylation mutants have increased glutathione metabolism and lipid biosynthesis.
A) Barplot representation of significantly enriched top GO terms of upregulated proteins in the comparison QYS vs FYS. BP= biological process; MF= molecular function; KEGG= KEGG pathway. GO terms sorted by adjusted p-value. B) Volcano plot of DEPs in QYS vs FYS highlights upregulated proteins related to glutathione metabolism. C) Volcano plot of DEPs in QYS vs FYS highlights upregulated proteins involved in lipid biosynthesis. Significance (adjusted p-value) and fold-change are converted to −Log10(adj. p-value) and Log2(fold-change), respectively. The horizontal dotted line shows the cut-off of p-value = 0.05. n=4 genotypes per genotypes and diet. | Abbreviations: QC= dFOXO-5KQ flies, control diet; FC= dFOXO-FLAG flies, control diet; QYS= dFOXO-5KQ flies, yeast starvation; FYS= dFOXO-FLAG flies, yeast starvation.

3.3.4.3 Acetylation of FOXO reduces oogenesis and upregulates cholesterol transport proteins during yeast starvation

Worth noting, GO enrichment from the comparison FLAG - 5KQ in the starved condition demonstrated that, besides a very drastic reduction of the term “cytoplasmatic translation”, proteins related to oogenesis were also down-regulated (Figure 3.17A), with yolk proteins (Yp1, Yp2, Yp3) among the most significant DEPs (Figure 3.17B). This is in line with the observation of the acetylation mutant flies having a reduced egg laying on a yeast deficient diet. The reduced egg production seemed to be specifically associated to yeast starvation, as 5KQ mutants in the continuous feeding condition did not show a reduction in YP proteins or fertility hormones compared to control flies (Figure S6).

The production of the three yolk proteins is under the control of steroid hormones, the synthesis of which requires cholesterol (Sieber et al., 2015; Niwa et al., 2011). Based on that and on previous observations that acetylation mutants were showing a similar starvation phenotype in case of cholesterol deficiency, we also looked at expression levels of cholesterol transport proteins in 5KQ flies compared to control in the yeast starvation condition. 4 proteins (Lsd-1, Apoltp, dSLC5A1 and norpA) were found to be upregulated in the mutant upon starvation (Figure 3.17C, Figure S7), suggesting that regulation of cholesterol transport proteins is altered in response to nutritional deprivation of yeast, and possibly of cholesterol, in the context of dFOXO acetylation.

We hypothesize that acetylation of dFOXO could lead to impaired cholesterol transport, resulting in a compensatory increase in cholesterol transport proteins and decreased production of fertility hormones and yolk proteins (Figure 16D).

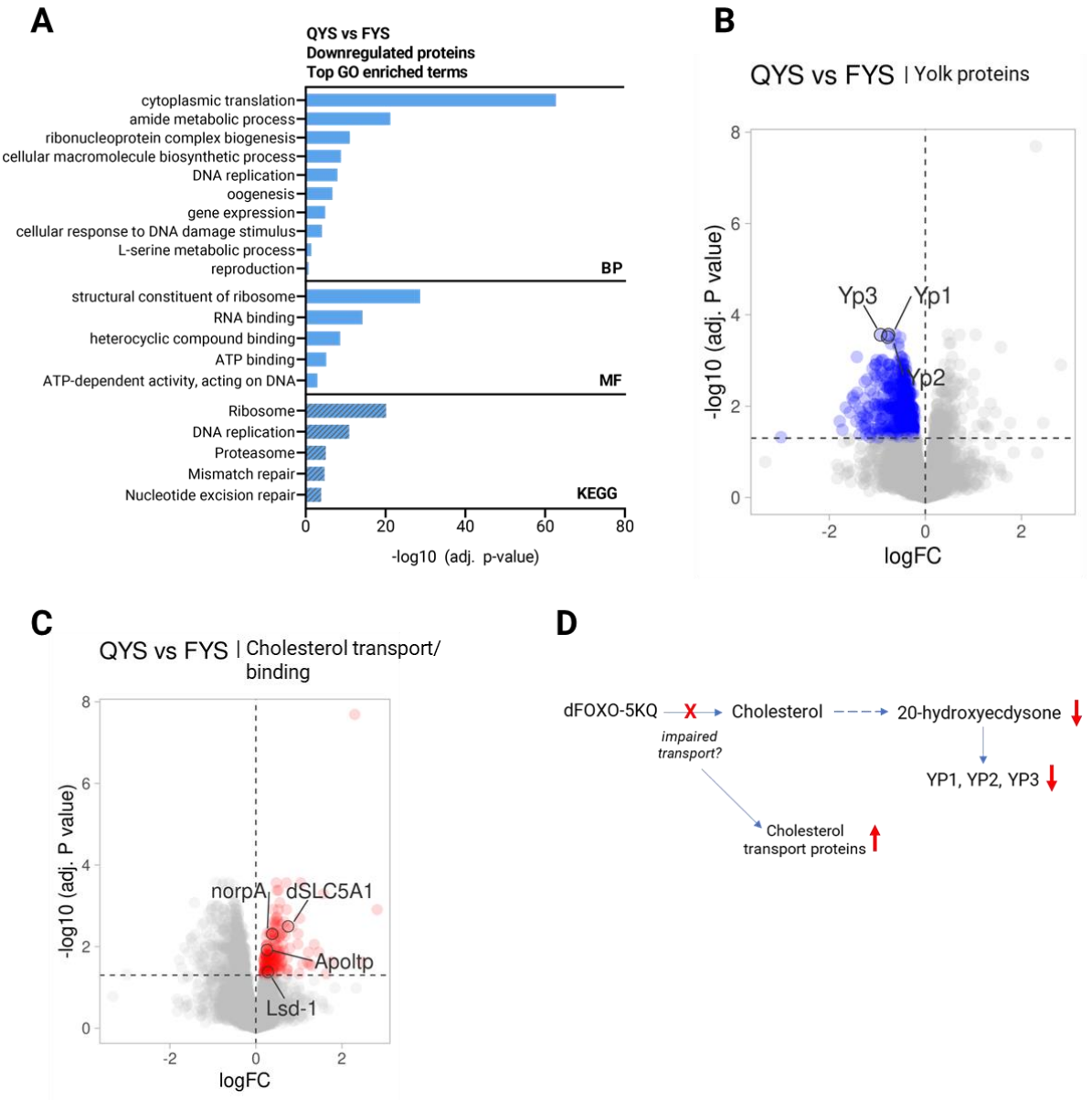

Figure 3.17: FOXO acetylation mutants have reduced oogenesis and upregulated cholesterol transport proteins upon yeast starvation.
A) Barplot representation of significantly enriched top GO terms of downregulated proteins in the comparison QYS vs FYS. BP= biological process; MF= molecular function; KEGG= KEGG pathway. GO terms sorted by adjusted p-value. B) Volcano plot of DEPs in QYS vs FYS highlights downregulation of yolk proteins YP1, YP2 and YP3. C) Volcano plot of DEPs in QYS vs FYS highlights upregulation of proteins involved in cholesterol transport. Cut-off of p-

value = 0.05. D) Schematic showing a possible link between impaired cholesterol transport and decreased oogenesis in dFOXO acetyl-lysine mutant flies.

In conclusion, this analysis identified specific proteomic profiles associated with dFOXO acetylation mutants depending on the nutritional state. On the one hand, in normal feeding conditions, acetylation of FOXO resulted in global downregulation of ion transport proteins as well as increased glutathione metabolism. On the other hand, in the context of yeast starvation, proteins involved in glutathione metabolism underwent a further increase in expression, which was accompanied by upregulation in lipid biosynthesis and cholesterol transport proteins and reduced oogenesis. The hypothesis that the observed proteomic profile is a consequence of reduced cholesterol levels will have to be validated by measuring the cholesterol content of yeast-starved dFOXO acetylation mutants.

3.3.5 Role of dFOXO acetylation in the response to excess of dietary sugar

3.3.5.1 Introduction

High-sugar diets are responsible for obesity and dysregulation of metabolism, which have an impact on health and survival (Chatterjee et al., 2017; De Fronzo et al., 2015), also in the fly (Morris et al., 2012; Skorupa et al., 2008; Lushchak et al., 2014). In this context, FOXO has been shown to be required to support resistance upon sugar-excess (van Dam et al., 2020). Moreover, in a study conducted in mice, acetylation of FOXO1 was associated to glucose metabolism, and in particular constitutive deacetylation of the transcription factor was shown to drive metabolism toward gluconeogenesis (Banks et al., 2011), suggesting the idea that FOXO in its acetylated state might promote glucose utilization. However, constitutively-acetylated-dFOXO mice were embryonic lethal (Banks et al., 2011), and the role of dFOXO acetylation in the context of a dysregulated glucose metabolism remains to be addressed directly.

3.3.5.2 dFOXO acetylation mediates survival upon high-sugar diet

To assess whether acetylation of FOXO was involved in regulation of survival on a high-sugar diet, we fed female control (FLAG) and acetyl-mimicking (5KQ) flies a standard food containing 5% sucrose (S5%), a diet with 2-fold increased levels of sucrose (S10%) or a diet with 4-fold increased sucrose (S20%). Yeast was maintained constant throughout all experiments (see Table 2 for SYA media recipes). Lifespan assays showed that excess of sugar shortened the lifespan of female control flies in a dose-dependent manner (Figure 3.18A-C), with 7% and 27% reduction on a S10% or S20% diet, respectively (Figure 3.18D). In contrast, dFOXO acetylation mutant flies did not show sensitivity to a 2-fold increase in sugar, and had a 17% reduction in lifespan on the diet with 4-fold increase. Comparing the survival of mutant and control animals, the 5KQ flies appeared to be longer-lived (+10%) on the S20%.

Next, to verify whether the better performance of 5KQ mutants on this diet was specifically attributable to the acetylation state of FOXO, we repeated the lifespan assays on the diet with highest concentration of sugar (S20%) and also included fly lines harboring different mutations of FOXO. As shown in Figure 3.18E-F, the assay

confirmed that 5KQ complete acetylation mutants had the longest lifespan on S20% compared to FLAG control flies. *foxo-null* mutants (ΔV3) had the lowest median lifespan, in line with previous findings (van Dam et al., 2020), and mutation of the DNA-binding site (DBD2) also drastically reduced fly survival, suggesting that the transcriptional ability of dFOXO is essential to mediate survival upon sugar excess. On the other hand, both acetyl-null (5KR) and partial acetylation (2KQ) mutants were not longer-lived than control flies, indicating that only acetyl-mimicking mutations of FOXO including more than two lysine residues were associated with improved survival on the high-sugar diet.

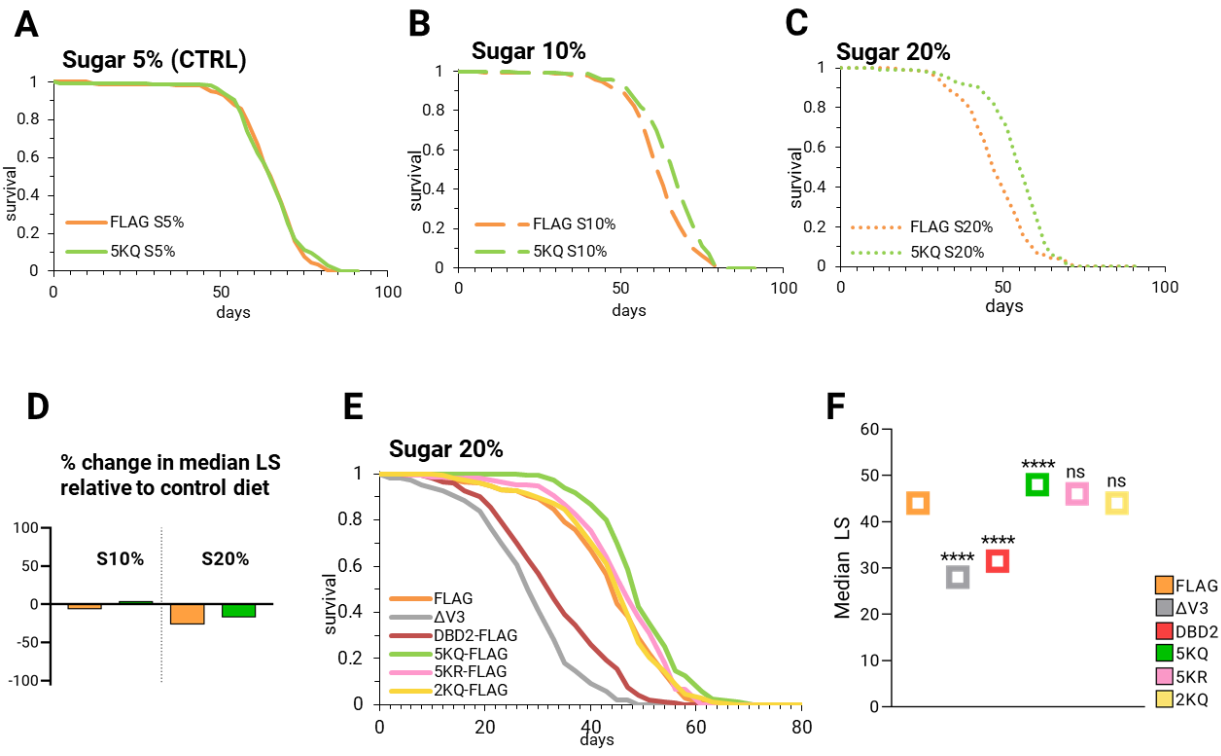

Figure 3.18: dFOXO complete acetylation mutants live longer than control flies when fed a sugar-rich diet.
A) Lifespan on 5% sugar diet (standard food) showed no significant difference between female control (FLAG) and dFOXO acetyl-lysine (5KQ) mutant flies. ns p>0.05 log-rank test. B) On a 10% sugar diet, control flies showed a reduced lifespan compared to dFOXO-5KQ mutants. ***p<0.001 log-rank test. C) Upon sugar increase to 20%, FLAG control flies showed a stronger reduction in lifespan with respect to dFOXO-5KQ mutants. ***p<0.001 log-rank test. D) Summary of the effect of sugar excess for each genotype presented as percentage change in median lifespan relative to control SYA diet. 2-fold increase of sugar (S10%) decreased lifespan of control flies by 7%, while it had no detrimental effect in FOXO 5KQ acetyl mutant

flies (+3%). The strongest reduction in lifespan for both FLAG control and 5KQ flies was observed in the S20% diet (-27% and -17%, respectively). E) Female lifespan assays of different dFOXO mutants on a 20% sugar diet. F) Summary of median LS from experiment shown in (E). ΔV3 (dfoxo-null) and DBD2 (DNA-binding mutant) flies are shorter lived than FLAG control flies, while partial acetyl-lysine (2KQ) and acetyl-null (5KR) mutants live as long as FLAG control flies on a 20% sugar diet. Complete acetyl-lysine (5KQ) mutants have a significantly increased lifespan compared to control flies. log-rank test, ns p>0.05, 2KQ and 5KR vs FLAG; ****p<0.0001, ΔV3 and DBD2 vs FLAG; ****p<0.0001, 5KQ vs FLAG. N= 150/genotype in each assay.

3.3.5.3 Metabolic phenotypes induced by high sugar intake are not influenced by dFOXO acetylation

Dietary sugars are sensed through the IIS pathway, with FOXO being the major downstream effector (Partridge et al., 2011) and, like humans, flies fed a sugar-rich diet can develop metabolic defects such as insulin resistance, increased glycation and adiposity (Morris et al., 2012; Skorupa et al., 2008). Therefore, we were interested to investigate how the high-sugar-induced metabolic phenotypes would correlate with the rescued lifespan of the acetylation mutants.

First, we monitored the molecular effect of high-sugar feeding on the IIS pathway, by measuring the expression levels of dFOXO by western blotting in control flies fed the S20% diet. We found that increasing dietary sugar to 10% reduced FOXO protein expression, while a diet with 20% of sugar determined a substantial increase in expression of the protein (Figure 3.19A-B). This observation suggests that, while a small increase in dietary sugar intake may lead to FOXO downregulation through activation of the IIS pathway, higher amounts of sucrose lead to insulin resistance (as evidenced by increased FOXO levels).

Next, we were interested in testing the molecular response of dFOXO mutant flies to the highest sugar diet. Again, we found that feeding excess sugar resulted in higher FOXO protein expression compared to control feeding, with a trend or significant increase in DBD2 mutants and FLAG control flies, respectively, and the highest increase observed in 5KQ acetylation mutants (Figure 3.19 E-F).

Glycation damage is a side effect of high sugar consumption and diabetes (Goh and Cooper, 2008), shown to increase in flies with high dietary sugar (Garrido et al., 2015). Based on that, we then checked levels of glycation upon increased sugar consumption and observed high glycation in whole flies in response to S20% feeding, but not S10% (Figure 3.19C-D). In the context of FOXO mutations, 4-fold increase in sugar intake confirmed a significant increase in glycation adducts in FLAG control flies but only showed a trend of increase in the other mutants (probably because of the high variability) (Figure 3.19G and Figure S8A). Consistent with previous reports (Morris et al., 2012; Skorupa et al., 2008), we also observed increased adiposity in all flies subjected to the S20% food, regardless of genotype (Figure S8B).

Overall, these data indicate that flies exposed to a high-sugar diet are prone to develop insulin resistance and high glycation levels, and that this is independent of the genetic mutation. This reveals that the survival of 5KQ flies in this condition is uncoupled from the development of these hallmarks of metabolic disease.

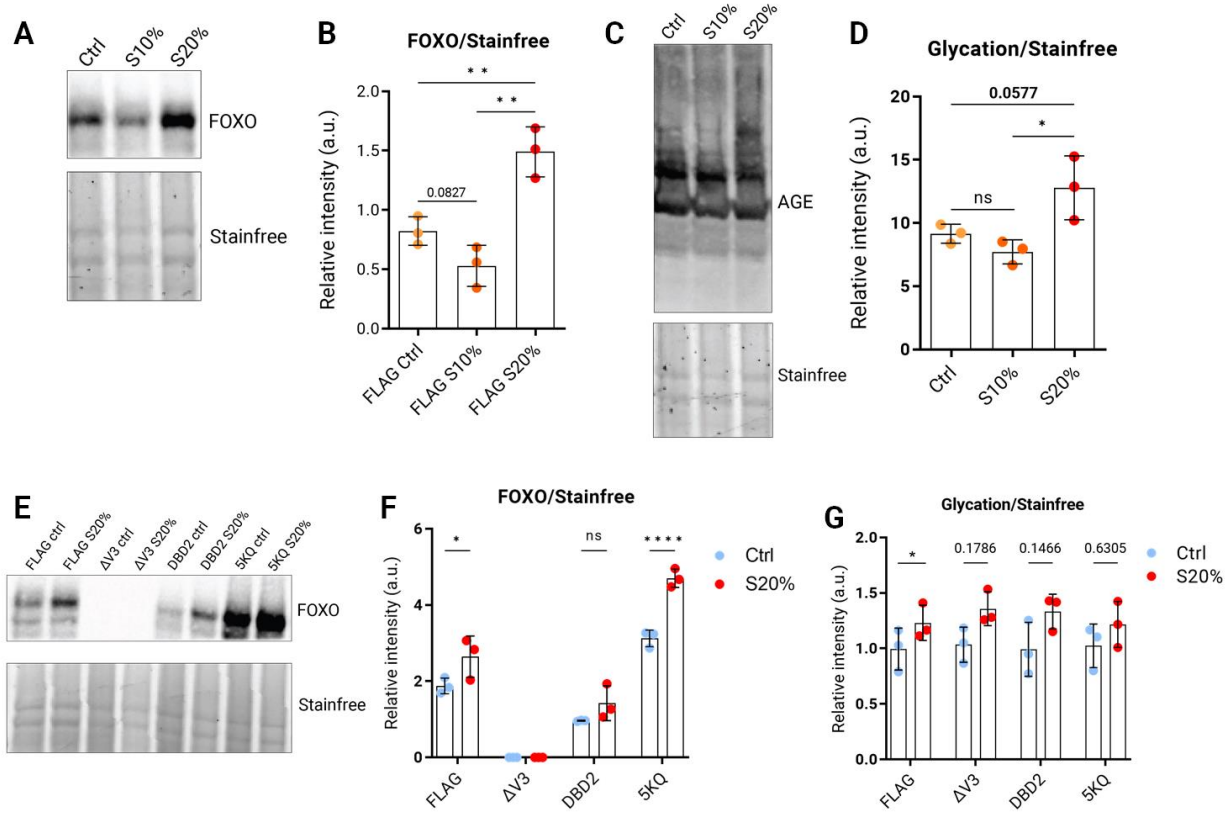

Figure 3.19: Molecular effects of a high-sucrose diet in WT and FOXO mutant flies.
A) Immunoblotting of whole-body protein extracts from female dFOXO-FLAG flies showing expression level of FOXO protein during continuous feeding regime (Ctrl) or upon sugar increase (S10% and S20%). Flies were fed on the diets for 7 days. B) Quantification of FOXO band over stainfree from blot shown in (A). FOXO expression showed a trend of decrease in S10% diet (p=0.0827) and it's significantly increased in S20%. **p<0.01, one-way ANOVA followed by Tukey's multiple comparisons test. N=3 biological replicates/condition, 20 flies each. C) Immunoblotting of whole-body protein extracts showing glycation damage in female dFOXO-FLAG flies fed for 7 days on Ctrl, S10% or S20% diet. D) Quantification of anti-AGE antibody over stainfree from blot shown in (C) revealed a trend of increase (p= 0.0577) in glycation damage in response to S20% feeding. Compared to S10%, the increase in S20% is statistically significant. ns p>0.05, *p<0.05, one-way ANOVA followed by Tukey's multiple comparisons test. N=3 biological replicates/condition, 20 flies each. E) Immunoblotting of whole-body protein extracts showing expression level of FOXO in different dFOXO mutants subjected to a control (Ctrl) or 20% sugar (S20%) diet. Flies were fed on the diets for 7 days. Extract from FOXO ΔV3 mutant flies included as negative control. F) Quantification of FOXO band over stainfree from blot shown in (E). Upon S20% diet, FOXO expression is significantly increased in FLAG control and, even more strongly, in 5KQ acetylation mutant flies. ns p>0.05, *p<0.05, ****p<0.0001, two-way ANOVA followed by Bonferroni's multiple comparisons test. N=3 biological replicates/condition, 20 flies each. G) Quantification of anti-AGE antibody over stainfree from blot shown in Figure S4 indicated a trend of increase in all tested genotypes upon S20% diet, with only FLAG control flies showing a significant increase. *p<0.05, two-way

ANOVA followed by Bonferroni's multiple comparisons test. N=3 biological replicates/condition, 20 flies each.

3.3.5.4 Acetylation of dFOXO attenuates dysregulation of purine metabolism and kidney stone formation

High-sugar diets promote increased purine catabolism, which in turn promotes uric acid accumulation and the formation of kidney stones (van Dam et al., 2020; Lang et al., 2019). The observation that deletion of *foxo* makes flies more sensitive to dietary sugar (van Dam et al., 2020) and that a SNP in human FOXO3 was found to be significantly associated with serum uric acid levels (Lang et al., 2019) suggests a potential role for the downstream effector of the IIS pathway in the regulation of purine metabolism.

With this in mind, and given the increased resistance of acetylation mutants to sugar excess, we next examined the tendency for stone formation in the Malpighian tubules (Figure 3.20A) of control and acetylation mutant flies kept on the S20% diet for 28 days. These tubular structures represent the fly equivalent of the kidney and are involved in urine excretion and osmoregulation (Rodan, 2019). An efficient method to visualize tubule stones is through polarized light microscopy, as it captures crystal refraction (Chen et al., 2012). At 28 days of age, control flies had a stronger tubule stones phenotype when fed the high sugar diet (S20%) compared to the control diet. Interestingly, while there was also a significant increase of tubule stones in 5KQ flies between the high sugar and standard diet, 5KQ mutants showed reduced tubular stones compared to control flies both in terms of severity (Figure 3.20C-B) and size (Figure S10).

The presence of tubule crystals may be an indication of uric acid accumulation (Ghimire et al., 2019). Thus, we next assessed uric acid content from whole FLAG and 5KQ flies fed the sugar-rich diet for 28 days. Remarkably, we found significantly increased levels in the controls, while the dFOXO acetylation mutants showed a lower and not significant rise in uric acid content (Figure 3.20D). This suggests that dietary sugars fuel purine metabolism, leading to increased uric acid levels and ultimately

stone formation. Consistent with previous observations linking FOXO to reduced uric acid metabolism and concretion formation (Lang et al., 2019), we find here that acetylation of dFOXO in particular appears to play a role in attenuating this phenotype.

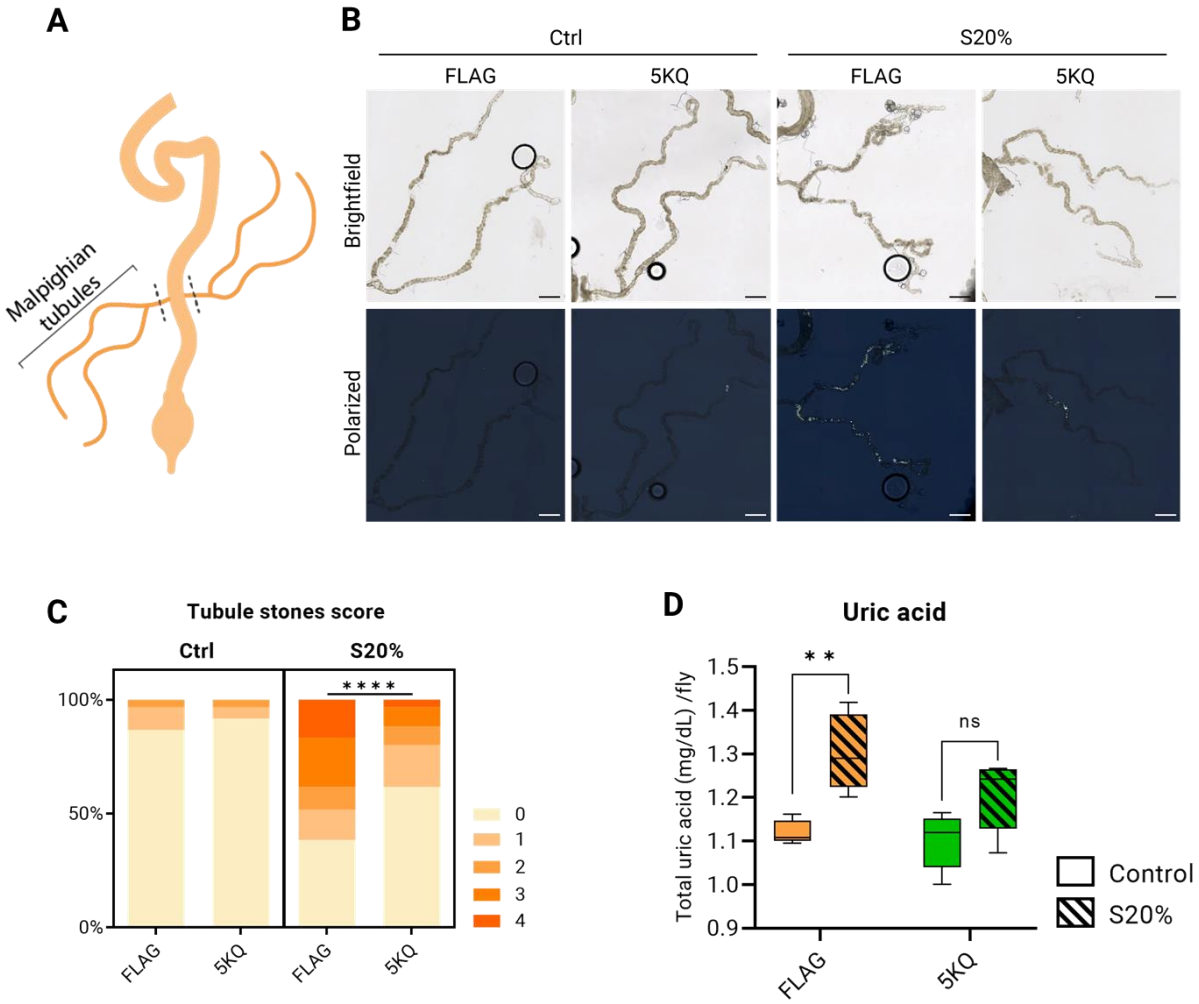

Figure 3.20: FOXO acetylation attenuates the dysfunctional phenotypes associated with high-sugar consumption.
A) Diagram of the fly lower gut, showing the Malpighian tubules pair and point of dissection. B) Representative images in brightfield and polarized light of dissected microtubules from FLAG control and 5KQ acetylation mutant females fed for 28 days on S5% (ctrl food) or 20%S. Scale bar= 200 µm. C) Tubule stones scoring of FLAG control and 5KQ acetylation mutant flies maintained for 28 days on control or S20% diet, presented as stacked bar plot. On control food, FLAG flies had an increase of mild stones formation (****p<0.0001, classification 1, FLAG ctrl vs 5KQ ctrl), but severity did not increase. 5KQ mutants treated with S20% food showed decreased accumulation and severity of the tubule stones. ****p<0.0001, classification 1-4, FLAG S20% vs 5KQ S20%, multiple linear regression. See FigureS9 for the scoring scale. N=15/genotype and condition. D) Total uric acid content of FLAG and 5KQ females fed for 28 days on S5% (control diet) or S20% (n=5 replicates per condition, each with 5 flies per sample). FLAG control flies had a significantly higher amount of uric acid upon high-sugar feeding. Diet: ***p<0.01; genotype: *p<0.05; interaction: *p<0.05 two-way ANOVA. N=5 replicates/genotype and condition, with 5 flies each.

In conclusion, these data show that flies exposed to a high-sugar diet have a shorter lifespan and that the phenotype is exacerbated by complete knock-out of FOXO or mutation of its binding site, whereas its complete acetylation leads to increased resistance. At the molecular level, excessive sugar exposure translates as increased FOXO expression, glycation levels, uric acid levels and stone formation, all side effects that appear to be alleviated in the acetylation mutants. In the future, it would be interesting to investigate how acetylation of FOXO is involved in purine catabolism and why this results in increased resistance to metabolic stress.

SECTION II: dFOXO DNA BINDING

4. Section II: dFOXO DNA binding

4.1 Introduction

It was suggested that FOXO transcription factors may have both DNA-binding dependent and independent functions (Matsumoto et al., 2006; Rudd et al., 2007). For example, fly lifespan and fecundity are strictly dependent on their transcriptional ability, whereas body size and development appear to be independent (Dr. Victor Bustos, unpublished data). FOXO transcription factors are also mediators of the starvation response (Chatterjee et al., 2014; Teleman et al., 2008) and integrate insulin signaling to modulate glucose and lipid metabolism (Kim et al., 2011; Kousteni, 2012). However, it remains unclear whether regulation of these pathways in *Drosophila* requires FOXO's binding to the DNA. In this part of the study, we used dFOXO DNA-binding domain mutant flies to separate metabolic functions that are dependent and independent of DNA binding.

4.2 Results

4.2.1 dFOXO is involved in the utilization of fat stores independent of its DNA-binding ability

Mouse studies have highlighted the ability of FOXO to modulate liver fat utilization, independent of its ability to directly bind to the DNA (Cook et al., 2015; Matsumoto et al., 2006). Thus, to investigate whether dFOXO transcriptional ability is required to mediate survival in *Drosophila* upon complete nutrient deprivation, we tested the resistance of *foxo-null* and DNA-binding domain (DBD2) mutants to a starvation diet. Remarkably, DBD2 mutants completely rescued the starvation phenotype of ΔV3 flies (Figure 4.21A), suggesting that survival in this condition is independent of DNA binding.

Feeding flies on the S20% diet had resulted in increased TAGs in all genotypes (Figure S8B), suggesting that lipid synthesis or storage is not affected by the loss of FOXO. Therefore, we next examined the TAGs content of control, ΔV3 and DBD2 female flies before and after starvation. As shown in Figure 4.21B, both control and DNA-binding

domain mutants have a substantial reduction in TAGs content post-mortem, whereas *foxo-null* flies cannot fully mobilize their fat stores. This suggests that dFOXO function, independent of its DNA-binding ability, is required for lipolysis under starvation.

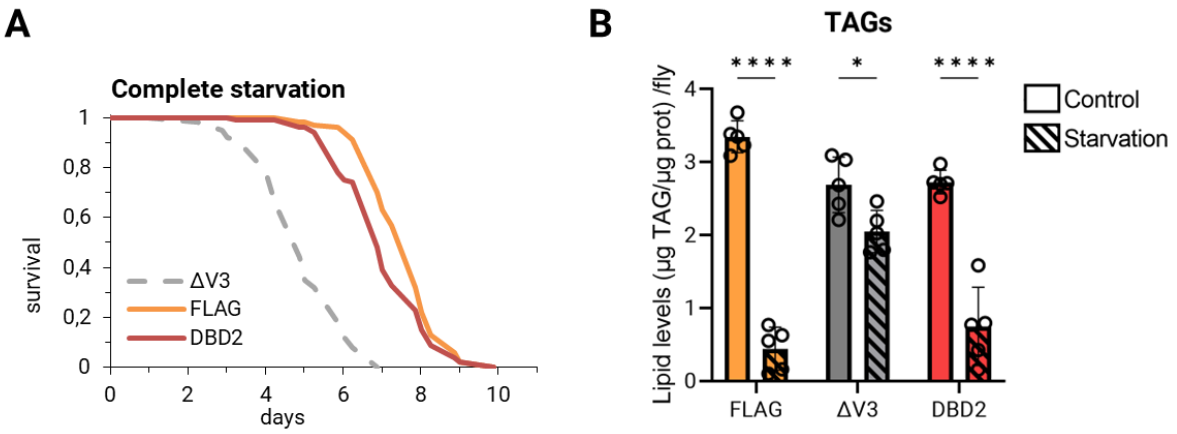

Figure 4.21: dFOXO regulates mobilization of fat stores under starvation independent of DNA binding.
A) Complete starvation assay showed that only ΔV3 flies were shorter-lived when compared to controls. ***p<0.001, ΔV3 vs FLAG flies; ns p>0.05, DBD2 vs FLAG; log-rank test. N= 150/genotype. B) TAG content of female flies continuously fed or starved to death showed that FLAG control and DBD2 mutant flies could mobilize their fat stores (FLAG control vs FLAG starvation, ****p<0.0001; DBD2 control vs DBD2 starvation, ****p<0.0001, Bonferroni multiple comparison test), while ΔV3 *dfoxo-null* exhibited higher TAGs level after starvation (ΔV3 control vs ΔV3 starvation, *p<0.0001, Bonferroni multiple comparison test). Diet: ****p<0.0001; genotype: ***p<0.001; interaction: ****p<0.0001 two-way ANOVA. N=5 biological replicates per assay, each with 5 flies/genotype.

These results confirm previous observations from our lab (Dr. Victor Bustos, unpublished data) and underly that dFOXO promotes lipid utilization independently of its direct binding to the DNA. Other studies have already proposed a role for FOXO as a transcriptional co-regulator (Cook et al., 2015; Czymai et al., 2010; Ramaswamy et al., 2002; Fan et al., 2009). Moreover, the observation that FOXO localizes to the nucleus of fat body cells during starvation (Figure 3.12; Chatterjee et al., 2014) supports the idea that FOXO might be involved in the response to starvation as a co-factor.

4.2.2 DNA binding of FOXO is necessary to respond to sugar starvation

FOXO transcription factor plays a critical role in maintaining whole body energy metabolism, and regulation of glucose levels is essential for survival during prolonged fasting. FOXO1 has been shown to adjust hepatic glucose metabolism in response to starvation by downregulating the expression of glycolytic genes and upregulating gluconeogenesis (Gross et al., 2008; Puigserver et al., 2003).

Since it has been shown that the response to complete starvation does not require direct binding of FOXO to the DNA (Ramaswamy et al., 2002; Fan et al., 2009), we wondered whether the ability of FOXO to maintain energy homeostasis in the absence of glucose relies on the presence of a functional DNA-binding domain. We tested the response of the mutant flies to a diet only composed of yeast and agar, but not sugar; along with *foxo-null* and DBD mutants, we also included acetyl-mimicking and acetyl-null flies in order to test whether acetylation of dFOXO influences sensitivity to the no-sugar food. As shown in Figure 4.22A, flies required a functional dFOXO-DBD to survive under sugar deprivation, and this was completely independent from the acetylation state of the protein. We then repeated the starvation assay using a defined medium without sugar (YAA -sugar) to verify the results from the yeast-based diet. Consistent with previous results, both *foxo-null* and DBD mutants were significantly shorter-lived compared to control or acetylation mutants (Figure 4.22B) and the difference was even more obvious on this diet than on the SYA -sugar food, possibly indicating sensitivity of the KO and DNA-binding deficient mutants to lack of some components in the defined medium. Interestingly, fecundity of all tested flies increased in the absence of sugar (Figure 4.22C), probably for an increased proportion in yeast compared to the control diet. This is in line with the observation that yeast supplementation increases fecundity in *Drosophila* (Bass et al., 2007) while excess of sugar decreases it (Chandegra et al., 2017), and suggests that dFOXO is not responsible for mediating this biological function upon sugar starvation.

Next, to examine the effect of sugar depletion on a molecular level, we performed western blotting from whole body lysates of FLAG control and DBD2 mutants and observed an increase in dFOXO protein levels upon sugar deprivation (Figure 4.22D).

Although the increase was significant in both genotypes, control flies showed a higher level of expression compared to DNA-binding deficient mutants (Figure 4.22E).

In response to starvation, FOXO redirects cellular metabolism towards lipolysis (Chatterjee et al., 2021) and, as we showed, dFOXO-mediated fat utilization is independent of its ability to bind DNA. To investigate whether this is the case also in the context of specific sugar starvation, we measured the lipid levels of flies subjected for 7 days to a food lacking sugar but containing yeast, a source of lipids (Jach et al., 2022). Consistent with the results from the complete starvation assays, both FLAG and DBD mutants were able to utilize their fat stores, whereas *foxo-null* flies showed same level of TAGs before and after starvation. Notably, DBD mutants showed a drastic reduction in their lipid levels compared to FLAG flies upon the same condition (Figure 4.22F), suggesting that the impaired transcriptional ability of FOXO may result in disproportionate lipolysis.

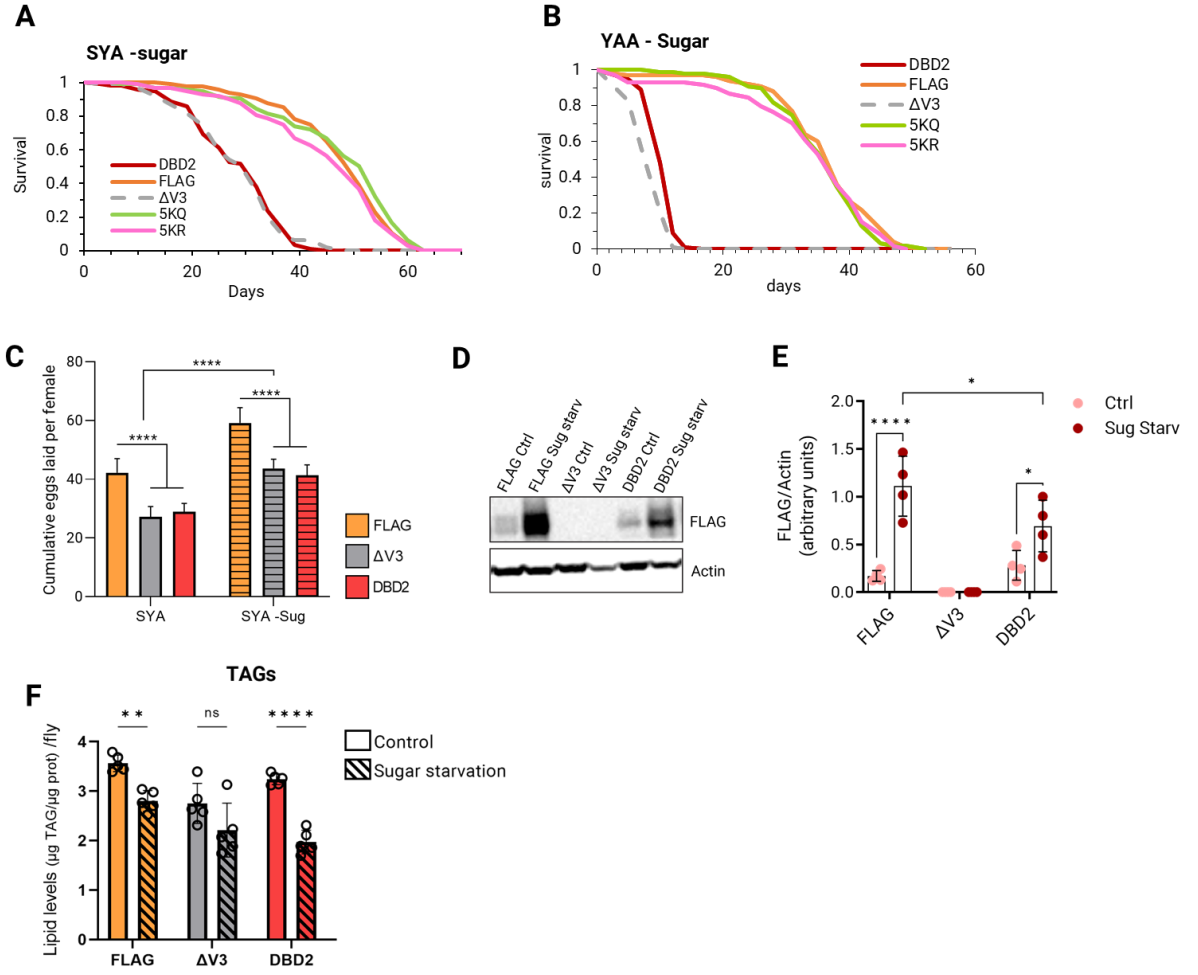

Figure 4.22: FOXO DBD mutants are sensitive to sugar deprivation.
A) On a no-sugar diet (SYA), ΔV3 and DBD2 mutant flies were sensitive whereas acetylation mutants (5KR and 5KQ) behaved as gene replacement flies (FLAG). The same was observed in a defined medium without sugar (B). ***p<0.001 log-rank test, ΔV3 or DBD2 vs FLAG flies. N= 150/genotype in each assay. C) In all tested genotypes, sugar deprivation led to a significant increase in egg production. ****p<0.0001, SYA vs SYA -Sugar; two-way ANOVA followed by Bonferroni's multiple comparisons tests. N= 10 biological replicates/genotype, 5 flies each. D-E) Immunoblotting (D) and respective quantification (E) of whole-body protein extracts showing expression level of FOXO in dFOXO mutants subjected to a control (Ctrl) or sugar-deprived (Sug starv) diet. Upon sugar starvation, DNA-binding mutant flies had a reduced expression of dFOXO compared to FLAG control flies. *p<0.05, one-way ANOVA followed by Bonferroni's multiple comparisons test. Flies were fed on the diets for 7 days. Extract from FOXO ΔV3 mutant flies included as negative control. N=4 biological replicates, 20 flies each. F) TAG content of female flies kept for 7 days on control or sugar starvation food showed that DNA-binding mutants utilized more lipids upon sugar deprivation. Diet: ****p<0.0001; genotype: ****p<0.0001; interaction: **p<0.01, two-way ANOVA. Bonferroni multiple comparison test: ns p>0.05, *p<0.05, ***p<0.001, ****p<0.0001. N=5 biological replicates per assay, each with 5 flies/genotype.

Taken together, these findings implicate that dFOXO is responsible for maintaining fecundity and survival upon sugar starvation, and that this is dependent on its DNA-binding ability, but not on its acetylation state. Moreover, an integral DNA-binding domain might be required to ensure normal activation of FOXO expression and balanced lipid utilization in case of sugar starvation.

4.2.3 Proteome profiling of FOXO DNA-binding domain mutants upon complete starvation

To better understand the biological changes that occur in the fly during total starvation, both in the context of a wild-type and dFOXO-mutated genetic background, we performed whole proteome analysis by mass spectrometry on FLAG control, ΔV3 and DBD2 whole flies that were either continuously fed (FC, DC, ΔC) or starved (FS, DS, ΔS) for 3 days. Preliminary analysis confirmed the good quality of the data, with approximately 4000 detected proteins per sample (Figure S11A). Principal component analysis showed a clear separation of the samples by genotype and, mostly, by diet. Interestingly, DBD and *foxo-null* mutants cluster together under normal feeding conditions, whereas they clearly separate under starvation (Figure S11B). This indicates that under normal feeding conditions they share a largely similar proteome, whereas their (biological) differences mainly emerge in response to starvation. Moreover, visualization of differentially expressed proteins also confirmed normal expression of FOXO in controls and DBD lines, but not in the *foxo-null* mutants, in both feeding conditions (Figure S12 A-F).

Comparing the starved to the fed condition, we observed that nutrient deprivation induced marked differential changes in protein expression in all three genotypes, with 843 (207/636), 771 (187/584) and 1048 (388/660) up- and down-regulated proteins in control, DBD and ΔV3 flies, respectively. Remarkably, the majority of differentially expressed proteins were observed in *foxo-null* flies, suggesting that these mutants must drastically alter their proteome in order to adapt to starvation without FOXO protein (Figure 4.23A-B). Gene Ontology enrichment for shared DEPs revealed that, under starvation, all three genotypes redirect some biological processes towards the

production of metabolites and energy at the expense of protein synthesis, which is drastically halted (Figure S14).

To gain more insight into the biological processes that are affected by the nutritional stress dependently of FOXO mutation, we performed Gene Ontology analysis for DEPs in the three genotypes upon starvation (Figure 4.23D). Comparison of the top 40 significantly enriched GO terms showed that, among the up-regulated proteins, the term “lipid oxidation” was more significantly enriched in DBD2 and ΔV3 flies compared to control flies, with a higher fold enrichment in DNA-binding deficient mutants. Similarly, the term “catabolic process” was significantly enriched only in the dFOXO mutants. On the other hand, processes that generate energy through oxidative phosphorylation seemed to be mainly upregulated in FLAG and *foxo-null* flies. Lastly, it is worth noting that gene expression and translation, as well as “regulation of amide metabolic process”, were mainly downregulated in the mutants.

The observation that DBD and *foxo-null* mutants had higher expression of proteins for lipid catabolism was confirmed by GO analysis of 42 proteins that were upregulated under starvation in both DBD2 and ΔV3, but not control flies (Figure 4.23C).

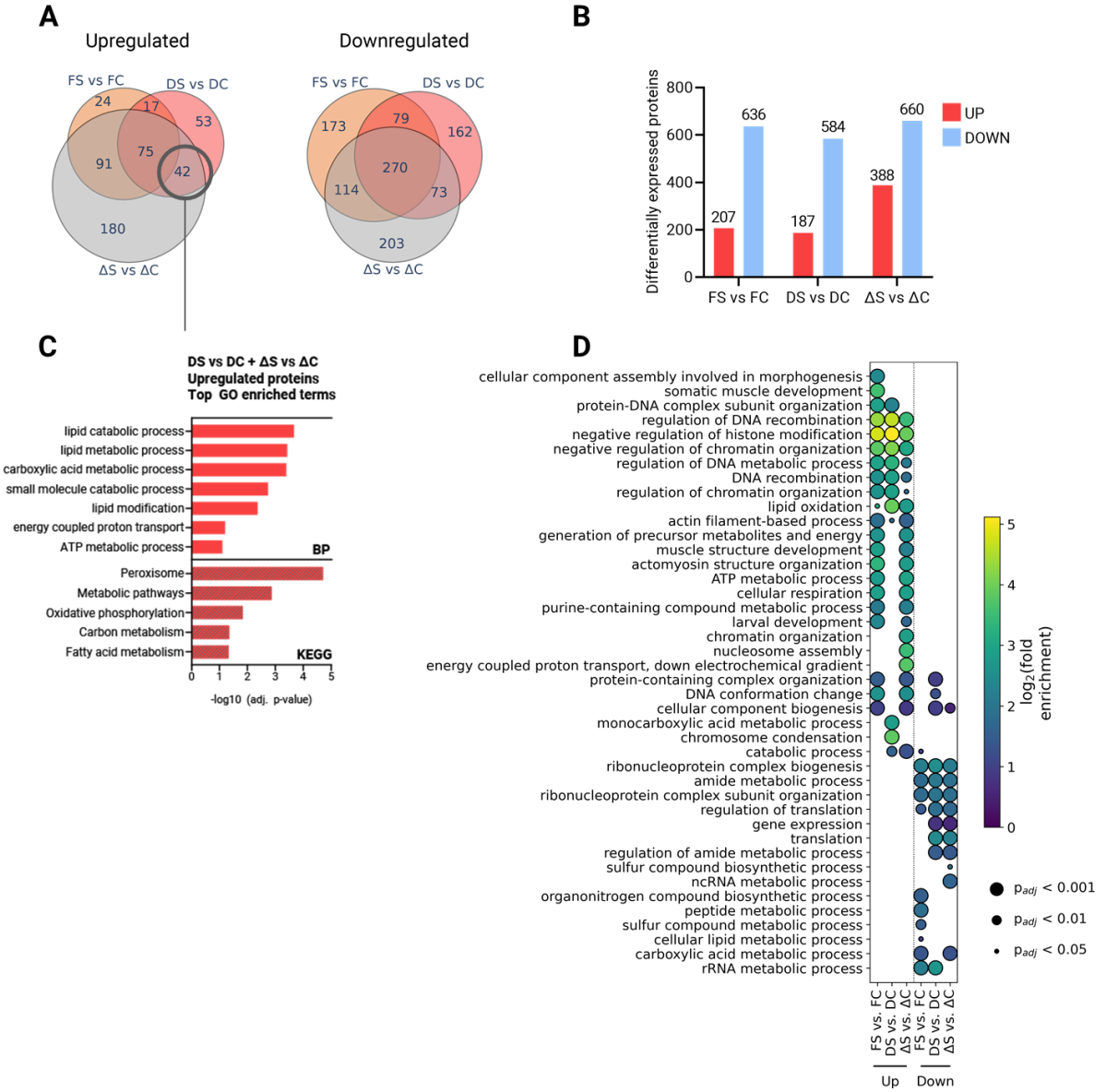

Figure 4.23: Proteome profiling of *dfoxo-null* and DBD2 mutants upon complete starvation. A) Venn diagram of upregulated (left) and downregulated (right) differentially expressed proteins (DEPs) in the comparisons FS vs FC (orange), DS vs DC (red) and ΔS vs ΔC (grey). B) Barplot representation of DEPs in the comparisons FS vs FC, DS vs DC and ΔS vs ΔC. The exact number of DEPs is indicated above the bar. C) Barplot representation of significantly enriched top GO terms of the 42 upregulated proteins shared between DS vs DC and ΔS vs ΔC. BP= biological process; KEGG= KEGG pathway. GO terms sorted by adjusted p-value. D) Dotplot representation of significantly enriched top GO terms sorted by adjusted p-value of up- and downregulated proteins in the comparisons FS vs FC, DS vs DC and ΔS vs ΔC. Dotplot was created by Dennis Gadalla. | Abbreviations: FC= dFOXO-FLAG flies, control diet; FS= dFOXO-FLAG flies, starvation diet; DC= dFOXO-DBD2 flies, control diet; DS= dFOXO-DBD2 flies, starvation diet; ΔC= dfoxo-null flies, control diet; ΔS= dfoxo-null flies, starvation diet.

4.2.3.1 FOXO DNA-binding domain mutants respond to starvation switching metabolism from carbohydrate to lipid utilization

Next, we analyzed the direct comparison between control flies and DBD mutants in the starved condition, in order to verify whether the resulting enriched GO terms would explain the phenotype observed in the DBD mutants. In line with the known function of dFOXO transcription factor in carbohydrate metabolism and with the observed phenotype of DBD mutants upon sugar starvation, the analysis revealed that sugar metabolism was downregulated in the mutants (Figure 4.24A, BP= carbohydrate metabolic process; KEGG= starch and sucrose metabolism). As a confirmation, the term “carbohydrate metabolic process” appeared to be downregulated also in starved *foxo-null* flies (Figure 4.24G). Interestingly, many amino acid biosynthesis-related proteins were significantly up-regulated in control flies compared to both FOXO DBD mutant flies (Figure 4.24A and S15) and *foxo-null*s (Figure 4.24E), suggesting that dFOXO transcriptional ability might be involved in this process.

Strikingly, the majority of GO terms of downregulated proteins in the comparison FS vs DS are related to fatty acid metabolism (Figure 4.24B and 4.24D), indicating an enhanced lipid catabolism in the dFOXO DNA-binding deficient mutants. Interestingly, the term “peroxisome” was also highly significant in the KEGG pathway category (Figure 4.24B), suggesting that this organelle might be involved in the utilization of lipids. Similarly, GO analysis of downregulated protein from the comparison FS vs ΔS revealed that the term “fatty acid metabolism” was significantly enriched (Figure 4.22F).

These results show that both *foxo-null* and DBD mutants can upregulate proteins required for fat degradation; however, previous assays had shown that the mutants have a very different sensitivity to starvation. With the aim of finding differentially expressed proteins that might explain this contradictory phenotype, we next compared the DEPs of *foxo-null* and DNA-binding domain mutants upon starvation and found that, while most proteins involved in lipid degradation were not differentially expressed, two proteins involved in lipid transfer (Apoltp, Apolipoprotein lipid transfer particle, and bgm, bubblegum) were significantly upregulated in DBD mutant flies (Figure S15B).

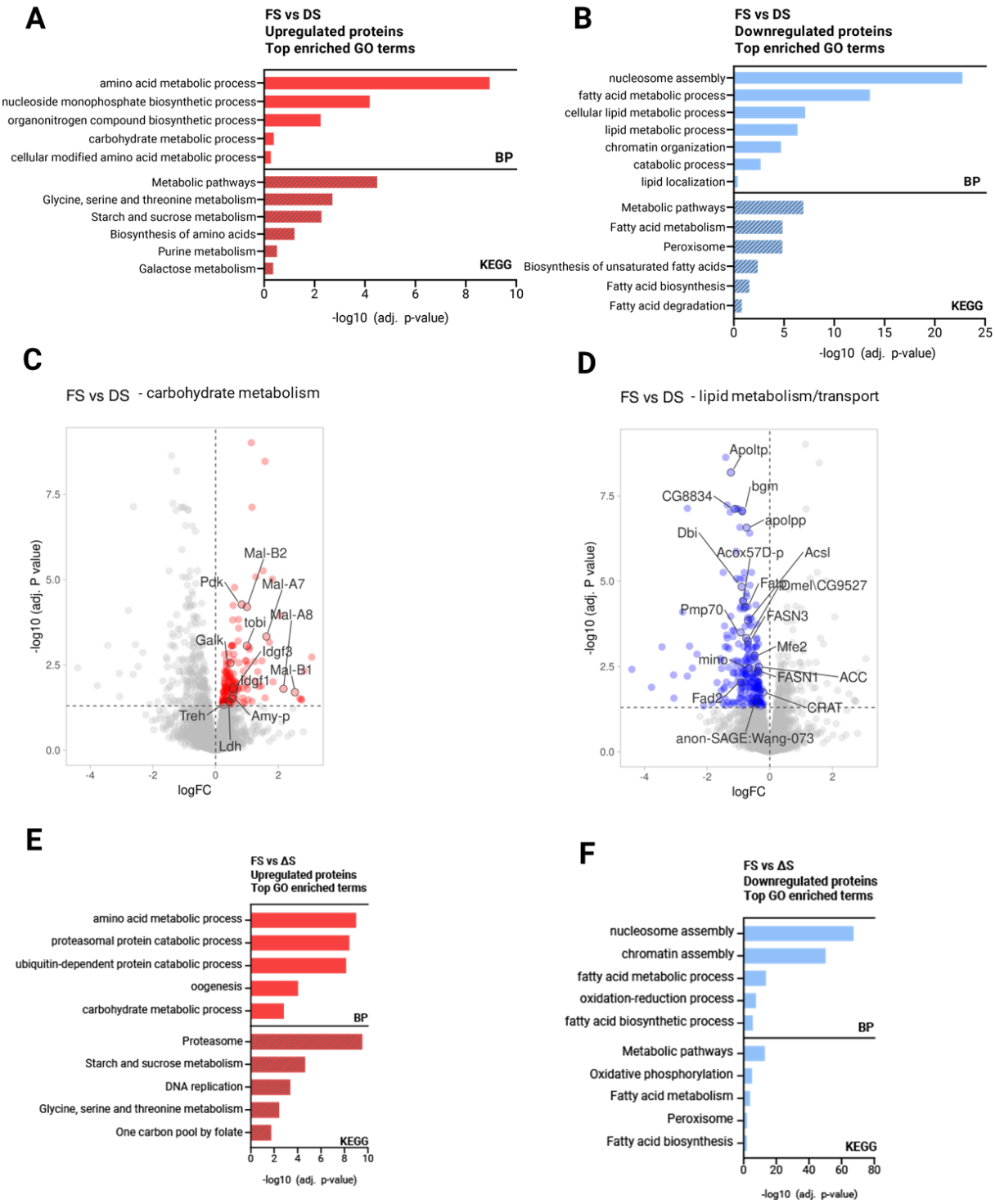

Figure 4.24: Upon starvation, DBD mutants have an upregulated lipid metabolism and downregulated AAs and sucrose metabolism.

A-B) Barplot representation of significantly enriched top GO terms of upregulated (A) or downregulated (B) proteins in the comparison FS vs DS. BP= biological process; KEGG= KEGG pathway. GO terms sorted by adjusted p-value. C) Volcano plot of DEPs in the comparison FS vs DS highlights upregulation of proteins involved in carbohydrate metabolism. D) Volcano plot of DEPs in the comparison FS vs DS shows that DBD2 mutants under starvation had an

upregulation of proteins related to lipid metabolism. E) Volcano plot shows DEPs related to lipid metabolism in the comparison DS vs ΔS. Cut-off of p-value = 0.05. F) Barplot representation of significantly enriched top GO terms sorted by adjusted p-value of upregulated (G) or downregulated (H) proteins in the comparison FS vs ΔS.

Taken together, these data suggest that, in the absence of dFOXO transcriptional output, flies respond to starvation by upregulating lipid catabolism and downregulating carbohydrate and amino acid metabolism. Since the proteomic analysis revealed high levels of catabolism even in *foxo-null* flies, we hypothesize that these flies are still able to upregulate proteins required for lipid degradation during starvation, but that they fail to mobilize fat in the absence of dFOXO, independently of its ability to bind DNA.

In the future, further experiments should be performed to test this hypothesis and to identify the putative dFOXO-dependent, DNA-binding independent mechanism of fat depletion during starvation stress.

DISCUSSION

5. Discussion

5.1 Role of dFOXO acetylation in the fly metabolism

FOXO transcription factors are involved in a wide range of evolutionarily conserved biological processes, including response to metabolic stress and survival. As a result, a number of post-translational modifications, including acetylation, tightly regulate FOXO activity (Daitoku et al., 2011; Wang et al., 2016). Some studies have discussed the role of FOXO acetylation in regulating some physiological functions *in vivo*. In a knock-in study, mice expressing constitutive acetyl-null FOXO1 exhibited increased lipid metabolism at the expense of glucose utilization (Banks et al., 2011). In *Drosophila*, dFOXO acetylation is implicated in the homeostatic response to starvation and changes depending on the nutritional state (Wang et al., 2011). dFOXO acetylation has been mainly connected to reduced transcriptional activity of the protein, both for decreased affinity to the DNA (Brunet et al., 2004; Daitoku et al., 2004; Matsuzaki et al., 2005) and preferential cytoplasmic retention (Banks et al., 2011; Qiang et al., 2010). This refers especially to acetylation of lysine residues buried within the NLS (Daitoku et al., 2004; Jing et al., 2007). However, the precise role of FOXO regulation by acetylation and its impact on longevity and metabolism have not been fully elucidated.

To investigate the function of acetylation *in vivo*, we used previously generated dFOXO acetylation mutant flies in which 5 lysine residues within the NLS are substituted with glutamine (5KQ), to mimic acetylation, or with arginine (5KR), to abolish it. These residues were chosen based on two criteria: evolutionary conservation, as acetylated residues are more likely to be conserved (Weinert et al., 2011), and likelihood of modification by acetylation, predicted by the bioinformatics tool LAceP (Hou et al., 2014). Mice expressing constitutively acetylated FOXO1 proteins die during development because of angiogenesis defects (Banks et al., 2011). In contrast, in our study both dFOXO acetyl-mimic and acetyl-null flies had wild-type-like phenotypes in terms of development, lifespan, body weight, fecundity and starvation resistance. On the other hand, *foxo-null* flies exhibit defects in all these phenotypes, confirming that dFOXO protein regulates these physiological functions *in vivo* (Slack et al., 2011), but

also suggesting that this is independent of the acetylation state. Of note, the constitutively acetylated mice have 7 lysine to glutamine substitutions (7KQ) (Banks et al., 2011), two of which are not conserved in *Drosophila*. This could be the reason behind the difference in viability, as the protein structure may too altered by the additional modifications. Moreover, this underlies the biological complexity of the murine system and the importance of a fully functioning FOXO in this context, consideration that should be taken into account for future studies.

5.1.1 FOXO acetylation mediates the response to starvation of specific (but not all) nutrients

Since FOXO primary role is to respond to situations of metabolic emergency, like nutrient deprivation, it is reasonable to think that the acetylation state of the protein would not majorly affect physiological functions under optimal conditions, but would do so in case of starvation. Thus, it is interesting to notice that the 5KQ acetylation mutants did not show reduced survival upon complete starvation, as we would expect. Acetylation was shown to have a primary role in regulating dFOXO activity during starvation in flies (Wang et al., 2011). However, the difference could be attributed to different factors. First, the fly strains used in this study may have a different sensitivity to starvation compared to flies used in other studies (Wang et al., 2011). Secondly, we can hypothesize that, although the activity of the protein is reduced by acetylation, it is not completely abolished by it and dFOXO would still be able to respond (at least partially) to extreme starvation. Furthermore, we are assuming that the 5 lysine residues substituted in our mutants are involved in the response to total starvation, but we still do not know exactly which ones are acetylated in *Drosophila* FOXO in this condition. It is possible that the key residues involved in this response are located in another region of the protein, or that complete starvation leads to a different pattern of acetylation, both possibilities that should be addressed in the future. Finally, the fact that both acetyl-null and acetyl-mimic flies are resistant to complete starvation suggests that this metabolic stress overrides the presence of the acetylation.

On the other hand, subjecting the flies to a specific diet allowed us to identify a physiological function that requires the presence of dFOXO in its deacetylated state: the response to yeast starvation. Both *foxo-null* and 5KQ acetylation mutants were shorter-lived on the diet, showed reduced fecundity and retained adiposity, whereas 5KR acetyl-null flies behaved exactly as controls. On the molecular level, response to yeast starvation appeared as increased dFOXO protein expression and decreased acetylation. This is in line with other studies showing a starvation-dependent increase in FOXO expression (Imae et al., 2003) and a prompt change in the acetylation state depending on the feeding condition (Wang et al., 2011), but it has not been investigated in the particular case of yeast deprivation. Early refeeding brought down FOXO protein levels and gradually increased acetylation, while, interestingly, two hours refeeding resulted in a sudden increase of the deacetylated protein. It's possible to interpret this as a mechanism that regulates the levels of acetylation and protein expression to restore normal activity after the metabolic stress has subsided and avoid excessive inhibition. To confirm this idea, it would be interesting to test the molecular response to refeeding after yeast starvation at later times points and check whether the protein reaches stable expression and acetylation levels.

Notably, the response to yeast starvation seemed to depend on dFOXO, but not on its ability to bind DNA, as dFOXO DNA-binding domain mutants (DBD) survived almost as controls on the starvation diet. This suggests that the response doesn’t depend on FOXO’s ability to directly activate target genes, but it might rather act as a co-activator to address the metabolic stress. Moreover, it implies that the putative interaction with transcriptional binding partners only happens when the protein is not acetylated. Supporting this idea, the interaction between FOXO1 and its co-binding partner HNF-4 only happens when FOXO1 is deacetylated (Matsuzaki et al., 2005). On the other hand, deacetylation might be a prerequisite to allow dFOXO preferential nuclear localization, that is affected by acetylation (Banks et al., 2011; Qiang et al., 2010). However, it is still to be verified whether the cellular response triggered by yeast starvation leads to dFOXO shuttling to the nucleus, and whether this would be compromised by acetylation of FOXO. Moreover, future studies should aim to identify the putative co-

factors whose binding with dFOXO allows the activation of a homeostatic response to yeast starvation.

5.1.2 Increased lipogenesis or decreased lipolysis?

Since starvation usually triggers utilization of the fat stores (Chatterjee et al., 2021), it's surprising to notice that the acetylation mutants are associated with a high adiposity upon the yeast starvation condition. It was reported that constitutively deacetylated FOXO1 mice display a phenotype of increased lipid catabolism (Banks et al., 2011); thus, it is possible that acetylation of the protein would drive metabolism towards the opposite direction. This finds confirmation in our proteomics data, that showed a drastic upregulation of fatty acid biosynthesis in the acetylation mutants compared to controls upon yeast starvation. Interestingly, this upregulation was accompanied by increased expression of proteins involved in glutathione metabolism, and in particular proteins from the glutathione S-transferase (GST) family.

GSTs are ubiquitous proteins that use glutathione as a substrate to carry out their enzymatic function of detoxification (Freitas et al., 2006). It was proposed that increased lipid biosynthesis could be induced, at least partially, by oxidative stress (Sekiya et al., 2008). In human hepatoma cells, treatment with hydrogen peroxide leads to the accumulation of triglycerides via up-regulation of sterol regulatory element-binding protein-1c (SREBP1c), involved in fatty acid metabolism (Sekiya et al., 2008). Interestingly, SREBP1c expression was found to inhibited by active FOXO1 (Deng et al., 2012). Furthermore, a study investigated the impact of glutathione deficiency on hepatic lipogenesis in rats and found that glutathione deficiency led to a significant reduction in the activity and expression of several lipogenic enzymes (Brandsch et al., 2010). A recent study showed that a decrease in the glutathione redox state leads to activation of Sirtuin-3 (Cortés-Rojo et al., 2020), a protein deacetylase that has been proved to regulate FOXO3a-mediated transcription of antioxidant genes (Sundaresan et al., 2009). The peculiar proteomic profile of the acetylation mutants might be explained as a result of lower affinity of dFOXO for DNA binding and consequent reduced ability to efficiently activate genes for oxidative stress resistance. The higher basal oxidative stress might contribute to enhanced lipid biosynthesis, and the

increased *Gst* gene expression could be an attempt to restore balanced redox homeostasis. Moreover, dFOXO acetylation might result in a lower inhibition of SREBP-1c, with consequent increase in the *de novo* lipogenesis. However, these hypotheses should be experimentally validated in the future.

Worth pointing out, *foxo-null* flies also show increased TAGs levels, both upon yeast starvation and complete starvation. However, we believe that the reason behind this phenotype differs from the case of the acetylation mutants. As will be discussed more in detail later, our results suggest that the complete loss of dFOXO compromises the mobilization of the fat stores; thus, the retained adiposity of *foxo-nulls* can probably be interpreted as decreased lipolysis ability rather than increased lipogenesis. However, this should be addressed directly in the future. For instance, in order to test which processes are affected by dFOXO loss of function compared to loss-of acetylation, it would help to repeat the proteomic analysis including the *foxo-null flies* in the experimental design.

Taken together, these findings are important as they demonstrate the crucial function of dFOXO acetylation as a key regulatory mechanism of lipid metabolism, and highlight the impact of this modification on the body homeostasis.

5.1.3 What's behind the response to yeast starvation? Cholesterol may be one of the answers

Much evidence pointed at amino acids starvation as the underlying cause of the yeast starvation phenotype seen in the *foxo-null* and 5KQ mutants. First, dFOXO had already been shown to mediate the response to total amino acid starvation, as *dfoxo-null* larvae did not develop on a food lacking amino acids (Slade et al., 2016; Kramer et al., 2008). The amino acid sensor GCN2, responsible for the response to essential amino acids starvation, was shown to promote FOXO activity (You et al., 2018), again suggesting a possible role of FOXO in this response. Moreover, proteins are the main component in yeast (Jach et al., 2022). Thus, we were surprised to see that a defined diet lacking both essential and non-essential amino acids did not reproduce the yeast starvation

phenotype of 5KQ mutants, since the *foxo-null* mutants were the only genotype whose lifespan was reduced. This result has different implications: on the one hand, it confirms the role of dFOXO in mediating response to amino acid starvation, in line with previous observations (Slade et al., 2016; Kramer et al., 2008). On the other hand, it doesn't exclusively implicate acetylation as the regulatory mechanism in play. In line with that idea, our proteomic results also suggest an involvement of dFOXO in processes related to amino acid metabolism, but those processes are downregulated in *foxo-null*, acetylation mutants and DBD mutants indistinctly. Additionally, it hints towards the presence of other nutritional components whose starvation is sensed through dFOXO, in an acetylation-state-dependent manner. One such component seems to be cholesterol, as the acetyl-mimicking mutants have reduced lifespan on a cholesterol deprived diet similar to *foxo-null* flies and display the biggest reduction in fecundity upon cholesterol deprivation.

Just like vertebrates, *Drosophila* needs cholesterol as a crucial component in their plasma membranes, as well as a building block for steroid hormones (Sieber et al., 2015; Niwa et al., 2011). For instance, cholesterol is needed for the production of a hormone, 20-hydroxyecdysone, involved in the production and delivery of yolk proteins, essential for the full development of the oocytes (Schonbaum et al., 2000; Bownes, 1986). Consistently with the idea of acetylation mutants having a dysregulated cholesterol metabolism, our proteomics data show a marked downregulation of yolk proteins (YP1, YP2, YP3) in these mutants upon yeast starvation. Moreover, GO analysis revealed a downregulation in terms related to vesicle-mediated transport. The endocytic machinery is heavily involved in the yolk uptake into the oocyte (Roth and Porter, 1964). Thus, a decrease efficiency in this mechanism is consistent with a reduced egg-laying phenotype.

Importantly, *Drosophila* cannot produce cholesterol on its own and needs to obtain it from the diet (Clark and Block, 1959). Thus, a functioning sterol sensing and transport mechanism is crucial for the maintenance of cholesterol homeostasis. Worth noting, FOXO has been implicated in cholesterol metabolism in different organisms and has been shown to activate transcription of a key nuclear receptor for cholesterol homeostasis in the fly (Afschar et al., 2015; Bujold et al., 2010). For instance,

mammalian FOXO1 regulates the expression of ABCA1 (Fukunaga et al., 2018) and ABCA6 (Gai et al., 2013), intracellular ATP-binding-cassette transporters responsive to cholesterol. In worms, daf-16/FOXO binds to the nuclear receptor NHR-8 to regulate cholesterol levels (Magner et al., 2013). In flies, dFOXO regulates the transcription of the nuclear receptor DHR96, which acts as a sentinel and responds to low cholesterol levels (Bujold et al., 2010). Moreover, DHR96 regulates the expression of the gene *npc1b,* which encodes a protein required for cholesterol transport (Bujold et al., 2010). Our results show that, upon yeast starvation, dFOXO acetylation mutants have an increased expression of some proteins involved in cholesterol transport. However, the upregulation of these proteins may indicate a compensatory mechanism in the fly that attempts to restore cholesterol homeostasis.

Based on this we hypothesize that dFOXO is involved in the regulation of cholesterol transport, which is impaired when the protein is acetylated. This results, on the one hand, in decreased expression of hormones necessary to ensure expression of fertility-related proteins, and on the other hand, in a compensatory upregulation of cholesterol transport proteins. While it is possible that the mutants have a dysregulated cholesterol metabolism also upon normal feeding, the effects would only become visible upon removal of this element from the diet. Interestingly, and consistently with this theory, we also detected lower levels of ion transport proteins in the 5KQ flies in normal feeding conditions, and it has already been proven that a variety of ion channels are regulated by changes in the level of membrane cholesterol (Levitan et al., 2010; Zhang et al., 2020; Garcia et al., 2019). Nevertheless, this hypothesis needs to be experimentally validated, for instance by measuring the cholesterol content of dFOXO acetylation mutants in both yeast-starved and fed condition. Moreover, it would be interesting to pinpoint the compounds that, when removed from the food together with cholesterol, cause a response in the mutants comparable to yeast starvation, as this would reveal the specific nutrients whose removal is sensed by dFOXO dependently of its acetylation state.

5.1.4 Complete vs partial acetylation

Of the five lysine residues harbored in the NLS, only three are actually conserved across species in *C. elegans*, *Drosophila* and mammals. Hence, it is possible that the response to yeast or cholesterol starvation in the fly preferentially involves the acetylation of these three residues. To test this hypothesis, we generated mutant flies in which only two (2KQ) or three (3KQ, 3KQII) lysines were mutated and observed completely overlapping phenotypes with the complete (5KQ) acetylation mutants. Under normal feeding conditions all partial acetylation mutants have normal lifespan, development, fecundity and body weight and show reduced survival and egg-laying when subjected to a no-yeast food. Moreover, the triple acetyl-mimic mutation of 3KQII mutant flies was sufficient to induce cytoplasmic localization of the dFOXO upon starvation, reproducing previous results using FOXO proteins carrying 7 acetyl-mimic point mutations (Banks et al., 2011; Qiang et al., 2010). On the other hand, when testing the resistance of acetyl-mimic flies to a high-sugar diet, the 2KQ partial acetylation mutants failed to provide the same increase in lifespan observed in the complete acetylation mutants. These results suggest that partial acetylation of dFOXO is sufficient to control some aspects of dFOXO regulation (response to starvation, cellular localization) but does not recapitulate all (protection from sugar excess). Thus, they raise new questions on the complexity of dFOXO regulation through acetylation. To have a deeper understanding of these molecular regulatory mechanisms, we should try to identify the key lysines residues that control FOXO activity. Moreover, it would be interesting to test whether different nutritional stress (cholesterol, yeast) correspond to different pattern of acetylation, and whether these modifications are mediated by specific deacetylases. In the fly, HDAC4 has been implicated in dFOXO deacetylation under full starvation (Wang et al., 2011); however, it would be important to determine if HDACs mediates dFOXO deacetylation also under yeast starvation, or if other deacetylases could regulate this modification (e.g., SIRTs).

5.1.5 FOXO as a potential sensor of methionine depletion

Although total amino acids restriction does not result in any visible phenotype in the acetylation mutants, specific removal or reduction of the EAA methionine determines

a consistent reduction in egg-laying and a slight delay in development in 5KQ flies and, more dramatically, in *foxo-nulls*. On the other hand, restriction of arginine affects fecundity of flies independently of their genotype. Several studies have demonstrated that restriction of specific amino acids extends lifespan in different species (Grandison et al., 2009; Miller et al., 2005; Juričić et al., 2020; Richardson et al., 2021). For instance, methionine restriction in particular has been associated with lifespan extension in both flies (Grandison et al., 2009) and rodents (Miller et al., 2005). Of note, the amino acid starvation sensor GCN2 has recently been described as a key mediator of the beneficial effects derived from restriction of all EAAs, except for methionine. GCN2 mutants show the strongest lifespan shortening upon arginine deprivation, whereas they are longer-lived than control flies under methionine restriction (Srivastava et al., 2022). This suggests the existence of at least two systems in the fly: a GCN2-independent mechanism, to sense absence of methionine, and a GCN2-dependent mechanism, to sense all other EAAs (Srivastava et al., 2022). Moreover, GCN2 acts as a kinase and was shown to promote FOXO activity by phosphorylation (You et al., 2018).

In light of these findings, our results may suggest a potential novel role of dFOXO as a molecular link between methionine restriction and regulation of physiological processes, including fecundity and development. However, further research is needed to clarify the mechanisms by which methionine influences dFOXO acetylation state and the subsequent metabolic response. Moreover, in order to investigate possible functions for dFOXO as an amino acid starvation sensor, we may also test these mutants on diets deficient in single or combinations of particular amino acids. Finally, the use of dFOXO mutants for the phosphorylation sites of GCN2 could be useful to unravel the link between this kinase and dFOXO and their interplay in EAA metabolism.

5.1.6 *Turn it off!* Inhibitory acetylation of FOXO protects against sugar excess

High-sugar consumption is associated with a variety of metabolic disfunctions, including insulin resistance, diabetes and obesity (DeFronzo et al., 2015), which have a strong impact on human health (Chatterjee et al., 2017; DeFronzo et al., 2015). Moreover, dysregulation of FOXO has been found to play a role in these metabolic

disorders (DeFronzo et al., 2015). In this context, the model organism *Drosophila* is a valid tool to study the consequences of excess dietary sugar (van Dam et al., 2020; Lennicke et al., 2020; Morris et al., 2012; Musselman et al., 2011; Rovenko et al., 2015), since it also develops high-sugar-related pathological phenotypes similar to humans (Morris et al., 2012; Skorupa et al., 2008; Lushchak et al., 2014). In a recent study, dFOXO was shown to be essential to mediate resistance upon excess of sugar (van Dam et al., 2020). On the other hand, a mice study had already found a link between FOXO1 acetylation and glucose metabolism *in vivo* (Banks et al., 2011). However, since constitutively acetylated FOXO1 animals were embryonically lethal (Banks et al., 2011), the relevance of FOXO acetylation in the context of dysregulated glucose metabolism had never been addressed directly.

In this study, we show that constitutively acetylated dFOXO mutant flies perform better than controls on a high-sugar diet, with an increase in lifespan of about 10%. We also show that high sugar-feeding causes the onset of three diabetic-like hallmarks (insulin resistance, glycation, adiposity) in both control and acetylation mutants, indicating that obesity and insulin resistance per se are not the cause for the lifespan shortening in control flies. Interestingly, 5KQ mutants showed the greatest increase in dFOXO expression as result of insulin resistance, which might be explained as increased stability and reduced susceptibility to degradation of acetylated FOXO proteins (Kitamura et al., 2005). Overall, these findings suggest that the increased resistance of 5KQ flies upon high-sugar diet is not impaired by the pathological traits. This is particularly interesting in the context of the “fat but fit” paradox (van Dam et al., 2020; Lennicke et al., 2020; Ortega et al., 2018), as it suggests that aging and disease phenotypes like obesity can be uncoupled. Moreover, our results match the previous observation that constitutively deacetylated FOXO promotes gluconeogenesis and lipid catabolism (Banks et al., 2011), as we find here that constitutive acetylation of pushes metabolism towards the opposite direction, promoting fat build up.

High-sugar diets represent a predisposing factor for hyperuricemia, as they provide exogenous sources for purine catabolism (Khitan et al., 2013). Uric acid is a metabolite derived from the oxidation of purine compounds (Keebaugh et al., 2010) and it has

been implicated in the development of human disorders suxh as insulin resistance, dyslipidemia, gout, and kidney stones (Lv et al., 2013; Keenan, et al., 2012; Barr et al., 1990; Moe et al., 2006). Recent studies in *Drosophila* have highlighted the importance of uric acid (Lang et al., 2019) and dysregulated purine metabolism (Lennicke et al., 2020) as factors that limit lifespan. Importantly, previous observations have found a connection between FOXO and uric acid metabolism. In *Drosophila*, FOXO overexpression protects against the formation of uric acid concretions on a high-yeast diet in a fly model of urate oxidase deficiency (Lang et al., 2019). A SNP in human FOXO3 was found to be significantly associated with serum uric acid levels (Lang et al., 2019). *foxo-null* flies fed a high-sugar diet displayed tubule dysfunction and dysregulation of purine catabolism, which led to formation of kidney stones (van Dam et al., 2020). Consistent with these observations, our results show that the lifespan-shortening effects of the S20% diet occur via increased uric acid accumulation and formation of renal stones. Moreover, they suggest for the first time that acetylation of FOXO specifically plays a role in attenuating hyperuricemia and renal stone formation. Control flies fed the diet for 28 days were characterized by high uric acids levels and evident concretion in the Malpighian stones. On the other hand, 5KQ acetylation mutants display lower uric acid accumulation and reduction in tubules stones, both in terms of number and size.

Considering other dFOXO mutant genotypes in the analysis, we observed that *foxo-null* flies are hypersensitive on the diet, confirming previous findings (van Dam et al., 2020). Additionally, mutation of the DNA binding site of FOXO significantly decreased fly survival, and neither acetyl-null (5KR) nor partial acetyl-mimicking (2KQ) mutants outlived control flies. Taken together, our results indicate that, in order to promote survival on the excess sugar diet, FOXO must be 1) completely acetylated within its NLS and 2) have a functional DNA binding domain.

This finding is especially intriguing, because acetylation of FOXO in the NLS has mainly been associated with nuclear exclusion (Banks et al., 2011; Qiang et al., 2010) (also observed by us) and reduced DNA-binding (Tsai et al., 2007). However, previous experiments from our lab had also shown that acetylated FOXO still maintains the

ability to initiate transcription, as dFOXO-5KQ cells were able to induce expression of two different *dfoxo* luciferase reporter constructs to the same extent as dFOXO wild type proteins (Dr. Victor Bustos, unpublished data). This implies that, even though acetylated dFOXO proteins are preferentially retained in the cytosol, the acetylation-mimicking mutations do not fully prevent nuclear localization. Based on these observations, the mechanism behind the protective role of FOXO acetylation in a diabetic-like context could be explained by a combination of different factors:

1) the preferential cytoplasmatic localization of acetylated dFOXO reduces the overall number of nuclear FOXO proteins;

2) acetylated FOXO drives metabolism towards lipogenesis.

In its deacetylated state, dFOXO promotes the transcription of genes for glucose biosynthesis (Gross et al., 2008; Puigserver et al., 2003; Frescas et al., 2005; Xiong et al., 2011) and lipid utilization (Banks et al., 2011). On the other hand, we have shown here that constitutively acetylated FOXO is associated with increased lipid biosynthesis. In the context of sugar-induced insulin resistance, the chronic activation of FOXO and consequent hyperactivation of the gluconeogenetic pathways can be deleterious, as they fuel the metabolic pathway of purines, leading to increased uric acid. Indeed, type 2 diabetes has been associated with an increased risk for uric acid kidney stones (Daudon et al., 2006). Thus, the removal of FOXO from the nucleus would prevent the excessive activation of genes for gluconeogenesis, consequently reducing the amount of circulating glucose and the resulting increase in uric acid. Moreover, the metabolic shift from glucose metabolism to lipid biosynthesis provided by dFOXO acetylation could contribute to the utilization of glucose as a substrate to build fat (Parks et al., 2008), resulting in reduction of free glucose levels. Supporting this hypothesis, constitutively acetylated FOXO mice are characterized by hyperglycemia (Banks et al., 2011). This may explain why dFOXO-5KQ flies show reduced hyperuricemia and are longer-lived compared to control flies, but still retain their adiposity.

2) modification of FOXO through acetylation may mediate the interaction with different binding partners, leading to activation of a specific subset of genes necessary to survive the metabolic stress.

Supporting this theory, acetylation of FOXO has already been described to modulate the interaction with other proteins (Zhao e al., 2010; Kitamura et al., 2005), and some studies had also found a positive association between FOXO acetylation and transcriptional activity (Motta et al., 2004; Perrot and Rechler, 2005; Dr. Victor Bustos, unpublished data). Some target genes of dFOXO are involved in purine metabolism (e.g., *Ade2, Ade5, Aprt, Veil*) (Alic et al., 2014); however, no significant change in the expression level of these genes was observed in response to increased uric acid levels (Lang et al., 2019), suggesting that dFOXO-mediated beneficial effects might pass through another transcriptional output, that is yet to be investigated. This might explain why 5KQ acetylation flies survive better on the sugar diet compared to DBD mutants, that completely lose the ability to bind DNA and activate specific survival gene programs.

In summary, it's possible that the health benefits observed in the 5KQ mutants result from a reduced transcription for gluconeogenetic genes and/or preferential transcription of genes necessary to survive upon high-sugar-induced metabolic stress. However, these hypotheses need experimental proof and should be further investigated in the future. For instance, it would be useful to investigate the cellular localization of (fully/partial) acetylated dFOXO upon high-sugar stimulation, as well as evaluating their transcriptional ability in the context of chronic high-sugar feeding. The inclusion of both *foxo-null* and DBD-mutants in the experimental design, would allow to identify dFOXO-dependent genes program that are activated upon sugar excess. It would be also interesting to verify whether, as expected, DBD mutants exhibit an increase in tubule stones. Moreover, the assessment of glucose levels would also be useful to test our hypothesis that dFOXO acetylation mutants preferentially utilize glucose as a source to build fatty acids. Last, the generation of a double 5KQ-DBD mutant could confirm the importance of both acetylation and DNA-binding as essential features to promote survival in the context of high-sugar feeding.

Overall, our study highlights the role of dFOXO acetylation in mediating lifespan in a context of dysregulated purine catabolism, also showing that longevity can be

uncoupled from obesity and insulin resistance, in accordance with other studies (van Dam et al., 2020; Lennicke et al., 2020). Given the over-nutrition encountered in developed countries, the causal nexus uncovered here has potential medical implications. Thus, having a better understanding of FOXO regulation through acetylation under high-sugar conditions could lead to the development of therapeutic strategies aimed at reducing the side effects of insulin resistance or other metabolic diseases.

5.2 Role of FOXO DNA-binding ability in the fly metabolism

FOXO proteins are mostly known for their role as transcription factors, which involves the binding to the DNA to activate gene programs. However, several studies have described both DNA-binding dependent and independent functions associated with FOXO. Previous research in our lab has also shown that the presence of a functional DBD is necessary for fly lifespan and fecundity, while body size and development are independent from DNA-binding (Dr. Victor Bustos, unpublished data). The DNA-binding independent functions of FOXO could underlie both a role as a co-factor, or a cytoplasmatic role. In fact, FOXO can regulate transcriptional responses independent of DNA-binding, interacting with a variety of transcription factors that help specifying target-gene expression (Cook et al., 2015; Czymai et al., 2010; Ramaswamy et al., 2002). Moreover, FOXO can exert cytoplasmic functions that are completely independent from its ability to activate transcription (Pan et al., 2017; Zhao et al., 2010).

While FOXOs play a key role in energy homeostasis by modulating glucose and lipid metabolism, the specific role of their DNA-binding ability in this context remains unclear. In this study, we investigated the metabolic functions that require FOXO DNA-binding or are regulated independently using endogenous mutants with two-point substitutions within the DBD. Mutations of one (Ramaswamy et al., 2002) or two (Matsumoto et al., 2006) homologous residues in FOXO1 was proven sufficient to abolish DNA binding *in vitro*.

5.2.1 DNA-binding independent lipids utilization

By transcriptionally controlling several metabolic pathways involved in the regulation of food intake, *Drosophila* FOXO acts as a crucial coordinator in systemic energy balance and nutrient sensing (Demontis and Perrimon, 2010; Hong et al., 2012; Wang et al., 2011). A recent study confirmed the role of dFOXO in starvation-dependent mobilization of the fat stores in the fat body (Zhao et al., 2021). Intriguingly, mouse studies have highlighted the ability of FOXO1 to modulate liver fat utilization

independent of its ability to directly bind DNA (Cook et al., 2015; Matsumoto et al., 2006). Moreover, it was proposed that direct FOXO DNA-binding is not necessary for the response to total starvation (Ramaswamy et al., 2002; Fan et al., 2009). In line with these findings, our results show that dFOXO promotes lipid utilization independently of its direct binding to the DNA. Indeed, *foxo-null* mutants appeared to be shorter-lived on a complete starvation medium, whereas DBD2 mutants were able to completely rescue the starvation phenotype. The sensitivity to starvation of *foxo-null* flies could be explained both as reduced lipid synthesis or impaired mobilization of the fat stores. However, experiments using a high-sugar diet had resulted in increased lipid levels in all genotypes tested, including *foxo-nulls*, suggesting that fat synthesis and storage are not affected by the absence of FOXO and that the difference relies on the ability to mobilize TAGs. This finding is consistent with a fly study showing that lipid biosynthesis is independent of dFOXO regulation (DiAngelo and Birnbaum, 2009). However, in contrast, some mouse studies have highlighted the role of FOXO in lipid biosynthesis. For instance, lipid synthesis in the mouse liver seems to be regulated by FOXO1, partially independent of DNA binding (Cook et al., 2015). The discrepancy could arise from tissue-specific functions of FOXO in the mammalian system, which are not recapitulated in the simplified *Drosophila* model with only one FOXO protein.

The mechanisms by which dFOXO regulates lipid mobilization during starvation are still not fully understood. TAGs can be utilized either via a “canonical pathway”, that relies on the enzymatic action of lipases (Zimmermann et al., 2004), or an alternative cytoplasmic process known as lipophagy, that makes use of the autophagic machinery to breakdown lipid droplets (Lettieri-Barbato et al., 2013). In mammals and flies, FOXO was found to be involved in both processes. In the liver and fat of mice, FOXO1 directly regulates the expression of ATGL, a key limiting enzyme in lipolysis (Chakrabarti et al., 2009; Lettieri-Barbato et al., 2014). Similarly, dFOXO drives the starvation-induced expression of the lipolytic gene *bmm* (Wang et al., 2011). On the other hand, FOXO has also been implicated in the expression of autophagic and lysosomal-associated proteins that can participate in lipid catabolism. In the mouse liver and adipose tissue, FOXO1 was shown to regulate the expression of the lysosomal lipase Lipa (Lettieri-

Barbato et al., 2013) and of autophagy-related genes such as *atg5*, *atg8* and *atg14* (Webb and Brunet, 2014). ATG14 expression was found to be critical for the induction of hepatic lipophagy under starvation (Xiong et al., 2012). Similar to mammalian FOXO, dFOXO also controls the expression of autophagic proteins (Bai et al., 2013) and lipases (Vihervaara and Puig, 2008; Hänschke et al., 2022). A study had reported that lip4 is upregulated upon starvation in a dFOXO-dependent manner (Vihervaara and Puig, 2008).

Based on the current knowledge and on our results, two models could explain the FOXO DNA-binding independent utilization of the fat stores:

1) FOXO as a co-factor (transcription dependent, DNA-binding independent)

FOXO could bind to another DNA-binding protein and promote transcription of indirect target genes necessary for lipid mobilization. Supporting this hypothesis, cell culture and *in vivo* studies have suggested that FOXO1 regulates lipid metabolism independent of DNA binding (Cook et al, 2015; Matsumoto et al., 2006), and have proposed that it may act as co-activator of another transcription factor (Cook et al., 2015). Previous experiments conducted in our lab had also demonstrated that the transcription of lip3, a lysosomal-associated lipase homologous of Lipa and involved in lipid usage (Hänschke et al., 2022), is dependent on dFOXO but not its ability to bind DNA, as DBD mutants were able to induce normal gene expression levels upon starvation (Dr. Victor Bustos, unpublished data).

2) FOXO as cytoplasmic mediator of lipid utilization (transcription independent, DNA-binding independent).

Supporting this hypothesis, a previous mouse study had reported that FOXO1 interacts with ATG7 in the cytoplasm (Zhao et al., 2010). In particular, the authors found that FOXO1 nuclear localization is not required for autophagy induction and tumor suppression, as cytosolic FOXO1 is sufficient to induce autophagy and inhibit tumor growth *in vivo* (Zhao et al., 2010). Recently, it was also observed that mutations in FOXO1-DBD did not disrupt the interaction with the autophagic protein (Sablon et al., 2022), underlying that FOXO exerts a cytoplasmatic role independent of its DNA-binding.

Since starvation induces nuclear retention of FOXO, the first model of FOXO acting as a co-factor appears more likely. However, a cytoplasmic role of FOXO under nutrient deprivation has already been described (Zhao et al., 2010), leaving open the possibility of a yet not described cytoplasmic role of FOXO as mediator of fat store utilization. Of note, in response to the starvation stress, FOXO1 appeared to be acetylated, which facilitated FOXO1 cytosolic retention and binding to ATG7 (Zhao et al., 2010).

In summary, it is possible that, in parallel with the conventional pathway of lipid utilization, part of the TAGs are hydrolyzed via FOXO-dependent, DNA-binding-independent mechanisms, which could include both the indirect transcription of target genes involved in lipid utilization (e.g., lipases, autophagic proteins) as well as a direct role of dFOXO protein in the cytosol. In this last case, cytosolic FOXO could interact with autophagic proteins (e.g., ATG7) to mediate access to the fat stores.

This proposed model of dFOXO action could explain the starvation sensitivity phenotype observed in *foxo-null* flies in contrast to DBD mutants. In the absence of FOXO, there is no proper induction of starvation-associated genes, nor of lipophagy, which leads to fatty acid accumulation. On the other hand, DBD mutants retain their ability to utilize TAGs through the autophagic machinery. Preliminary findings of our lab support this model, as *foxo-null* flies have an accumulation of protein Ref2p, which is indicative of dysfunctional autophagy. In the DBD mutants the accumulation is significantly reduced, indicating that dFOXO, but not its DNA-binding activity, plays a key role in the starvation induced autophagy (Dr. Victor Bustos, unpublished data).

However, this model needs to be further tested in the future. In particular, we should try to understand how dFOXO and starvation-induced autophagy are related. Moreover, we should aim to identify 1) the potential binding partners that may mediate the transcription of genes indirectly activated by dFOXO and/or 2) putative cytosolic proteins whose interaction with dFOXO provides access to lipid deposits.

5.2.2 DNA-binding dependent response to sugar starvation

During the starvation response, FOXO takes on the role of regulator of energy balance, adjusting glucose levels according to the physiological needs. In particular, in the case of nutrient deficiency, it enhances the expression of gluconeogenic genes at the expense of glycolytic genes (Gross et al., 2008; Puigserver et al., 2003). Previous studies (Ramaswamy et al., 2002; Fan et al., 2009) and ours have highlighted that the response to complete starvation relies on the presence of FOXO, but not of a functional DNA-binding domain. However, whether the ability of FOXO to regulate energy homeostasis in the specific context of glucose starvation relies on its DNA-binding ability has not yet been investigated.

In our study, we showed that DBD mutant flies subjected to a no sugar diet are as short-lived as *foxo-null* flies, highlighting the importance not only of FOXO, but also of its direct transcriptional ability, in survival upon sugar starvation. This result is expected, as FOXO1 has been described as the main regulator of hepatic gluconeogenesis in the mouse liver during starvation (Kodani and Nakae, 2020). In particular, the fasting state induces expression of *pgc1*, which binds to and activates FOXO1, triggering the expression of gluconeogenic genes such as glucose-6-phosphatase (G6pc) (Lin et al., 2005; Puigserver et al., 2003) and the consequent release of glucose from glycogen (van Schaftingen and Gerin, 2002). Thus, the transcriptional output of FOXO is essential to address sugar deficiency.

On the other hand, fecundity, which is reduced in both dFOXO mutants under normal feeding conditions, is not further decreased on a no-sugar diet, showing that this biological function is not mediated by FOXO in these nutritional conditions. On the contrary, egg laying is increased in all genotypes upon removal of sugar. This can be explained as the effect of an increased proportion in yeast compared to the control diet, in accordance with previous observations that yeast supplementation, opposite to sugar implementation, increases fecundity in *Drosophila* (Bass et al., 2007; Chandegra et al., 2017).

The observation that DBD mutants have a different response to complete and sugar-specific starvation is intriguing, as it underlies different FOXO-mediated strategies of coping with the metabolic stress. Glucose is an essential, not replaceable source of energy for the organism, stored in the body as glycogen and then liberated again in times of fasting (Hantzidiamantis and Lappin, 2022). The same applies to flies, where fat body glycogen serves as a metabolic safeguard for the maintenance of sugar levels (Yamada et al., 2018). In the specific case of sugar deprivation, the transcriptional ability of FOXO might be indispensable to activate gluconeogenesis and release sugar to the organs in need (Kodani and Nakae, 2020). Mice with a liver-specific FOXO1 deletion showed reduced hepatic glucose production and reduced expression of glucose production genes (Haeusler et al., 2010). Triple ablation of *foxo* genes causes more pronounced fasting hypoglycemia (Haeusler et al., 2010), as it impacts on glucose metabolism through downregulation of gluconeogenesis and upregulation of glycolysis (Xiong et al., 2013). In a yeast-based, no-sugar food, all other components are present in the diet, so what really makes a difference in the phenotype that we observe is the lack of sugar, and it underlies the flies' inability to survive upon sugar deficiency when FOXO cannot bind to the DNA. Our results from the defined diet confirm the phenotype found in the FOXO-DBD mutants and underpin an even greater vulnerability to sugar deficiency. This also implies that the KO and DNA-binding mutants are sensitive to the lack of certain components in the medium, which would be interesting to investigate further. On the other hand, in the case of complete starvation, the system prioritizes pathways for lipid utilization (Chatterjee et al., 2021), which do not exclusively rely on dFOXO transcriptional ability, as discussed earlier. Thus, in this context of complete nutrient deficiency, DBD mutants can live longer than *foxo-null* flies.

Interestingly, dFOXO acetylation mutants were not affected by the sugar deprivation, suggesting that survival upon this metabolic stress is completely independent from the acetylation state of the protein. This result is interesting, as it seems to confirm our previous hypothesis that acetylated FOXO, although preferentially localized in the cytosol, still retain (at least partially) the ability to activate gene transcription. As the

regulation of glucose metabolism does not rely exclusively on dFOXO, but also includes the action of co-binding partners (Kodani and Nakae, 2020), it is possible that a low number of nuclear dFOXO proteins is still sufficient to enable basal transcription of gluconeogenetic genes. The finding that a knock-in mouse bearing a FOXO1 acetyl-null mutant relies on fat, and not sugar, as an energy source (Banks et al., 2011) and our previous observation that dFOXO acetylated flies prioritize lipid biosynthesis instead of gluconeogenesis seem to be in contrast with this hypothesis. However, the discrepancy could lie in the different metabolic context analyzed – complete starvation, sugar excess, or sugar starvation – which would provide the cells with a different set of co-binding partners.

On the molecular level, depletion of sugar leads to an increase in protein expression of both wild type and DBD-mutated dFOXO proteins, consistent with the observation that that *foxo* transcription is increased upon starvation (Imae et al., 2003). Control flies show a higher expression level compared to DNA-binding deficient mutants, suggesting that the transcriptional ability of FOXO is somehow necessary to promote its protein expression/activation under sugar starvation. A possible explanation is that one/multiple targets of FOXO activated upon the starvation stress are also responsible for inducing its transcription or increasing its stability. For instance, it was already reported that, upon starvation, active FOXO1 binds to the *pgc1* promoter to enhance its transcription (Daitoku et al.,2003); in turn, PGC1 binding to FOXO1 is essential to ensure FOXO activation and expression of gluconeogenetic genes (Puigserver et al., 2003). In B cells, FOXO1 activates the expression of EBF1, which in turn activates the expression of FOXO1 in a positive feedback loop (Mansson et al., 2012). This kind of regulation has also been described recently between FOXO1 and Bnip3, a direct target gene of FOXO (He et al., 2022).

Upon 7 days of sugar deprivation, dFOXO mutants are characterized by very different TAGs profiles: control flies display a mild reduction in whole body lipid content, *foxo-null* flies retain their fat stores and dFOXO DBD-deficient flies have the strongest reduction. The observation that DNA-binding mutants utilize more lipids than controls

upon sugar starvation suggests that, while a functioning DNA-binding domain in not essential to mobilize fat, the lack of it results in excessive lipid catabolism. Worth noting, the difference in lipolysis between control and DBD mutant flies was not observed on a complete starvation medium; this suggests that the disproportionate lipolysis triggered by the absence of sugar could be a compensatory mechanism to their inability to produce glucose through activation of gluconeogenic pathways. Moreover, the fast and abrupt utilization of energy stores might be the reason behind the shorter lifespan of these flies on the no sugar diet.

5.2.3 FOXO-DBD mutation induces a carbo-lipid switch

Performing proteomics analysis of control, *foxo-null* and DBD mutant flies gave us an overview of the biological changes that occur in the mutants upon complete nutrient deprivation. Strikingly, GO analysis revealed that both DBD mutants and KO flies are characterized by a very specific proteomic profile: compared to controls, they display an increased expression of proteins involved in lipid catabolism and a downregulation of carbohydrate metabolism-related proteins. This finding may seem counterintuitive, as our experiments have shown that *foxo-null* flies are hypersensitive to starvation and unable to mobilize their fat stores. Moreover, FOXO transcription factors have previously been shown to be required for the transcriptional control of lipolytic genes such as *bmm* (Chakrabarti and Kandror, 2009; Wang et al., 2011; Zhang et al., 2016). A possible explanation could be that *foxo-null* flies are still able to upregulate proteins necessary for lipid degradation in response to starvation, but that the presence of FOXO is important to provide access to the fat stores. This would be in line with the previously suggested role for FOXO in mediating lipophagy (Webb and Brunet, 2014) and would further validate our hypothesis of a FOXO putative cytoplasmatic role or as a co-factor in coordinating the autophagy-mediated access to lipid stores. Furthermore, this would suggest that the expression of lipolytic genes is not under exclusive control of dFOXO. Supporting this idea, in a previous observation both *foxo-null* flies and DBD mutants were found able to increase transcription of *bmm* after starvation, even though it was reduced compared to control flies (Dr. Victor Bustos, unpublished data). Moreover, the greater expression of lipolytic proteins in the *foxo-*

nulls could be seen as a compensatory mechanism due to inefficient usage of fat stores. On the other hand, the downregulation of proteins involved in regulation of amide metabolism in the dFOXO mutants matches its known role in carbohydrate metabolism (Gross et al., 2008; Puigserver et al., 2003; Frescas et al., 2005) and with the sugar-starvation phenotype observed in the DBD mutants.

Overall, these findings have two important implications:

1) as an adaptation to the impossibility of successfully activating gluconeogenesis, DBD mutants switch from carbohydrates to lipids as their preferred energy source. The fact that FOXO1 may be involved in promoting the switch from carbohydrate to fatty acid metabolism during starvation was already proposed by another study (Gross et al., 2008). In the specific case of DBD mutants, the metabolic shift may be even more extreme because, without a functional DBD, they can only rely on this source of energy. Interestingly, a recent study based on the use of bmm mutant flies showed the exact opposite switch, as impairment of fatty acid mobilization as an energy source resulted in upregulation of compensatory carbohydrate catabolism (Nazario-Yepiz et al., 2021).

2) the upregulation of genes necessary to prepare the cell to the utilization of lipids is not exclusively under transcriptional control of FOXO, but FOXO plays an essential role in mediating access to the fat stores.

Besides the already discussed possible mechanisms of lipid utilization (transcription-mediated lipophagy or cytoplasmatic-mediated lipophagy), another possibility lies in the regulation of proteins involved in lipid transport. DBD mutants show an increased expression of lipid transport proteins (eg., Apoltp, apolpp, bgm) compared to both control and *foxo-null* flies. In particular, the lipid transfer particle Apoltp is between the most significantly upregulated proteins. Apoltp, homologous of ApoB in vertebrates (Palm et al., 2012), is synthesized in the fat body and required for bulk lipid transport by facilitating lipid transfer between apolpp and tissues (Heier and Kühnlein, 2018). In previous studies, mammalian FOXO1 has been shown to regulate the expression of various apolipoproteins, including ApoB (Zhang et al., 2012), ApoC3 (Altomonte et al., 2004), ApoA1 (Shavva et al., 2017), and ApoM (Izquierdo et al., 2022). However,

whether this regulation is conserved in the fly or whether it strictly requires direct FOXO DNA-binding has still not been elucidated. With an increased expression in the DBD-deficient mutants compared to control and *foxo-nulls*, our findings suggest that regulation of Apoltp in particular does not require FOXO DNA-binding ability.

In conclusion, the increased transport of lipids to the tissues may reflect an adaptation to the DBD mutant inability to produce and use carbohydrate substrates to sustain the energy demand, and fits into the carbo-lipid switch phenotype that characterize these mutants. Nevertheless, it is difficult to determine whether the increase in lipid transport proteins is a consequence of the transition to a lipolytic metabolism, or whether it is a cause of it. In the future, it would be interesting to further investigate this aspect and research the causal link of this lipid transport-lipid catabolism upregulation.

5.3 Conclusions

FOXO proteins have evolved to control different aspects of body homeostasis and metabolism, and they do so by exerting their transcriptional ability, interacting with specific binding partners or via post-translational modifications. In this study, we have attempted to shed some light on the different physiological processes regulated by dFOXO in *Drosophila*. By using mutants in which either the DNA-binding ability of FOXO or its acetylation state were compromised, we could pinpoint the specific contribution of one or the other in the regulation of dFOXO-related metabolic functions. Interestingly, we found that specific features of FOXO are in charge of regulating different processes.

The DNA-binding ability of FOXO seems to be mainly involved in the response to sugar starvation, which is totally dependent on its ability to transactivate target genes and promote carbohydrate metabolism. On the contrary, DBD mutants are resistant to complete starvation diet, as only the presence of dFOXO, but not a functional DBD, is required to ensure survival by inducing a carbo-lipid switch and full mobilization of the fat stores. This suggests a putative role of dFOXO as a co-factor or as a cytoplasmic protein in the regulation of lipid utilization.

On the other hand, partial acetylation of dFOXO seems to play a primary role in the response to yeast starvation – which may be partially interpreted as deprivation of cholesterol – and in the shift to a pro-lipogenic metabolism. As this function does not depend on dFOXO direct binding to the DNA, it points again towards the possible role of the protein as a co-factor. Moreover, complete acetylation has a protective effect against the high-sugar-induced hyperuricemia. Our observations suggest that, in a diabetes-like context, decreased transcriptional activity of the protein is beneficial to attenuate the side effects of excess sugar exposure. Finally, acetylation of dFOXO might specifically mediate the sensing of methionine deprivation, whereas the response to total amino acid starvation depends on both the transcriptional ability and the deacetylated state of the protein.

Overall, our results have several implications. First, the complexity of the phenotypes observed in each of the dFOXO mutants reflects the intricacy of the metabolic pathways regulated by the protein. Second, they show that specific FOXO features (DNA-binding, acetylation) are associated with specific functions, which are dictated by the metabolic context. Third, they indicate that a particular function can both be detrimental or beneficial depending on the nutritional state (e.g., dFOXO acetylation in yeast starvation/sugar excess), possibly suggesting a novel approach for targeted therapeutic strategies aimed at enhancing one or the other function depending on the nutritional context. However, they also highlight some enigmas (e.g., role of dFOXO acetylation in transcription), that will need to be elucidated in the future.

Finally, although preliminary, our study brings us a step closer to understanding the complex mechanisms that regulate FOXO in healthy and pathological metabolic contexts.

SUPPLEMENT

6. SUPPLEMENT

Supplemental Figure 1 (related to Figure 3.4)

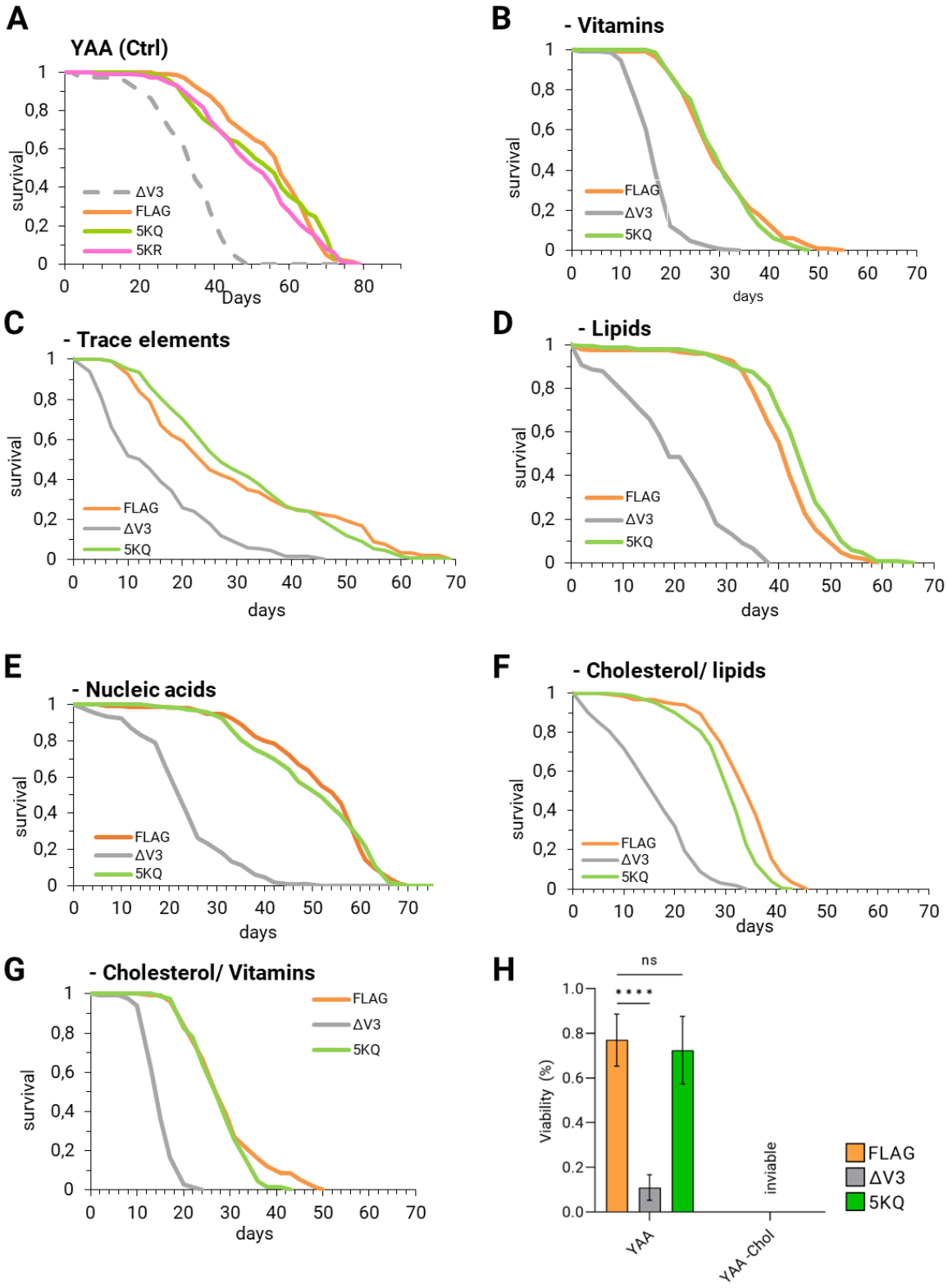

Figure S1: Effect of different nutrients starvation on lifespan and development (related to Figure 3.4).
A) Female lifespan assay showed *dfoxo-null* Δ3 flies were shorter lived than FLAG control flies on a standard defined diet (YAA), whereas acetylation mutants (5KQ and 5KR) lived as long as control. log-rank test, ***p<0.001 ΔV3 vs FLAG flies; ns p>0.05 5KQ and 5KR vs FLAG. B-G) Female dFOXO mutants flies were tested on diets lacking single elements - vitamins (B), trace elements (C), lipids (D), nucleic acids (E) - or a combination of two - cholesterol + lipids (F) and cholesterol + vitamins (G). In all conditions, ΔV3 mutants showed a consistently reduced lifespan with respect to FLAG control flies. 5KQ acetylation mutants showed significant lifespan shortening upon cholesterol + lipids starvation, whereas they lived longer than control upon lipid starvation only. log-rank test, ***p<0.001 ΔV3 vs FLAG flies in all diets; ***p<0.001, 5KQ vs FLAG in YAA -Cholesterol/lipids and YAA -Lipids. N=150/genotype in each assay. H) Deprivation of cholesterol was developmentally lethal for all tested genotypes. ns p>0.05, ****p<0.0001, Student's t-test.

Supplemental Figure 2 (related to Figure 3.8)

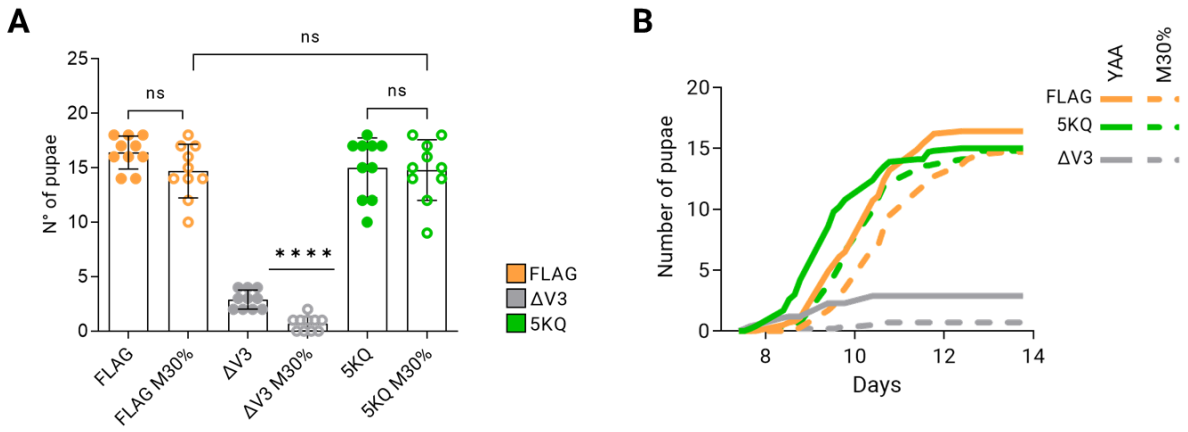

Figure S2: dFOXO acetylation does not affect viability and pupation upon methionine restriction (related to Figure 3.8).

A-B) Effect of methionine reduction (YAA M30%) on development of FLAG, ΔV3 and 5KQ mutant flies, expressed as total number of pupae (A) and pupae formation over time (B). *dfoxo-null* ΔV3 flies showed significantly reduced total number of pupae compared to control flies (****p<0.0001, one-way ANOVA), while 5KQ flies subjected to the starvation diet showed unchanged phenotype with respect to control flies. N=10 replicates/genotype, 20 eggs each.

Supplemental Figure 3 (related to Chapter 3.3.3)

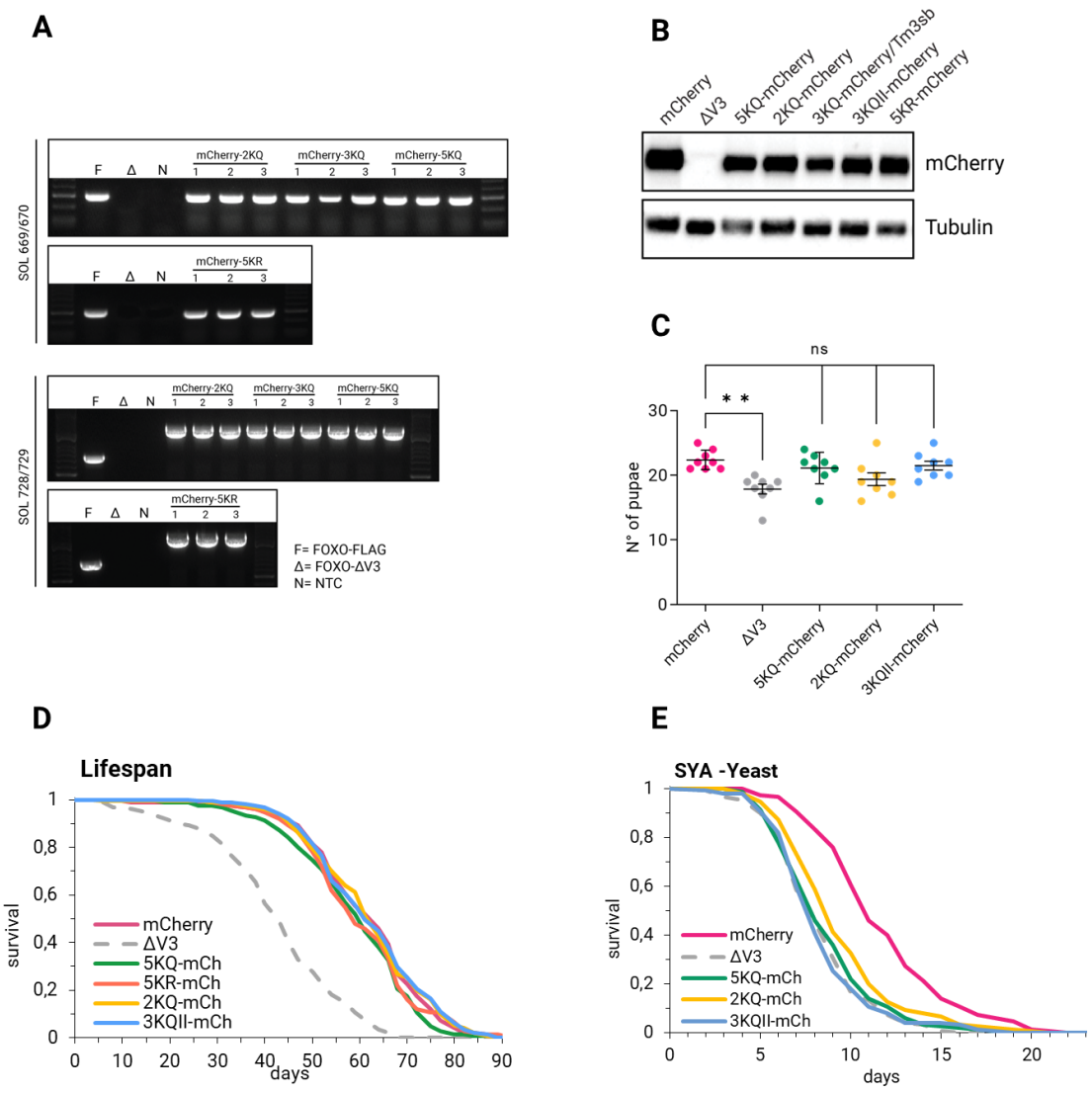

Figure S3: Molecular validation and phenotyping of novel mCherry-tagged FOXO acetylation mutants (related to Chapter 3.3.3).

A) PCR analysis confirmed the correct reinsertion of mCherry-tagged V3 gene replacement constructs. SOL669/670 primers were used to amplify the V3 region, and SOL728/729 were used to amplify the TAG sequence. B) Immunoblotting on whole-body protein extracts from female flies confirmed dFOXO expression in the newly generated mCherry-tagged partial acetylation mutants (2KQ, 3KQII, 5KQ, 5KR). 3KQ-mCherry was only viable in the heterozygous state and showed reduced protein. C) All partial and complete mCherry-tagged dFOXO acetylation mutants showed similar development as control. ns p>0.05, one-way ANOVA followed by Bonferroni's multiple comparisons test vs control (mCherry). D) On a standard SYA diet, mCherry-tagged partial (2KQ, 3KQII) or complete acetyl-mimicking (5KQ) or acetyl-null (5KR) mutants showed similar lifespan as dFOXO-mCherry control flies. ns p>0.05 log-rank test, 2KQ, 3KQII and 5KQ vs mCherry. E) Upon yeast deprivation, both *dfoxo-null* flies (ΔV3) and complete/partial acetyl-lysine mutants (5KQ, 2KQ, 3KQII) are shorter-lived than mCherry control flies. ***p<0.001 log-rank test.

Supplemental Figure 4 (related to Chapter 3.3.4)

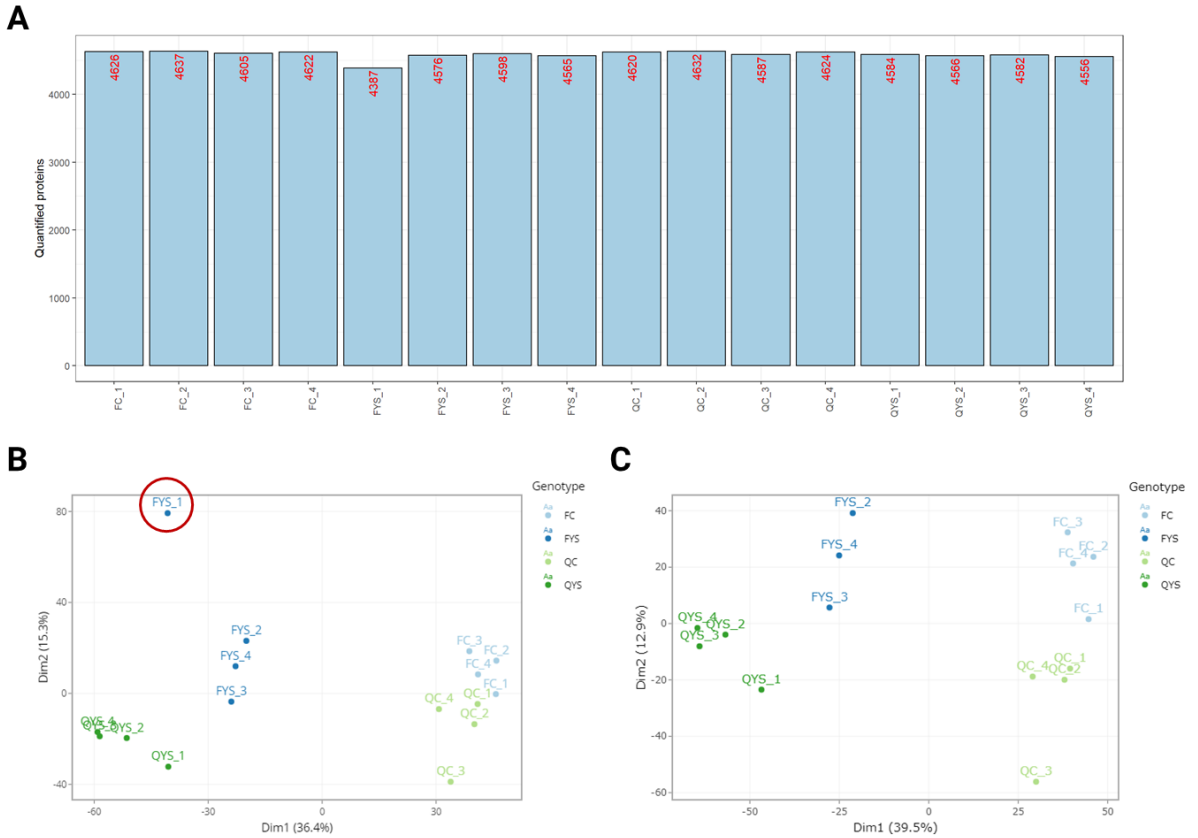

Figure S4: Quantified proteins and PCA analysis of FLAG and 5KQ total proteome samples, in control and yeast starvation condition (related to Chapter 3.3.4).
A) Barplot showing quantified proteins obtained from MS analysis of FC, QC, QYS and FYS. B-C) Principal component analysis (PCA) of quantified proteins from 2 genotypes (FLAG, 5KQ) and 2 conditions (control, yeast starvation). n=4 samples per genotype per condition. FYS was an outlier in the PCA and removed from subsequential analysis (C). Analysis was performed by Dr. Ilian Atanassov. | Abbreviations: QC= dFOXO-5KQ flies, control diet; FC= dFOXO-FLAG flies, control diet; QYS= dFOXO-5KQ flies, yeast starvation; FYS= dFOXO-FLAG flies, yeast starvation.

Supplemental Figure 5 (related to Figure 3.14)

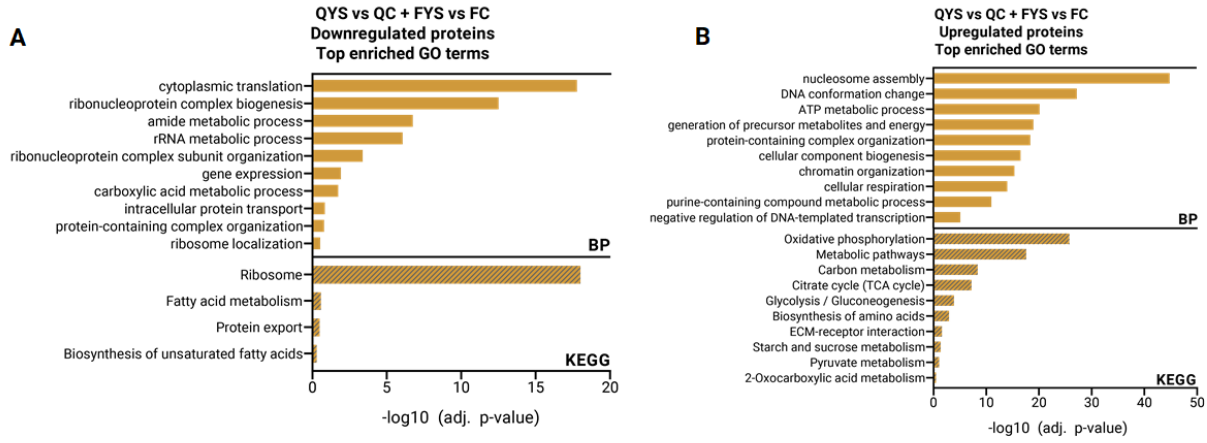

Figure S5: GO enrichment analysis from proteomic analysis of FLAG and 5KQ flies upon yeast starvation (related to Figure 3.14).
A-B) Barplot representation of significantly enriched top GO terms of upregulated (A) and downregulated (B) proteins shared between the comparisons QYS vs QC and FYS vs FC. BP= biological process; KEGG= KEGG pathway. GO terms sorted by adjusted p-value. Abbreviations: QC= dFOXO-5KQ flies, control diet; FC= dFOXO-FLAG flies, control diet; QYS= dFOXO-5KQ flies, yeast starvation; FYS= dFOXO-FLAG flies, yeast starvation.

Supplemental Figure 6 (related to Figure 3.17)

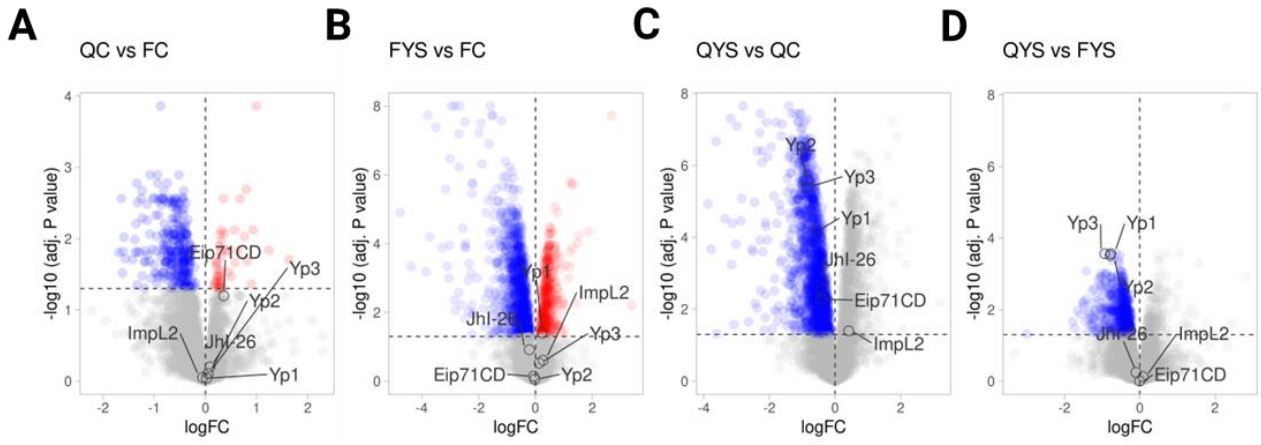

Figure S6: Yolk proteins and ecdysone/juvenile hormone induced proteins are downregulated in QYS (related to Figure 3.17).
A-D) Volcano plot of DEPs in QC vs FC (A), FYS vs FC (B), QYS vs QC (C) and QYS vs FYS (D) showed a downregulation of proteins implicated in oogenesis in 5KQ mutants upon yeast starvation. Abbreviations: QC= dFOXO-5KQ flies, control diet; FC= dFOXO-FLAG flies, control diet; QYS= dFOXO-5KQ flies, yeast starvation; FYS= dFOXO-FLAG flies, yeast starvation.

Supplemental Figure 7 (related to Figure 3.17)

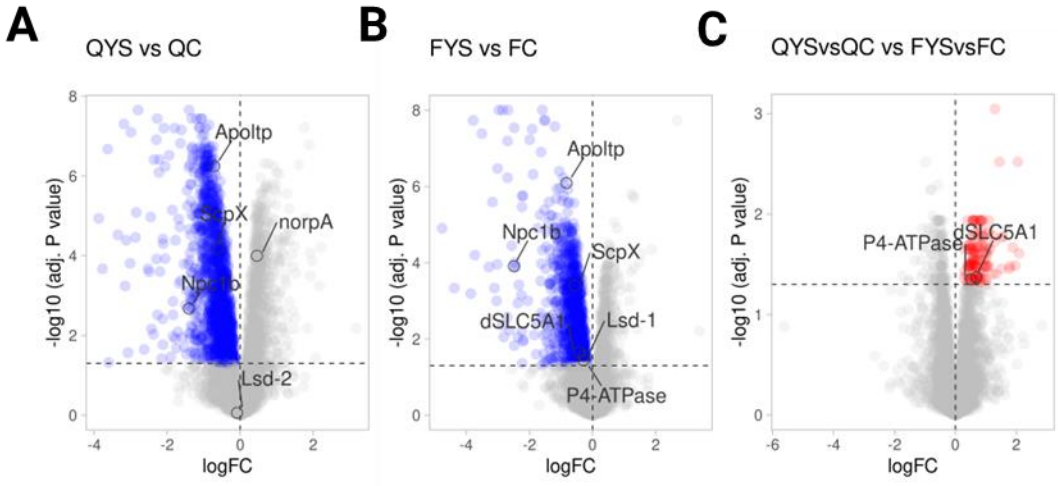

Figure S7: Differential expression of cholesterol transport/ binding proteins in QYS and FYS samples (related to Figure 3.17).
A-C) Volcano plot of DEPs in QYS vs QC (A), FYS vs FC (B) and the double comparison QYSvsQC vs FYSvsFC. Cholesterol transport proteins were downregulated in both genotypes in the yeast-starved condition vs control diet, but 5KQ acetylation mutants maintained a higher expression of those proteins upon yeast deprivation compared to FLAG control flies. Abbreviations: QC= dFOXO-5KQ flies, control diet; FC= dFOXO-FLAG flies, control diet; QYS= dFOXO-5KQ flies, yeast starvation; FYS= dFOXO-FLAG flies, yeast starvation.

Supplemental Figure 8 (related to Figure 3.19)

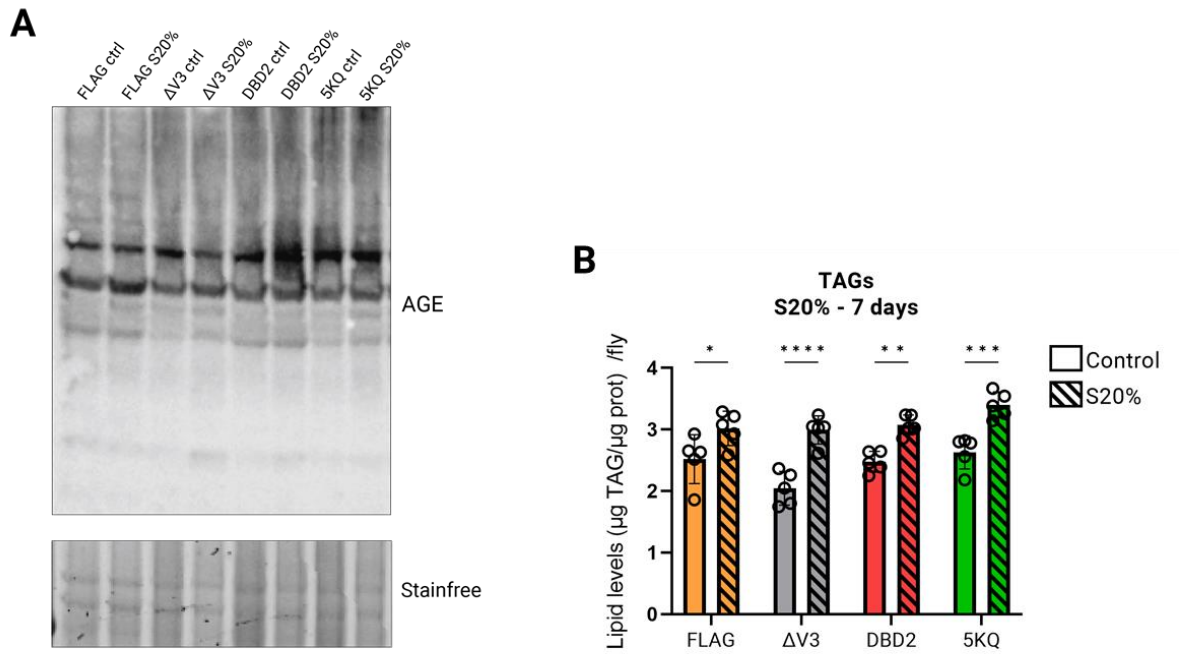

Figure S8: Metabolic effect of high sucrose diet on glycation and lipid accumulation (related to Figure 3.19)

A) Immunoblotting of whole-body protein extracts showing glycation damage in female dFOXO-FLAG, ΔV3, DBD2 and 5KQ flies fed for 7 days on control (Ctrl) or 20% sugar (S20%) diet. Anti-AGE western blot and stain-free gel image of total protein related to the quantification in Figure 3.19G. N=3 biological replicates/condition, 20 flies each. B) TAG content of female flies kept for seven days on control (SYA) and high sugar (S20%) diet showed that all genotypes were able to accumulate fat as TAGs. Diet: ****p<0.0001; genotype: **p<0.01; interaction: ns p>0.05, two-way ANOVA. Bonferroni multiple comparison test: *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. N=5 biological replicates per assay, each with 5 flies/genotype.

Supplemental Figure 9 (related to Figure 3.20)

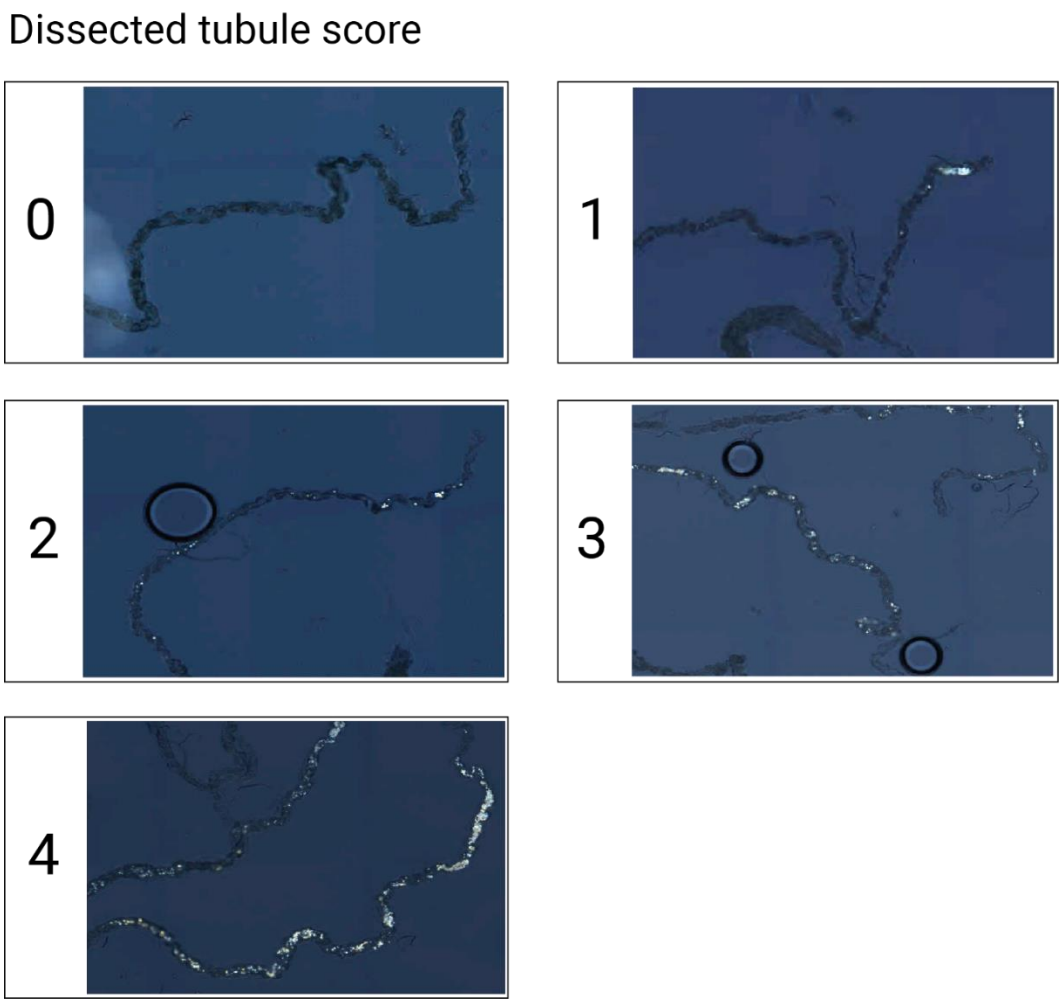

Figure S9: Tubule stones scoring system (related to Figure 3.20).
Scoring of the tubule stone phenotype based on polarized light microscopy imaging of dissected tubules. Scoring: 0 = clear (~0%), 1 = mild (< 25%), 2 = moderate (25–50%), 3 = strong (50–75%), 4 = severe (> 75%). The score was assigned to each Malpighian tubule and then averaged by 4. n=15 flies per genotype per tested diet.

Supplemental Figure 10 (related to Chapter 3.3.5.4)

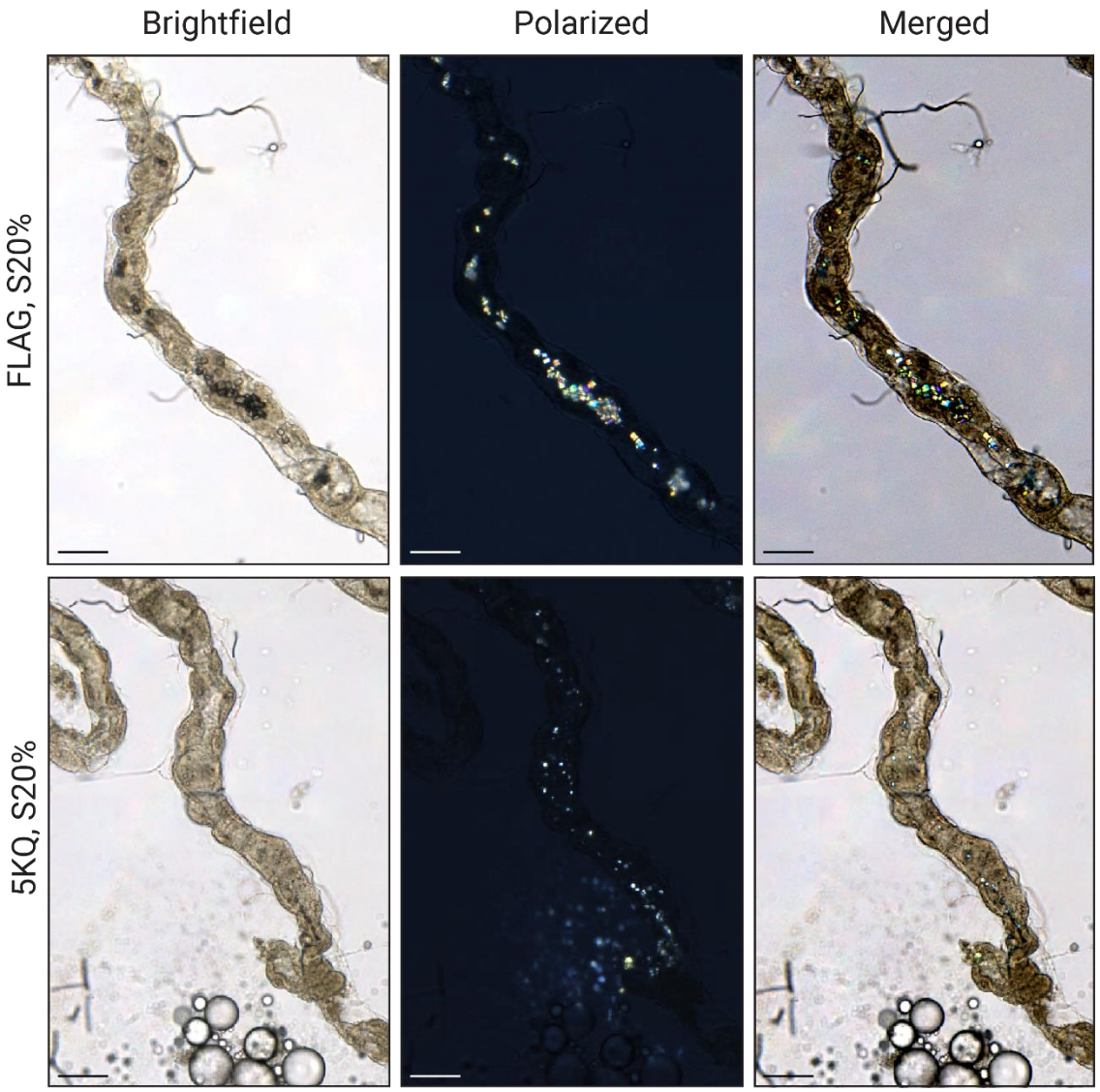

Figure S10: FOXO acetylation attenuates the dysfunctional phenotypes associated with high-sugar consumption (related to Chapter 3.3.5.4).
The images show representative brightfield, polarized light and their merged pictures of dissected Malpighian tubules from FLAG control and 5KQ acetylation mutant females, fed for 28 days on 20%S. FLAG flies showed bigger crystal deposits compared to 5KQ acetylation mutants. Scale bar=50 µm.

Supplemental Figure 11 (related to Chapter 4.2.3)

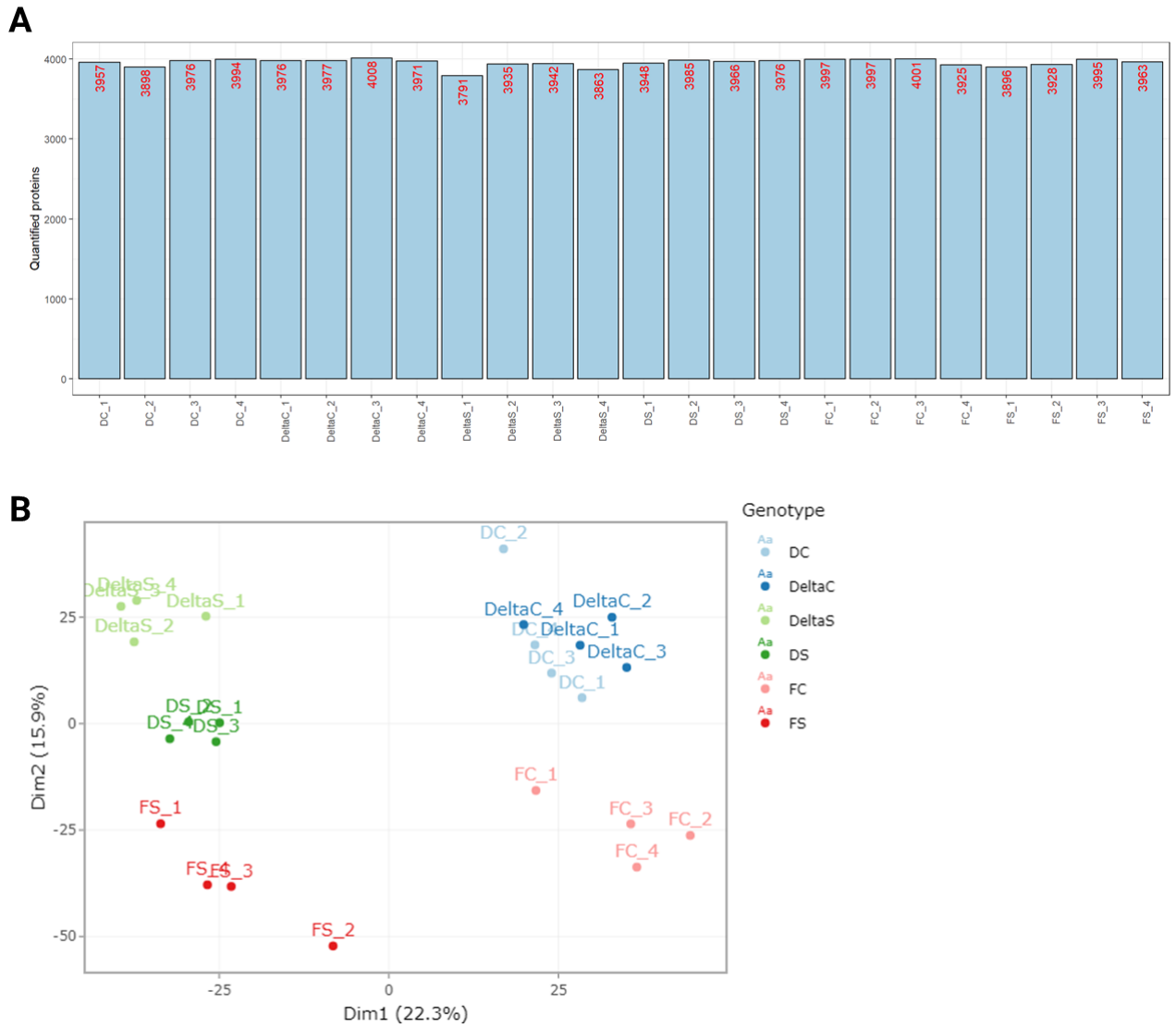

Figure S11: Quantified proteins and PCA analysis of FLAG, DBD2 and ΔV3 flies total proteome, in control and starvation condition (related to Chapter 4.2.3).
A) Barplot of quantified proteins obtained from MS analysis of fed or starved FLAG, DBD2 and ΔV3 mutant flies. B) Principal component analysis (PCA) of quantified proteins from 3 genotypes (FLAG, DBD2, ΔV3) and 2 conditions (control, starvation). n=4 samples per genotype per condition. Analysis was performed by Dr. Ilian Atanassov. | Abbreviations: FC= dFOXO-FLAG flies, control diet; FS= dFOXO-FLAG flies, starvation diet; DC= dFOXO-DBD2 flies, control diet; DS= dFOXO-DBD2 flies, starvation diet; ΔC= *dfoxo-null* flies, control diet; ΔS= *dfoxo-null* flies, starvation diet.

Supplemental Figure 12 (related to Chapter 4.2.3)

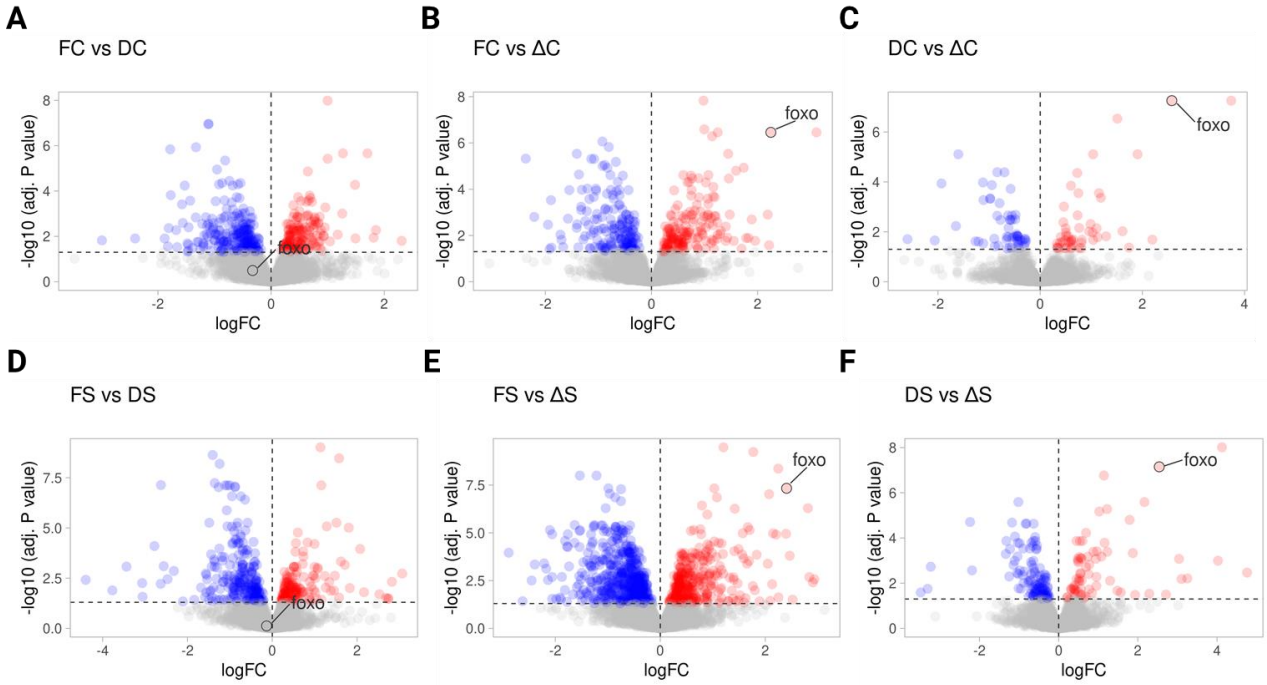

Figure S12: Differential expression of FOXO in FLAG, DBD and ΔV3 mutants, upon control and starved condition (related to Chapter 4.2.3).
A-F) Volcano plots of DEPs in FLAG, DBD2 and ΔV3 flies upon continuous feeding or starvation highlight expression of dFOXO in all comparisons, confirming knockout of the protein in ΔV3 flies.

Supplemental Figure 13 (related to Figure 4.23)

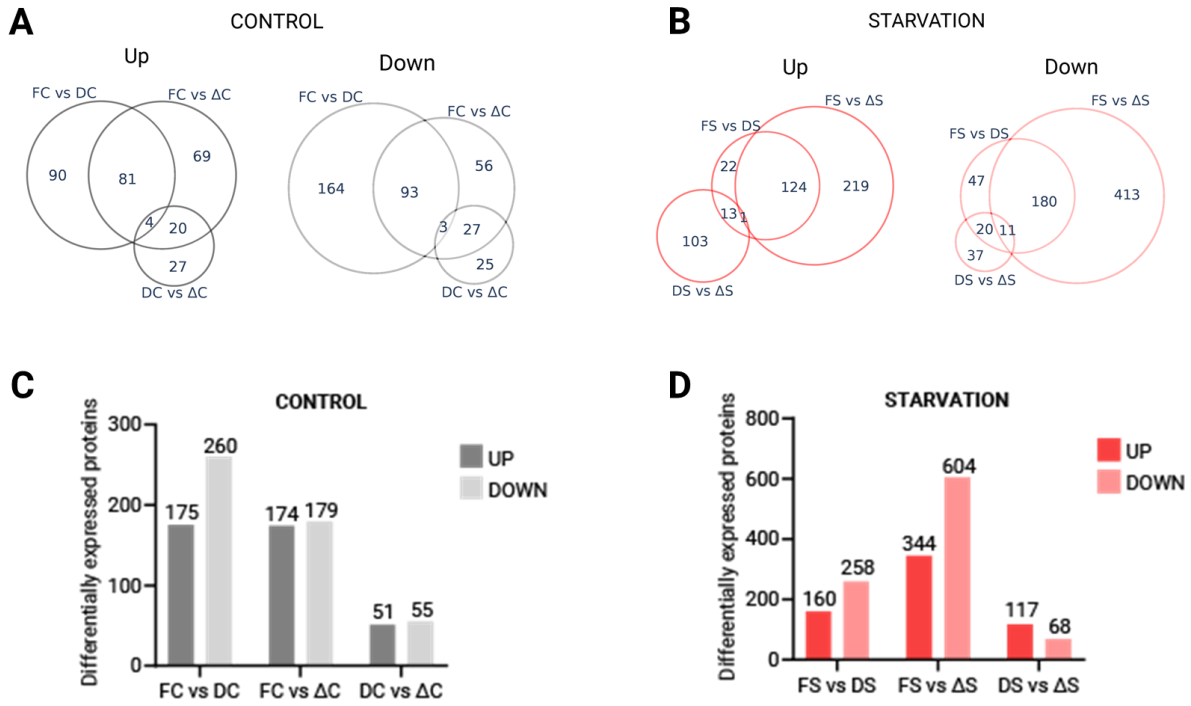

Figure S13: Proteome profiling of control, *dfoxo-null* and DBD2 mutants upon normal feeding or complete starvation (related to Figure 4.23).
A-B) Venn diagrams of upregulated (left) and downregulated (right) differentially expressed proteins (DEPs) comparing the 3 genotypes (FLAG, DBD2 and ΔV3) in control (A) or starved (B) feeding condition. C-D) Barplot representations of DEPs shown in (A) and (B), respectively. The exact number of DEPs is indicated above the bar. Abbreviations: FC= dFOXO-FLAG flies, control diet; FS= dFOXO-FLAG flies, starvation diet; DC= dFOXO-DBD2 flies, control diet; DS= dFOXO-DBD2 flies, starvation diet; ΔC= *dfoxo-null* flies, control diet; ΔS= *dfoxo-null* flies, starvation diet.

Supplemental Figure 14 (related to Figure 4.23)

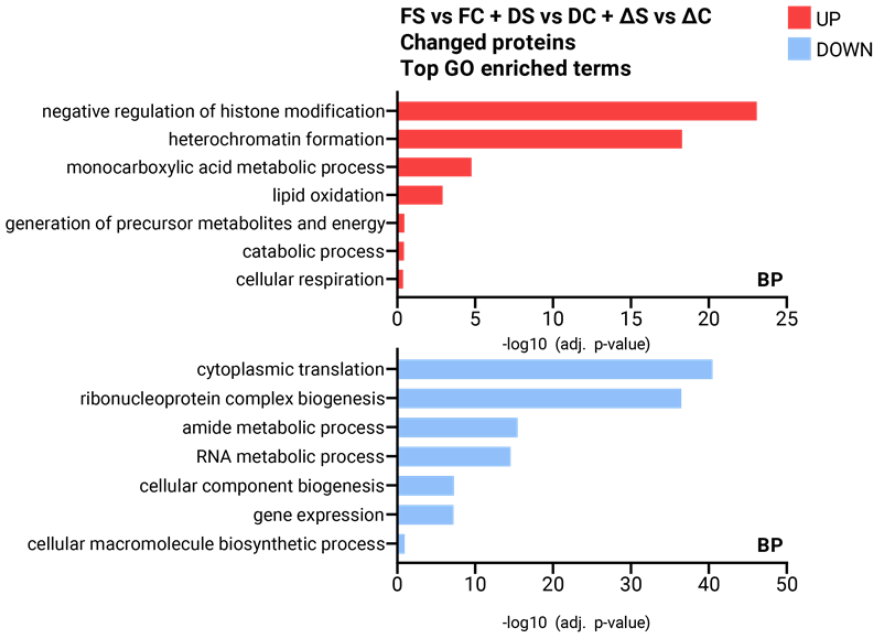

Figure S14: GO enrichment analysis from proteomic analysis of FLAG, DBD2 and ΔV3 mutant flies upon starvation (related to Figure 4.23).
Barplot representation of significantly enriched top GO terms of upregulated (top) or downregulated (bottom) proteins shared between FLAG, DBD2 and ΔV3 mutants upon starvation. FS vs DS. BP= biological process; KEGG= KEGG pathway. GO terms sorted by adjusted p-value.

Supplemental Figure 15 (related to Figure 4.24)

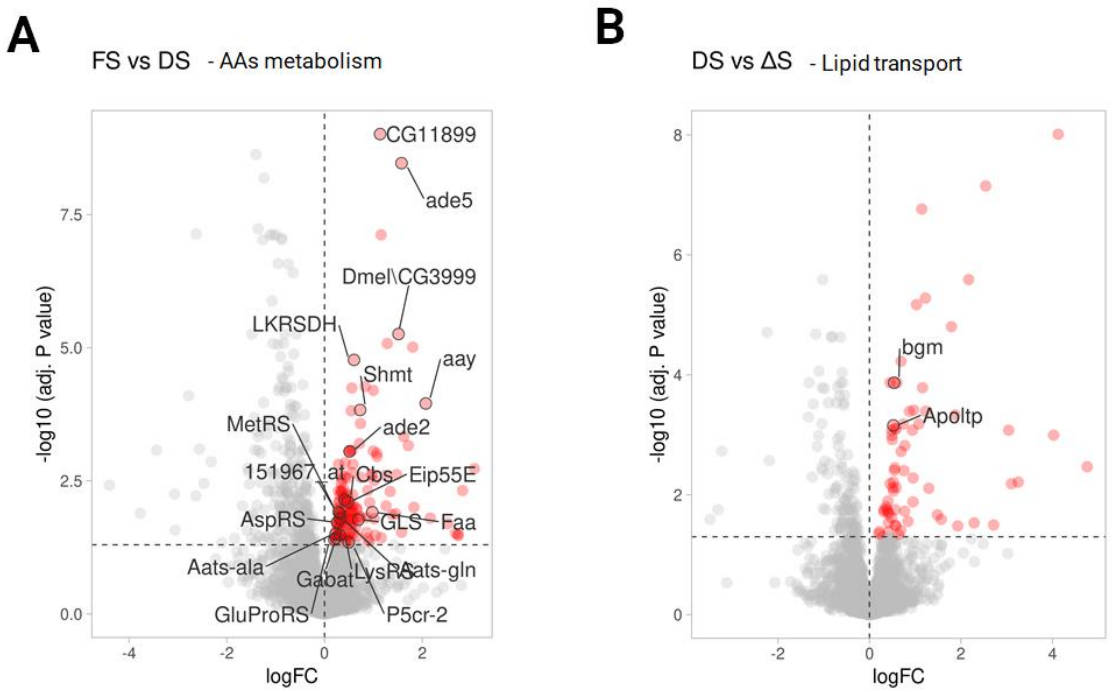

Figure S15: Differentially expressed proteins in FLAG vs DBD or DBD vs ΔV3 upon starvation (related to Figure 4.24).
A) Volcano plot of DEPs in the comparison FS vs DS highlights upregulation of proteins involved in amino acids metabolism. B) Volcano plot of DEPs in the comparison DS vs ΔS highlights upregulation of two proteins involved lipid transport.

List of figures

List of tables

Bibliography

Afschar S, Toivonen JM, Hoffmann JM, Tain LS, Wieser D, Finlayson AJ, Driege Y, Alic N, Emran S, Stinn J, Froehlich J, Piper MD, Partridge L. Nuclear hormone receptor DHR96 mediates the resistance to xenobiotics but not the increased lifespan of insulin-mutant Drosophila. Proc Natl Acad Sci U S A. 2016 Feb 2;113(5):1321-6. doi: 10.1073/pnas.1515137113

Altomonte J, Cong L, Harbaran S, Richter A, Xu J, Meseck M, Dong HH. Foxo1 mediates insulin action on apoC-III and triglyceride metabolism. J Clin Invest. 2004 Nov;114(10):1493-503. doi: 10.1172/JCI19992

Aoki M, Jiang H, Vogt PK. Proteasomal degradation of the FoxO1 transcriptional regulator in cells transformed by the P3k and Akt oncoproteins. Proc Natl Acad Sci U S A. 2004 Sep 14;101(37):13613-7. doi: 10.1073/pnas.0405454101

Atanesyan L, Günther V, Dichtl B, Georgiev O, Schaffner W. Polyglutamine tracts as modulators of transcriptional activation from yeast to mammals. Biol Chem. 2012 Jan;393(1-2):63-70. doi: 10.1515/BC-2011-252

Baghdadi M, Nespital T, Mesaros A, Buschbaum S, Withers DJ, Grönke S, Partridge L. Reduced insulin signaling in neurons induces sex-specific health benefits. Sci Adv. 2023 Feb 22;9(8):eade8137. doi: 10.1126/sciadv.ade8137

Bai H, Kang P, Tatar M. Drosophila insulin-like peptide-6 (dilp6) expression from fat body extends lifespan and represses secretion of Drosophila insulin-like peptide-2 from the brain. Aging Cell. 2012 Dec;11(6):978-85. doi: 10.1111/acel.12000

Baker KD, Thummel CS. Diabetic larvae and obese flies-emerging studies of metabolism in Drosophila. Cell Metab. 2007 Oct;6(4):257-66. doi: 10.1016/j.cmet.2007.09.002

Banks AS, Kim-Muller JY, Mastracci TL, Kofler NM, Qiang L, Haeusler RA, Jurczak MJ, Laznik D, Heinrich G, Samuel VT, Shulman GI, Papaioannou VE, Accili D. Dissociation of the glucose and lipid regulatory functions of FoxO1 by targeted knockin of acetylation-defective alleles in mice. Cell Metab. 2011 Nov 2;14(5):587-97. doi: 10.1016/j.cmet.2011.09.012

Bär C, Povedano JM, Serrano R, Benitez-Buelga C, Popkes M, Formentini I, Bobadilla M, Bosch F, Blasco MA. Telomerase gene therapy rescues telomere length, bone marrow aplasia, and survival in mice with aplastic anemia. Blood. 2016 Apr 7;127(14):1770-9. doi: 10.1182/blood-2015-08-667485

Barr WG. Uric Acid. In: Walker HK, Hall WD, Hurst JW, editors. Clinical Methods: The History, Physical, and Laboratory Examinations. 3rd edition. Boston: Butterworths; 1990. Chapter 165. Available from: https://www.ncbi.nlm.nih.gov/books/NBK273/

Barthel A, Schmoll D, Unterman TG. FoxO proteins in insulin action and metabolism. Trends Endocrinol Metab. 2005 May-Jun;16(4):183-9. doi: 10.1016/j.tem.2005.03.010. Erratum in: Trends Endocrinol Metab. 2005 Aug;16(6):253.

Bass TM, Grandison RC, Wong R, Martinez P, Partridge L, Piper MD. Optimization of dietary restriction protocols in Drosophila. J Gerontol A Biol Sci Med Sci. 2007 Oct;62(10):1071-81. doi: 10.1093/gerona/62.10.1071

Bauer JH, Goupil S, Garber GB, Helfand SL. An accelerated assay for the identification of lifespan-extending interventions in Drosophila melanogaster. Proc Natl Acad Sci U S A. 2004 Aug 31;101(35):12980-5. doi: 10.1073/pnas.0403493101

Benedetti MG, Foster AL, Vantipalli MC, White MP, Sampayo JN, Gill MS, Olsen A, Lithgow GJ. Compounds that confer thermal stress resistance and extended lifespan. Exp Gerontol. 2008 Oct;43(10):882-91. doi: 10.1016/j.exger.2008.08.049

Beurel E, Grieco SF, Jope RS. Glycogen synthase kinase-3 (GSK3): regulation, actions, and diseases. Pharmacol Ther. 2015 Apr;148:114-31. doi: 10.1016/j.pharmthera.2014.11.016

Bhardwaj G, Penniman CM, Klaus K, Weatherford ET, Pan H, Dreyfuss JM, Nair KS, Kahn CR, O'Neill BT. Transcriptomic Regulation of Muscle Mitochondria and Calcium Signaling by Insulin/IGF-1 Receptors Depends on FoxO Transcription Factors. Front Physiol. 2022 Feb 4;12:779121. doi: 10.3389/fphys.2021.779121

Bjedov I, Toivonen JM, Kerr F, Slack C, Jacobson J, Foley A, Partridge L. Mechanisms of life span extension by rapamycin in the fruit fly Drosophila melanogaster. Cell Metab. 2010 Jan;11(1):35-46. doi: 10.1016/j.cmet.2009.11.010

Bownes M. The regulation of the yolk protein genes, a family of sex differentiation genes in Drosophila melanogaster. Bioessays. 1994 Oct;16(10):745-52. doi: 10.1002/bies.950161009

Brandsch C, Schmidt T, Behn D, Weisse K, Mueller AS, Stangl GI. Glutathione deficiency down-regulates hepatic lipogenesis in rats. Lipids Health Dis. 2010 May 19;9:50. doi: 10.1186/1476-511X-9-50

Brent MM, Anand R, Marmorstein R. Structural basis for DNA recognition by FoxO1 and its regulation by posttranslational modification. Structure. 2008 Sep 10;16(9):1407-16. doi: 10.1016/j.str.2008.06.013

Broer L, Buchman AS, Deelen J, Evans DS, Faul JD, Lunetta KL, Sebastiani P, Smith JA, Smith AV, Tanaka T, Yu L, Arnold AM, Aspelund T, Benjamin EJ, De Jager PL, Eirkisdottir G, Evans DA, Garcia ME, Hofman A, Kaplan RC, Kardia SL, Kiel DP, Oostra BA, Orwoll ES, Parimi N, Psaty BM, Rivadeneira F, Rotter JI, Seshadri S, Singleton A, Tiemeier H, Uitterlinden AG, Zhao W, Bandinelli S, Bennett DA, Ferrucci L, Gudnason V, Harris TB, Karasik D, Launer LJ, Perls TT, Slagboom PE, Tranah GJ, Weir DR, Newman AB, van Duijn CM, Murabito JM. GWAS of longevity in CHARGE consortium confirms APOE and FOXO3 candidacy. J Gerontol A Biol Sci Med Sci. 2015 Jan;70(1):110-8. doi: 10.1093/gerona/glu166

Brogiolo W, Stocker H, Ikeya T, Rintelen F, Fernandez R, Hafen E. An evolutionarily conserved function of the Drosophila insulin receptor and insulin-like peptides in growth control. Curr Biol. 2001 Feb 20;11(4):213-21. doi: 10.1016/s0960-9822(01)00068-9

Brown MK, Evans JL, Luo Y. Beneficial effects of natural antioxidants EGCG and alpha-lipoic acid on life span and age-dependent behavioral declines in Caenorhabditis elegans. Pharmacol Biochem Behav. 2006 Nov;85(3):620-8. doi: 10.1016/j.pbb.2006.10.017

Brownawell AM, Kops GJ, Macara IG, Burgering BM. Inhibition of nuclear import by protein kinase B (Akt) regulates the subcellular distribution and activity of the forkhead transcription factor AFX. Mol Cell Biol. 2001 May;21(10):3534-46. doi: 10.1128/MCB.21.10.3534-3546.2001

Brunet A, Bonni A, Zigmond MJ, Lin MZ, Juo P, Hu LS, Anderson MJ, Arden KC, Blenis J, Greenberg ME. Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor. Cell. 1999 Mar 19;96(6):857-68. doi: 10.1016/s0092-8674(00)80595-4

Brunet A, Kanai F, Stehn J, Xu J, Sarbassova D, Frangioni JV, Dalal SN, DeCaprio JA, Greenberg ME, Yaffe MB. 14-3-3 transits to the nucleus and participates in dynamic nucleocytoplasmic transport. J Cell Biol. 2002 Mar 4;156(5):817-28. doi: 10.1083/jcb.200112059

Brunet A, Park J, Tran H, Hu LS, Hemmings BA, Greenberg ME. Protein kinase SGK mediates survival signals by phosphorylating the forkhead transcription factor FKHRL1 (FOXO3a). Mol Cell Biol. 2001 Feb;21(3):952-65. doi: 10.1128/MCB.21.3.952-965.2001

Brunet A, Sweeney LB, Sturgill JF, Chua KF, Greer PL, Lin Y, Tran H, Ross SE, Mostoslavsky R, Cohen HY, Hu LS, Cheng HL, Jedrychowski MP, Gygi SP, Sinclair DA, Alt FW, Greenberg ME. Stress-dependent regulation of FOXO transcription factors by the SIRT1 deacetylase. Science. 2004 Mar 26;303(5666):2011-5. doi: 10.1126/science.1094637

Bujold M, Gopalakrishnan A, Nally E, King-Jones K. Nuclear receptor DHR96 acts as a sentinel for low cholesterol concentrations in Drosophila melanogaster. Mol Cell Biol. 2010 Feb;30(3):793-805. doi: 10.1128/MCB.01327-09

Bullock M. FOXO factors and breast cancer: outfoxing endocrine resistance. Endocr Relat Cancer. 2016 Feb;23(2):R113-30. doi: 10.1530/ERC-15-0461

Calnan DR, Brunet A. The FoxO code. Oncogene. 2008 Apr 7;27(16):2276-88. doi: 10.1038/onc.2008.21

Chakrabarti P, English T, Karki S, Qiang L, Tao R, Kim J, Luo Z, Farmer SR, Kandror KV. SIRT1 controls lipolysis in adipocytes via FOXO1-mediated expression of ATGL. J Lipid Res. 2011 Sep;52(9):1693-701. doi: 10.1194/jlr.M014647

Chakrabarti P, Kandror KV. FoxO1 controls insulin-dependent adipose triglyceride lipase (ATGL) expression and lipolysis in adipocytes. J Biol Chem. 2009 May 15;284(20):13296-13300. doi: 10.1074/jbc.C800241200

Chandegra B, Tang JLY, Chi H, Alic N. Sexually dimorphic effects of dietary sugar on lifespan, feeding and starvation resistance in Drosophila. Aging (Albany NY). 2017 Dec 4;9(12):2521-2528. doi: 10.18632/aging.101335

Chapman T, Partridge L. Female fitness in Drosophila melanogaster: an interaction between the effect of nutrition and of encounter rate with males. Proc Biol Sci. 1996 Jun 22;263(1371):755-9. doi: 10.1098/rspb.1996.0113

Chatterjee D, Katewa SD, Qi Y, Jackson SA, Kapahi P, Jasper H. Control of metabolic adaptation to fasting by dILP6-induced insulin signaling in Drosophila oenocytes. Proc Natl Acad Sci U S A. 2014 Dec 16;111(50):17959-64. doi: 10.1073/pnas.1409241111

Chatterjee N, Perrimon N. What fuels the fly: Energy metabolism in *Drosophila* and its application to the study of obesity and diabetes. Sci Adv. 2021 Jun 9;7(24):eabg4336. doi: 10.1126/sciadv.abg4336

Chatterjee S, Khunti K, Davies MJ. Type 2 diabetes. Lancet. 2017 Jun 3;389(10085):2239-2251. doi: 10.1016/S0140-6736(17)30058-2

Chen CC, Jeon SM, Bhaskar PT, Nogueira V, Sundararajan D, Tonic I, Park Y, Hay N. FoxOs inhibit mTORC1 and activate Akt by inducing the expression of Sestrin3 and Rictor. Dev Cell. 2010 Apr 20;18(4):592-604. doi: 10.1016/j.devcel.2010.03.008

Chen H, Tini M, Evans RM. HATs on and beyond chromatin. Curr Opin Cell Biol. 2001 Apr;13(2):218-24. doi: 10.1016/s0955-0674(00)00200-3

Chen WC, Lin WY, Chen HY, Chang CH, Tsai FJ, Man KM, Shen JL, Chen YH. Melamine-induced urolithiasis in a Drosophila model. J Agric Food Chem. 2012 Mar 14;60(10):2753-7. doi: 10.1021/jf204647p

Cheng Z, White MF. Targeting Forkhead box O1 from the concept to metabolic diseases: lessons from mouse models. Antioxid Redox Signal. 2011 Feb 15;14(4):649-61. doi: 10.1089/ars.2010.3370

Cheong MC, Lee HJ, Na K, Joo HJ, Avery L, You YJ, Paik YK. NSBP-1 mediates the effects of cholesterol on insulin/IGF-1 signaling in Caenorhabditis elegans. Cell Mol Life Sci. 2013 May;70(9):1623-36. doi: 10.1007/s00018-012-1221-0

Cho SMJ, Lee HJ, Shim JS, Song BM, Kim HC. Associations between age and dyslipidemia are differed by education level: The Cardiovascular and Metabolic Diseases Etiology Research Center (CMERC) cohort. Lipids Health Dis. 2020 Jan 18;19(1):12. doi: 10.1186/s12944-020-1189-y

Chu Y, Gómez Rosso L, Huang P, Wang Z, Xu Y, Yao X, Bao M, Yan J, Song H, Wang G. Liver Med23 ablation improves glucose and lipid metabolism through modulating FOXO1 activity. Cell Res. 2014 Oct;24(10):1250-65. doi: 10.1038/cr.2014.120

Clancy DJ, Gems D, Harshman LG, Oldham S, Stocker H, Hafen E, Leevers SJ, Partridge L. Extension of life-span by loss of CHICO, a Drosophila insulin receptor substrate protein. Science. 2001 Apr 6;292(5514):104-6. doi: 10.1126/science.1057991

Clark AJ, Block K. The absence of sterol synthesis in insects. J Biol Chem. 1959 Oct;234:2578-82.

Colman RJ, Anderson RM, Johnson SC, Kastman EK, Kosmatka KJ, Beasley TM, Allison DB, Cruzen C, Simmons HA, Kemnitz JW, Weindruch R. Caloric restriction delays disease onset and mortality in rhesus monkeys. Science. 2009 Jul 10;325(5937):201-4. doi: 10.1126/science.1173635

Cook JR, Matsumoto M, Banks AS, Kitamura T, Tsuchiya K, Accili D. A mutant allele encoding DNA binding-deficient FoxO1 differentially regulates hepatic glucose and lipid metabolism. Diabetes. 2015 Jun;64(6):1951-65. doi: 10.2337/db14-1506

Cortés-Rojo C, Vargas-Vargas MA, Olmos-Orizaba BE, Rodríguez-Orozco AR, Calderón-Cortés E. Interplay between NADH oxidation by complex I, glutathione redox state and sirtuin-3, and its role in the development of insulin resistance. Biochim Biophys Acta Mol Basis Dis. 2020 Aug 1;1866(8):165801. doi: 10.1016/j.bbadis.2020.165801

Crimmins EM. Lifespan and Healthspan: Past, Present, and Promise. Gerontologist. 2015 Dec;55(6):901-11. doi: 10.1093/geront/gnv130

Cryan JF, O'Riordan KJ, Cowan CSM, Sandhu KV, Bastiaanssen TFS, Boehme M, Codagnone MG, Cussotto S, Fulling C, Golubeva AV, Guzzetta KE, Jaggar M, Long-Smith CM, Lyte JM, Martin JA, Molinero-Perez A, Moloney G, Morelli E, Morillas E, O'Connor R, Cruz-Pereira JS, Peterson VL, Rea K, Ritz NL, Sherwin E, Spichak S, Teichman EM, van de Wouw M, Ventura-Silva AP, Wallace-Fitzsimons SE, Hyland N, Clarke G, Dinan TG. The Microbiota-Gut-Brain Axis. Physiol Rev. 2019 Oct 1;99(4):1877-2013. doi: 10.1152/physrev.00018.2018

Daemen S, Kutmon M, Evelo CT. A pathway approach to investigate the function and regulation of SREBPs. Genes Nutr. 2013 May;8(3):289-300. doi: 10.1007/s12263-013-0342-x

Daitoku H, Hatta M, Matsuzaki H, Aratani S, Ohshima T, Miyagishi M, Nakajima T, Fukamizu A. Silent information regulator 2 potentiates Foxo1-mediated transcription through its deacetylase activity. Proc Natl Acad Sci U S A. 2004 Jul 6;101(27):10042-7. doi: 10.1073/pnas.0400593101

Daitoku H, Kaneko Y, Yoshimochi K, Matsumoto K, Araoi S, Sakamaki JI, Takahashi Y, Fukamizu A. Non-transcriptional Function of FOXO1/DAF-16 Contributes to Translesion DNA Synthesis. Mol Cell Biol. 2016 Nov;36(21):2755-2766. doi: 10.1128/MCB.00265-16

Daitoku H, Yamagata K, Matsuzaki H, Hatta M, Fukamizu A. Regulation of PGC-1 promoter activity by protein kinase B and the forkhead transcription factor FKHR. Diabetes. 2003 Mar;52(3):642-9. doi: 10.2337/diabetes.52.3.642

Daudon M, Traxer O, Conort P, Lacour B, Jungers P. Type 2 diabetes increases the risk for uric acid stones. J Am Soc Nephrol. 2006 Jul;17(7):2026-33. doi: 10.1681/ASN.2006030262

DeFronzo RA, Ferrannini E, Groop L, Henry RR, Herman WH, Holst JJ, Hu FB, Kahn CR, Raz I, Shulman GI, Simonson DC, Testa MA, Weiss R. Type 2 diabetes mellitus. Nat Rev Dis Primers. 2015 Jul 23;1:15019. doi: 10.1038/nrdp.2015.19

Demontis F, Perrimon N. FOXO/4E-BP signaling in Drosophila muscles regulates organism-wide proteostasis during aging. Cell. 2010 Nov 24;143(5):813-25. doi: 10.1016/j.cell.2010.10.007

Deng X, Zhang W, O-Sullivan I, Williams JB, Dong Q, Park EA, Raghow R, Unterman TG, Elam MB. FoxO1 inhibits sterol regulatory element-binding protein-1c (SREBP-1c) gene expression via transcription factors Sp1 and SREBP-1c. J Biol Chem. 2012 Jun 8;287(24):20132-43. doi: 10.1074/jbc.M112.347211

DiAngelo JR, Birnbaum MJ. Regulation of fat cell mass by insulin in Drosophila melanogaster. Mol Cell Biol. 2009 Dec;29(24):6341-52. doi: 10.1128/MCB.00675-09

doi: 10.1016/j.bbalip.2014.08.004

Dong XC. FOXO transcription factors in non-alcoholic fatty liver disease. Liver Res. 2017 Sep;1(3):168-173. doi: 10.1016/j.livres.2017.11.004

Drewes T, Senkel S, Holewa B, Ryffel GU. Human hepatocyte nuclear factor 4 isoforms are encoded by distinct and differentially expressed genes. Mol Cell Biol. 1996 Mar;16(3):925-31. doi: 10.1128/MCB.16.3.925

Duran-Ortiz S, List EO, Ikeno Y, Young J, Basu R, Bell S, McHugh T, Funk K, Mathes S, Qian Y, Kulkarni P, Yakar S, Berryman DE, Kopchick JJ. Growth hormone receptor gene disruption in mature-adult mice improves male insulin sensitivity and extends female lifespan. Aging Cell. 2021 Dec;20(12):e13506. doi: 10.1111/acel.13506

Fan W, Imamura T, Sonoda N, Sears DD, Patsouris D, Kim JJ, Olefsky JM. FOXO1 transrepresses peroxisome proliferator-activated receptor gamma transactivation, coordinating an insulin-induced feed-forward response in adipocytes. J Biol Chem. 2009 May 1;284(18):12188-97. doi: 10.1074/jbc.M808915200

Fasano C, Disciglio V, Bertora S, Lepore Signorile M, Simone C. FOXO3a from the Nucleus to the Mitochondria: A Round Trip in Cellular Stress Response. Cells. 2019 Sep 19;8(9):1110. doi: 10.3390/cells8091110

Fontana L, Partridge L. Promoting health and longevity through diet: from model organisms to humans. Cell. 2015 Mar 26;161(1):106-118. doi: 10.1016/j.cell.2015.02.020

Freitas DR, Rosa RM, Moraes J, Campos E, Logullo C, Da Silva Vaz I Jr, Masuda A. Relationship between glutathione S-transferase, catalase, oxygen consumption, lipid peroxidation and oxidative stress in eggs and larvae of Boophilus microplus (Acarina: Ixodidae). Comp Biochem Physiol A Mol Integr Physiol. 2007 Apr;146(4):688-94. doi: 10.1016/j.cbpa.2006.04.032

Frescas D, Valenti L, Accili D. Nuclear trapping of the forkhead transcription factor FoxO1 via Sirt-dependent deacetylation promotes expression of glucogenetic genes. J Biol Chem. 2005 May 27;280(21):20589-95. doi: 10.1074/jbc.M412357200

Fröjdö S, Vidal H, Pirola L. Alterations of insulin signaling in type 2 diabetes: a review of the current evidence from humans. Biochim Biophys Acta. 2009 Feb;1792(2):83-92. doi: 10.1016/j.bbadis.2008.10.019

Fu Z, Tindall DJ. FOXOs, cancer and regulation of apoptosis. Oncogene. 2008 Apr 7;27(16):2312-9. doi: 10.1038/onc.2008.24

Fukunaga K, Imachi H, Lyu J, Dong T, Sato S, Ibata T, Kobayashi T, Yoshimoto T, Yonezaki K, Matsunaga T, Murao K. IGF1 suppresses cholesterol accumulation in the liver of growth hormone-deficient mice via the activation of ABCA1. Am J Physiol Endocrinol Metab. 2018 Dec 1;315(6):E1232-E1241. doi: 10.1152/ajpendo.00134.2018

Fukuoka M, Daitoku H, Hatta M, Matsuzaki H, Umemura S, Fukamizu A. Negative regulation of forkhead transcription factor AFX (Foxo4) by CBP-induced acetylation. Int J Mol Med. 2003 Oct;12(4):503-8.

Furuyama T, Kitayama K, Shimoda Y, Ogawa M, Sone K, Yoshida-Araki K, Hisatsune H, Nishikawa S, Nakayama K, Nakayama K, Ikeda K, Motoyama N, Mori N. Abnormal angiogenesis in Foxo1 (Fkhr)-deficient mice. J Biol Chem. 2004 Aug 13;279(33):34741-9. doi: 10.1074/jbc.M314214200

Furuyama T, Nakazawa T, Nakano I, Mori N. Identification of the differential distribution patterns of mRNAs and consensus binding sequences for mouse DAF-16 homologues. Biochem J. 2000 Jul 15;349(Pt 2):629-34. doi: 10.1042/0264-6021:3490629

Furuyama T, Yamashita H, Kitayama K, Higami Y, Shimokawa I, Mori N. Effects of aging and caloric restriction on the gene expression of Foxo1, 3, and 4 (FKHR, FKHRL1, and AFX) in the rat skeletal muscles. Microsc Res Tech. 2002 Nov 15;59(4):331-4. doi: 10.1002/jemt.10213

Gai J, Ji M, Shi C, Li W, Chen S, Wang Y, Li H. FoxO regulates expression of ABCA6, an intracellular ATP-binding-cassette transporter responsive to cholesterol. Int J Biochem Cell Biol. 2013 Nov;45(11):2651-9. doi: 10.1016/j.biocel.2013.08.020

Gajiwala KS, Burley SK. Winged helix proteins. Curr Opin Struct Biol. 2000 Feb;10(1):110-6. doi: 10.1016/s0959-440x(99)00057-3

Garcia A, Lev B, Hossain KR, Gorman A, Diaz D, Pham THN, Cornelius F, Allen TW, Clarke RJ. Cholesterol depletion inhibits Na+,K+-ATPase activity in a near-native membrane environment. J Biol Chem. 2019 Apr 12;294(15):5956-5969. doi: 10.1074/jbc.RA118.006223

Garrido D, Rubin T, Poidevin M, Maroni B, Le Rouzic A, Parvy JP, Montagne J. Fatty acid synthase cooperates with glyoxalase 1 to protect against sugar toxicity. PLoS Genet. 2015 Feb 18;11(2):e1004995. doi: 10.1371/journal.pgen.1004995

Ghimire S, Terhzaz S, Cabrero P, Romero MF, Davies SA, Dow JAT. Targeted renal knockdown of Na+/H+ exchanger regulatory factor Sip1 produces uric acid nephrolithiasis in Drosophila. Am J Physiol Renal Physiol. 2019 Oct 1;317(4):F930-F940. doi: 10.1152/ajprenal.00551.2018

Giannakou ME, Goss M, Jünger MA, Hafen E, Leevers SJ, Partridge L. Long-lived Drosophila with overexpressed dFOXO in adult fat body. Science. 2004 Jul 16;305(5682):361. doi: 10.1126/science.1098219

Giannakou ME, Goss M, Partridge L. Role of dFOXO in lifespan extension by dietary restriction in Drosophila melanogaster: not required, but its activity modulates the response. Aging Cell. 2008 Mar;7(2):187-98. doi: 10.1111/j.1474-9726.2007.00362.x

Giannakou ME, Partridge L. Role of insulin-like signalling in Drosophila lifespan. Trends Biochem Sci. 2007 Apr;32(4):180-8. doi: 10.1016/j.tibs.2007.02.007. Epub 2007 Apr 6. PMID: 17412594.

Goh SY, Cooper ME. Clinical review: The role of advanced glycation end products in progression and complications of diabetes. J Clin Endocrinol Metab. 2008 Apr;93(4):1143-52. doi: 10.1210/jc.2007-1817

Golson ML, Kaestner KH. Fox transcription factors: from development to disease. Development. 2016 Dec 15;143(24):4558-4570. doi: 10.1242/dev.112672

Grandison RC, Piper MD, Partridge L. Amino-acid imbalance explains extension of lifespan by dietary restriction in Drosophila. Nature. 2009 Dec 24;462(7276):1061-4. doi: 10.1038/nature08619

Greer EL, Brunet A. FOXO transcription factors in ageing and cancer. Acta Physiol (Oxf). 2008 Jan;192(1):19-28. doi: 10.1111/j.1748-1716.2007

Greer EL, Oskoui PR, Banko MR, Maniar JM, Gygi MP, Gygi SP, Brunet A. The energy sensor AMP-activated protein kinase directly regulates the mammalian FOXO3 transcription factor. J Biol Chem. 2007 Oct 12;282(41):30107-19. doi: 10.1074/jbc.M705325200

Grewal SS. Insulin/TOR signaling in growth and homeostasis: a view from the fly world. Int J Biochem Cell Biol. 2009 May;41(5):1006-10. doi: 10.1016/j.biocel.2008.10.010

Grönke S, Clarke DF, Broughton S, Andrews TD, Partridge L. Molecular evolution and functional characterization of Drosophila insulin-like peptides. PLoS Genet. 2010 Feb 26;6(2):e1000857. doi: 10.1371/journal.pgen.1000857

Gross DN, van den Heuvel AP, Birnbaum MJ. The role of FoxO in the regulation of metabolism. Oncogene. 2008 Apr 7;27(16):2320-36. doi: 10.1038/onc.2008.25

Haeusler RA, Kaestner KH, Accili D. FoxOs function synergistically to promote glucose production. J Biol Chem. 2010 Nov 12;285(46):35245-8. doi: 10.1074/jbc.C110.175851

Hantzidiamantis PJ, Lappin SL. Physiology, Glucose. 2022 Sep 19. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2022 Jan–.

Harrison DE, Strong R, Sharp ZD, Nelson JF, Astle CM, Flurkey K, Nadon NL, Wilkinson JE, Frenkel K, Carter CS, Pahor M, Javors MA, Fernandez E, Miller RA. Rapamycin fed late in life extends lifespan in genetically heterogeneous mice. Nature. 2009 Jul 16;460(7253):392-5. doi: 10.1038/nature08221

Hay N. Interplay between FOXO, TOR, and Akt. Biochim Biophys Acta. 2011 Nov;1813(11):1965-70. doi: 10.1016/j.bbamcr.2011.03.013

Hayhurst GP, Lee YH, Lambert G, Ward JM, Gonzalez FJ. Hepatocyte nuclear factor 4alpha (nuclear receptor 2A1) is essential for maintenance of hepatic gene expression and lipid homeostasis. Mol Cell Biol. 2001 Feb;21(4):1393-403. doi: 10.1128/MCB.21.4.1393-1403.2001

He XB, Huang H, Wu Y, Guo F. Acrylamide Induces Protein Aggregation in CNS through Suppression of FoxO1-mediated Autophagy. bioRxiv. 2022. doi: 10.1101/2022.07.29.498756

Hietakangas V, Cohen SM. Re-evaluating AKT regulation: role of TOR complex 2 in tissue growth. Genes Dev. 2007 Mar 15;21(6):632-7. doi: 10.1101/gad.416307

Hill R, Kalathur RK, Callejas S, Colaço L, Brandão R, Serelde B, Cebriá A, Blanco-Aparicio C, Pastor J, Futschik M, Dopazo A, Link W. A novel phosphatidylinositol 3-kinase (PI3K) inhibitor directs a potent FOXO-dependent, p53-independent cell cycle arrest phenotype characterized by the differential induction of a subset of FOXO-regulated genes. Breast Cancer Res. 2014 Dec 9;16(6):482. doi: 10.1186/s13058-014-0482-y

Hirota K, Daitoku H, Matsuzaki H, Araya N, Yamagata K, Asada S, Sugaya T, Fukamizu A. Hepatocyte nuclear factor-4 is a novel downstream target of insulin via FKHR as a signal-regulated transcriptional inhibitor. J Biol Chem. 2003 Apr 11;278(15):13056-60. doi: 10.1074/jbc.C200553200

Holzenberger M, Dupont J, Ducos B, Leneuve P, Géloën A, Even PC, Cervera P, Le Bouc Y. IGF-1 receptor regulates lifespan and resistance to oxidative stress in mice. Nature. 2003 Jan 9;421(6919):182-7. doi: 10.1038/nature01298

Hong SH, Lee KS, Kwak SJ, Kim AK, Bai H, Jung MS, Kwon OY, Song WJ, Tatar M, Yu K. Minibrain/Dyrk1a regulates food intake through the Sir2-FOXO-sNPF/NPY pathway in Drosophila and mammals. PLoS Genet. 2012;8(8):e1002857. doi: 10.1371/journal.pgen.1002857. Epub 2012 Aug 2. Erratum in: PLoS Genet. 2012 Sep;8(9). doi: 10.1371/annotation/8c2c8644-1beb-4410-8bef-c388b7256738

Hornsveld M, Dansen TB, Derksen PW, Burgering BMT. Re-evaluating the role of FOXOs in cancer. Semin Cancer Biol. 2018 Jun;50:90-100. doi: 10.1016/j.semcancer.2017.11.017

Hosaka T, Biggs WH 3rd, Tieu D, Boyer AD, Varki NM, Cavenee WK, Arden KC. Disruption of forkhead transcription factor (FOXO) family members in mice reveals their functional diversification. Proc Natl Acad Sci U S A. 2004 Mar 2;101(9):2975-80. doi: 10.1073/pnas.0400093101

Hou T, Zheng G, Zhang P, Jia J, Li J, Xie L, Wei C, Li Y. LAceP: lysine acetylation site prediction using logistic regression classifiers. PLoS One. 2014 Feb 20;9(2):e89575. doi: 10.1371/journal.pone.0089575

Hu W, Yang Z, Yang W, Han M, Xu B, Yu Z, Shen M, Yang Y. Roles of forkhead box O (FoxO) transcription factors in neurodegenerative diseases: A panoramic view. Prog Neurobiol. 2019 Oct;181:101645. doi: 10.1016/j.pneurobio.2019.101645

Huang J, Zhou W, Dong W, Watson AM, Hong Y. From the Cover: Directed, efficient, and versatile modifications of the Drosophila genome by genomic engineering. Proc Natl Acad Sci U S A. 2009 May 19;106(20):8284-9. doi: 10.1073/pnas.0900641106

Huang J, Zhou W, Watson AM, Jan YN, Hong Y. Efficient ends-out gene targeting in Drosophila. Genetics. 2008 Sep;180(1):703-7. doi: 10.1534/genetics.108.090563

Hwangbo DS, Gershman B, Tu MP, Palmer M, Tatar M. Drosophila dFOXO controls lifespan and regulates insulin signalling in brain and fat body. Nature. 2004 Jun 3;429(6991):562-6. doi: 10.1038/nature02549

Ihara A, Uno M, Miyatake K, Honjoh S, Nishida E. Cholesterol regulates DAF-16 nuclear localization and fasting-induced longevity in C. elegans. Exp Gerontol. 2017 Jan;87(Pt A):40-47. doi: 10.1016/j.exger.2016.10.011

Imae M, Fu Z, Yoshida A, Noguchi T, Kato H. Nutritional and hormonal factors control the gene expression of FoxOs, the mammalian homologues of DAF-16. J Mol Endocrinol. 2003 Apr;30(2):253-62. doi: 10.1677/jme.0.0300253

Iqbal, A., Duitama, C., Metge, F., Rosskopp, D., Boucas, J. Flaski. (2021). doi:10.5281/zenodo.4849515

Izquierdo MC, Shanmugarajah N, Lee SX, Kraakman MJ, Westerterp M, Kitamoto T, Harris M, Cook JR, Gusarova GA, Zhong K, Marbuary E, O-Sullivan I, Rasmus N, Camastra S, Unterman TG, Ferrannini E, Hurwitz BE, Haeusler RA. Hepatic FoxOs link insulin signaling with plasma lipoprotein metabolism through an apolipoprotein M/sphingosine-1-phosphate pathway. J Clin Invest. 2022 Apr 1;132(7):e146219. doi: 10.1172/JCI146219

Jach ME, Serefko A, Ziaja M, Kieliszek M. Yeast Protein as an Easily Accessible Food Source. Metabolites. 2022 Jan 11;12(1):63. doi: 10.3390/metabo12010063

Jackson BC, Carpenter C, Nebert DW, Vasiliou V. Update of human and mouse forkhead box (FOX) gene families. Hum Genomics. 2010 Jun;4(5):345-52. doi: 10.1186/1479-7364-4-5-345

Jagger C, Gillies C, Moscone F, Cambois E, Van Oyen H, Nusselder W, Robine JM; EHLEIS team. Inequalities in healthy life years in the 25 countries of the European Union in 2005: a cross-national meta-regression analysis. Lancet. 2008 Dec 20;372(9656):2124-31. doi: 10.1016/S0140-6736(08)61594-9

Jiang JC, Jaruga E, Repnevskaya MV, Jazwinski SM. An intervention resembling caloric restriction prolongs life span and retards aging in yeast. FASEB J. 2000 Nov;14(14):2135-7. doi: 10.1096/fj.00-0242fje

Jiang Y, Yan F, Feng Z, Lazarovici P, Zheng W. Signaling Network of Forkhead Family of Transcription Factors (FOXO) in Dietary Restriction. Cells. 2019 Dec 31;9(1):100. doi: 10.3390/cells9010100

Jing E, Gesta S, Kahn CR. SIRT2 regulates adipocyte differentiation through FoxO1 acetylation/deacetylation. Cell Metab. 2007 Aug;6(2):105-14. doi: 10.1016/j.cmet.2007.07.003

Jünger MA, Rintelen F, Stocker H, Wasserman JD, Végh M, Radimerski T, Greenberg ME, Hafen E. The Drosophila forkhead transcription factor FOXO mediates the reduction in cell number associated with reduced insulin signaling. J Biol. 2003;2(3):20. doi: 10.1186/1475-4924-2-20

Juričić P, Grönke S, Partridge L. Branched-Chain Amino Acids Have Equivalent Effects to Other Essential Amino Acids on Lifespan and Aging-Related Traits in Drosophila. J Gerontol A Biol Sci Med Sci. 2020 Jan 1;75(1):24-31. doi: 10.1093/gerona/glz080

Kaletsky R, Murphy CT. The role of insulin/IGF-like signaling in C. elegans longevity and aging. Dis Model Mech. 2010 Jul-Aug;3(7-8):415-9. doi: 10.1242/dmm.001040

Kamei Y, Mizukami J, Miura S, Suzuki M, Takahashi N, Kawada T, Taniguchi T, Ezaki O. A forkhead transcription factor FKHR up-regulates lipoprotein lipase expression in skeletal muscle. FEBS Lett. 2003 Feb 11;536(1-3):232-6. doi: 10.1016/s0014-5793(03)00062-0

Keebaugh AC, Thomas JW. The evolutionary fate of the genes encoding the purine catabolic enzymes in hominoids, birds, and reptiles. Mol Biol Evol. 2010 Jun;27(6):1359-69. doi: 10.1093/molbev/msq022

Keenan T, Blaha MJ, Nasir K, Silverman MG, Tota-Maharaj R, Carvalho JA, Conceição RD, Blumenthal RS, Santos RD. Relation of uric acid to serum levels of high-sensitivity C-reactive protein, triglycerides, and high-density lipoprotein cholesterol and to hepatic steatosis. Am J Cardiol. 2012 Dec 15;110(12):1787-92. doi: 10.1016/j.amjcard.2012.08.012

Kenyon C, Chang J, Gensch E, Rudner A, Tabtiang R. A C. elegans mutant that lives twice as long as wild type. Nature. 1993 Dec 2;366(6454):461-4. doi: 10.1038/366461a0

Khitan Z, Kim DH. Fructose: a key factor in the development of metabolic syndrome and hypertension. J Nutr Metab. 2013;2013:682673. doi: 10.1155/2013/682673

Kim DH, Park MH, Chung KW, Kim MJ, Jung YR, Bae HR, Jang EJ, Lee JS, Im DS, Yu BP, Chung HY. The essential role of FoxO6 phosphorylation in aging and calorie restriction. Age (Dordr). 2014;36(4):9679. doi: 10.1007/s11357-014-9679-3

Kim DH, Perdomo G, Zhang T, Slusher S, Lee S, Phillips BE, Fan Y, Giannoukakis N, Gramignoli R, Strom S, Ringquist S, Dong HH. FoxO6 integrates insulin signaling with gluconeogenesis in the liver. Diabetes. 2011 Nov;60(11):2763-74. doi: 10.2337/db11-0548

Kim SH, Kim BK, Park S, Park SK. Phosphatidylcholine Extends Lifespan via DAF-16 and Reduces Amyloid-Beta-Induced Toxicity in Caenorhabditis elegans. Oxid Med Cell Longev. 2019 Jul 11;2019:2860642. doi: 10.1155/2019/2860642

Kim Y, Sun H. ASM-3 acid sphingomyelinase functions as a positive regulator of the DAF-2/AGE-1 signaling pathway and serves as a novel anti-aging target. PLoS One. 2012;7(9):e45890. doi: 10.1371/journal.pone.0045890

Kimura KD, Tissenbaum HA, Liu Y, Ruvkun G. daf-2, an insulin receptor-like gene that regulates longevity and diapause in Caenorhabditis elegans. Science. 1997 Aug 15;277(5328):942-6. doi: 10.1126/science.277.5328.942

Kitamura YI, Kitamura T, Kruse JP, Raum JC, Stein R, Gu W, Accili D. FoxO1 protects against pancreatic beta cell failure through NeuroD and MafA induction. Cell Metab. 2005 Sep;2(3):153-63. doi: 10.1016/j.cmet.2005.08.004

Klass MR. Aging in the nematode Caenorhabditis elegans: major biological and environmental factors influencing life span. Mech Ageing Dev. 1977 Nov-Dec;6(6):413-29. doi: 10.1016/0047-6374(77)90043-4

Kodani N, Nakae J. Tissue-Specific Metabolic Regulation of FOXO-Binding Protein: FOXO Does Not Act Alone. Cells. 2020 Mar 13;9(3):702. doi: 10.3390/cells9030702

Kousteni S. FoxO1, the transcriptional chief of staff of energy metabolism. Bone. 2012 Feb;50(2):437-43. doi: 10.1016/j.bone.2011.06.034

Kramer JM, Slade JD, Staveley BE. foxo is required for resistance to amino acid starvation in Drosophila. Genome. 2008 Aug;51(8):668-72. doi: 10.1139/G08-047

Lam EW, Brosens JJ, Gomes AR, Koo CY. Forkhead box proteins: tuning forks for transcriptional harmony. Nat Rev Cancer. 2013 Jul;13(7):482-95. doi: 10.1038/nrc3539

Lang S, Hilsabeck TA, Wilson KA, Sharma A, Bose N, Brackman DJ, Beck JN, Chen L, Watson MA, Killilea DW, Ho S, Kahn A, Giacomini K, Stoller ML, Chi T, Kapahi P. A conserved role of the insulin-like signaling pathway in diet-dependent uric acid pathologies in Drosophila melanogaster. PLoS Genet. 2019 Aug 15;15(8):e1008318. doi: 10.1371/journal.pgen.1008318

Langlet F, Haeusler RA, Lindén D, Ericson E, Norris T, Johansson A, Cook JR, Aizawa K, Wang L, Buettner C, Accili D. Selective Inhibition of FOXO1 Activator/Repressor Balance Modulates Hepatic Glucose Handling. Cell. 2017 Nov 2;171(4):824-835.e18. doi: 10.1016/j.cell.2017.09.045

Lee C, Longo V. Dietary restriction with and without caloric restriction for healthy aging. F1000Res. 2016 Jan 29;5:F1000 Faculty Rev-117. doi: 10.12688/f1000research.7136.1

Lee SH, Lee SK, Paik D, Min KJ. Overexpression of fatty-acid-β-oxidation-related genes extends the lifespan of Drosophila melanogaster. Oxid Med Cell Longev. 2012;2012:854502. doi: 10.1155/2012/854502

Lehtinen MK, Yuan Z, Boag PR, Yang Y, Villén J, Becker EB, DiBacco S, de la Iglesia N, Gygi S, Blackwell TK, Bonni A. A conserved MST-FOXO signaling pathway mediates oxidative-stress responses and extends life span. Cell. 2006 Jun 2;125(5):987-1001. doi: 10.1016/j.cell.2006.03.046

Lennicke C, Dos Santos E, Cochemé HM. Sugar-induced dysregulation of purine metabolism impacts lifespan. Aging (Albany NY). 2020 Dec 27;12(24):24479-24480. doi: 10.18632/aging.104223

Lettieri Barbato D, Aquilano K, Baldelli S, Cannata SM, Bernardini S, Rotilio G, Ciriolo MR. Proline oxidase-adipose triglyceride lipase pathway restrains adipose cell death and tissue inflammation. Cell Death Differ. 2014 Jan;21(1):113-23. doi: 10.1038/cdd.2013.137

Lettieri Barbato D, Aquilano K, Ciriolo MR. FoxO1 at the nexus between fat catabolism and longevity pathways. Biochim Biophys Acta. 2014 Oct;1841(10):1555-1560.

Lettieri Barbato D, Tatulli G, Aquilano K, Ciriolo MR. FoxO1 controls lysosomal acid lipase in adipocytes: implication of lipophagy during nutrient restriction and metformin treatment. Cell Death Dis. 2013 Oct 17;4(10):e861. doi: 10.1038/cddis.2013.404

Lettieri-Barbato D, Ioannilli L, Aquilano K, Ciccarone F, Rosina M, Ciriolo MR. FoxO1 localizes to mitochondria of adipose tissue and is affected by nutrient stress. Metabolism. 2019 Jun;95:84-92. doi: 10.1016/j.metabol.2019.04.006

Levitan I, Fang Y, Rosenhouse-Dantsker A, Romanenko V. Cholesterol and ion channels. Subcell Biochem. 2010;51:509-49. doi: 10.1007/978-90-481-8622-8_19

Lin J, Handschin C, Spiegelman BM. Metabolic control through the PGC-1 family of transcription coactivators. Cell Metab. 2005 Jun;1(6):361-70. doi: 10.1016/j.cmet.2005.05.004

Lin K, Dorman JB, Rodan A, Kenyon C. daf-16: An HNF-3/forkhead family member that can function to double the life-span of Caenorhabditis elegans. Science. 1997 Nov 14;278(5341):1319-22. doi: 10.1126/science.278.5341.1319

Lin SJ, Defossez PA, Guarente L. Requirement of NAD and SIR2 for life-span extension by calorie restriction in Saccharomyces cerevisiae. Science. 2000 Sep 22;289(5487):2126-8. doi: 10.1126/science.289.5487.2126

López-Otín C, Blasco MA, Partridge L, Serrano M, Kroemer G. Hallmarks of aging: An expanding universe. Cell. 2023 Jan 19;186(2):243-278. doi: 10.1016/j.cell.2022.11.001

López-Otín C, Blasco MA, Partridge L, Serrano M, Kroemer G. The hallmarks of aging. Cell. 2013 Jun 6;153(6):1194-217. doi: 10.1016/j.cell.2013.05.039

Lushchak OV, Gospodaryov DV, Rovenko BM, Yurkevych IS, Perkhulyn NV, Lushchak VI. Specific dietary carbohydrates differentially influence the life span and fecundity of Drosophila melanogaster. J Gerontol A Biol Sci Med Sci. 2014 Jan;69(1):3-12. doi: 10.1093/gerona/glt077

Lustig RH, Schmidt LA, Brindis CD. Public health: The toxic truth about sugar. Nature. 2012 Feb 1;482(7383):27-9. doi: 10.1038/482027a

Lv Q, Meng XF, He FF, Chen S, Su H, Xiong J, Gao P, Tian XJ, Liu JS, Zhu ZH, Huang K, Zhang C. High serum uric acid and increased risk of type 2 diabetes: a systemic review and meta-analysis of prospective cohort studies. PLoS One. 2013;8(2):e56864. doi: 10.1371/journal.pone.0056864

Magner DB, Wollam J, Shen Y, Hoppe C, Li D, Latza C, Rottiers V, Hutter H, Antebi A. The NHR-8 nuclear receptor regulates cholesterol and bile acid homeostasis in C. elegans. Cell Metab. 2013 Aug 6;18(2):212-24. doi: 10.1016/j.cmet.2013.07.007

Mair W, Piper MD, Partridge L. Calories do not explain extension of life span by dietary restriction in Drosophila. PLoS Biol. 2005 Jul;3(7):e223. doi: 10.1371/journal.pbio.0030223

Mansson R, Welinder E, Åhsberg J, Lin YC, Benner C, Glass CK, Lucas JS, Sigvardsson M, Murre C. Positive intergenic feedback circuitry, involving EBF1 and FOXO1, orchestrates B-cell fate. Proc Natl Acad Sci U S A. 2012 Dec 18;109(51):21028-33. doi: 10.1073/pnas.1211427109

Martins R, Lithgow GJ, Link W. Long live FOXO: unraveling the role of FOXO proteins in aging and longevity. Aging Cell. 2016 Apr;15(2):196-207. doi: 10.1111/acel.12427

Matsumoto M, Han S, Kitamura T, Accili D. Dual role of transcription factor FoxO1 in controlling hepatic insulin sensitivity and lipid metabolism. J Clin Invest. 2006 Sep;116(9):2464-72. doi: 10.1172/JCI27047

Matsumoto M, Pocai A, Rossetti L, Depinho RA, Accili D. Impaired regulation of hepatic glucose production in mice lacking the forkhead transcription factor Foxo1 in liver. Cell Metab. 2007 Sep;6(3):208-16. doi: 10.1016/j.cmet.2007.08.006

Matsuzaki H, Daitoku H, Hatta M, Aoyama H, Yoshimochi K, Fukamizu A. Acetylation of Foxo1 alters its DNA-binding ability and sensitivity to phosphorylation. Proc Natl Acad Sci U S A. 2005 Aug 9;102(32):11278-83. doi: 10.1073/pnas.0502738102

Matsuzaki H, Daitoku H, Hatta M, Tanaka K, Fukamizu A. Insulin-induced phosphorylation of FKHR (Foxo1) targets to proteasomal degradation. Proc Natl Acad Sci U S A. 2003 Sep 30;100(20):11285-90. doi: 10.1073/pnas.1934283100

Mattison JA, Roth GS, Beasley TM, Tilmont EM, Handy AM, Herbert RL, Longo DL, Allison DB, Young JE, Bryant M, Barnard D, Ward WF, Qi W, Ingram DK, de Cabo R. Impact of caloric restriction on health and survival in rhesus monkeys from the NIA study. Nature. 2012 Sep 13;489(7415):318-21. doi: 10.1038/nature11432

McCay CM, Crowell MF, Maynard LA. The effect of retarded growth upon the length of life span and upon the ultimate body size. 1935. Nutrition. 1989 May-Jun;5(3):155-71; discussion 172.

Mihaylova MM, Vasquez DS, Ravnskjaer K, Denechaud PD, Yu RT, Alvarez JG, Downes M, Evans RM, Montminy M, Shaw RJ. Class IIa histone deacetylases are hormone-activated regulators of FOXO and mammalian glucose homeostasis. Cell. 2011 May 13;145(4):607-21. doi: 10.1016/j.cell.2011.03.043

Miller RA, Buehner G, Chang Y, Harper JM, Sigler R, Smith-Wheelock M. Methionine-deficient diet extends mouse lifespan, slows immune and lens aging, alters glucose, T4, IGF-I and insulin levels, and increases hepatocyte MIF levels and stress resistance. Aging Cell. 2005 Jun;4(3):119-25. doi: 10.1111/j.1474-9726.2005.00152.x

Mirzaei H, Suarez JA, Longo VD. Protein and amino acid restriction, aging and disease: from yeast to humans. Trends Endocrinol Metab. 2014 Nov;25(11):558-66. doi: 10.1016/j.tem.2014.07.002

Miyauchi T, Uchida Y, Kadono K, Hirao H, Kawasoe J, Watanabe T, Ueda S, Okajima H, Terajima H, Uemoto S. Up-regulation of FOXO1 and reduced inflammation by β-hydroxybutyric acid are essential diet

restriction benefits against liver injury. Proc Natl Acad Sci U S A. 2019 Jul 2;116(27):13533-13542. doi: 10.1073/pnas.1820282116

Moe OW. Kidney stones: pathophysiology and medical management. Lancet. 2006 Jan 28;367(9507):333-44. doi: 10.1016/S0140-6736(06)68071-9

Mori J, Patel VB, Ramprasath T, Alrob OA, DesAulniers J, Scholey JW, Lopaschuk GD, Oudit GY. Angiotensin 1-7 mediates renoprotection against diabetic nephropathy by reducing oxidative stress, inflammation, and lipotoxicity. Am J Physiol Renal Physiol. 2014 Apr 15;306(8):F812-21. doi: 10.1152/ajprenal.00655.2013

Morris SN, Coogan C, Chamseddin K, Fernandez-Kim SO, Kolli S, Keller JN, Bauer JH. Development of diet-induced insulin resistance in adult Drosophila melanogaster. Biochim Biophys Acta. 2012 Aug;1822(8):1230-7. doi: 10.1016/j.bbadis.2012.04.012

Motta MC, Divecha N, Lemieux M, Kamel C, Chen D, Gu W, Bultsma Y, McBurney M, Guarente L. Mammalian SIRT1 represses forkhead transcription factors. Cell. 2004 Feb 20;116(4):551-63. doi: 10.1016/s0092-8674(04)00126-6

Munekata K, Sakamoto K. Forkhead transcription factor Foxo1 is essential for adipocyte differentiation. In Vitro Cell Dev Biol Anim. 2009 Dec;45(10):642-51. doi: 10.1007/s11626-009-9230-5

Musselman LP, Fink JL, Narzinski K, Ramachandran PV, Hathiramani SS, Cagan RL, Baranski TJ. A high-sugar diet produces obesity and insulin resistance in wild-type Drosophila. Dis Model Mech. 2011 Nov;4(6):842-9. doi: 10.1242/dmm.007948

Nakae J, Kitamura T, Silver DL, Accili D. The forkhead transcription factor Foxo1 (Fkhr) confers insulin sensitivity onto glucose-6-phosphatase expression. J Clin Invest. 2001 Nov;108(9):1359-67. doi: 10.1172/JCI12876

Nässel DR, Vanden Broeck J. Insulin/IGF signaling in Drosophila and other insects: factors that regulate production, release and post-release action of the insulin-like peptides. Cell Mol Life Sci. 2016 Jan;73(2):271-90. doi: 10.1007/s00018-015-2063-3

Nazario-Yepiz NO, Fernández Sobaberas J, Lyman R, Campbell MR 3rd, Shankar V, Anholt RRH, Mackay TFC. Physiological and metabolomic consequences of reduced expression of the Drosophila brummer triglyceride Lipase. PLoS One. 2021 Sep 21;16(9):e0255198. doi: 10.1371/journal.pone.0255198

Newgard CB, Brady MJ, O'Doherty RM, Saltiel AR. Organizing glucose disposal: emerging roles of the glycogen targeting subunits of protein phosphatase-1. Diabetes. 2000 Dec;49(12):1967-77. doi: 10.2337/diabetes.49.12.1967

Nikolich-Žugich J, Goldman DP, Cohen PR, Cortese D, Fontana L, Kennedy BK, Mohler MJ, Olshansky SJ, Perls T, Perry D, Richardson A, Ritchie C, Wertheimer AM, Faragher RG, Fain MJ. Preparing for an Aging World: Engaging Biogerontologists, Geriatricians, and the Society. J Gerontol A Biol Sci Med Sci. 2016 Apr;71(4):435-44. doi: 10.1093/gerona/glv164

Niwa R, Niwa YS. The Fruit Fly Drosophila melanogaster as a Model System to Study Cholesterol Metabolism and Homeostasis. Cholesterol. 2011;2011:176802. doi: 10.1155/2011/176802

Obsil T, Obsilova V. Structural basis for DNA recognition by FOXO proteins. Biochim Biophys Acta. 2011 Nov;1813(11):1946-53. doi: 10.1016/j.bbamcr.2010.11.025

Obsilova V, Vecer J, Herman P, Pabianova A, Sulc M, Teisinger J, Boura E, Obsil T. 14-3-3 Protein interacts with nuclear localization sequence of forkhead transcription factor FoxO4. Biochemistry. 2005 Aug 30;44(34):11608-17. doi: 10.1021/bi050618r

Oeppen J, Vaupel JW. Demography. Broken limits to life expectancy. Science. 2002 May 10;296(5570):1029-31. doi: 10.1126/science.1069675

Oh SW, Mukhopadhyay A, Svrzikapa N, Jiang F, Davis RJ, Tissenbaum HA. JNK regulates lifespan in Caenorhabditis elegans by modulating nuclear translocation of forkhead transcription factor/DAF-16. Proc Natl Acad Sci U S A. 2005 Mar 22;102(12):4494-9. doi: 10.1073/pnas.0500749102

Olshansky SJ, Carnes BA. Inconvenient Truths About Human Longevity. J Gerontol A Biol Sci Med Sci. 2019 Nov 13;74(Suppl_1):S7-S12. doi: 10.1093/gerona/glz098

Orme MH, Alrubaie S, Bradley GL, Walker CD, Leevers SJ. Input from Ras is required for maximal PI(3)K signalling in Drosophila. Nat Cell Biol. 2006 Nov;8(11):1298-302. doi: 10.1038/ncb1493

Orr HT, Zoghbi HY. Trinucleotide repeat disorders. Annu Rev Neurosci. 2007;30:575-621. doi: 10.1146/annurev.neuro.29.051605.113042. PMID: 17417937.

Ortega-Molina A, Efeyan A, Lopez-Guadamillas E, Muñoz-Martin M, Gómez-López G, Cañamero M, Mulero F, Pastor J, Martinez S, Romanos E, Mar Gonzalez-Barroso M, Rial E, Valverde AM, Bischoff JR, Serrano M. Pten positively regulates brown adipose function, energy expenditure, and longevity. Cell Metab. 2012 Mar 7;15(3):382-94. doi: 10.1016/j.cmet.2012.02.001

Pajvani UB, Accili D. The new biology of diabetes. Diabetologia. 2015 Nov;58(11):2459-68. doi: 10.1007/s00125-015-3722-5

Palm W, Sampaio JL, Brankatschk M, Carvalho M, Mahmoud A, Shevchenko A, Eaton S. Lipoproteins in Drosophila melanogaster--assembly, function, and influence on tissue lipid composition. PLoS Genet. 2012;8(7):e1002828. doi: 10.1371/journal.pgen.1002828

Pan CW, Jin X, Zhao Y, Pan Y, Yang J, Karnes RJ, Zhang J, Wang L, Huang H. AKT-phosphorylated FOXO1 suppresses ERK activation and chemoresistance by disrupting IQGAP1-MAPK interaction. EMBO J. 2017 Apr 13;36(8):995-1010. doi: 10.15252/embj.201695534

Parks EJ, Skokan LE, Timlin MT, Dingfelder CS. Dietary sugars stimulate fatty acid synthesis in adults. J Nutr. 2008 Jun;138(6):1039-46. doi: 10.1093/jn/138.6.1039

Partridge L, Alic N, Bjedov I, Piper MD. Ageing in Drosophila: the role of the insulin/Igf and TOR signalling network. Exp Gerontol. 2011 May;46(5):376-81. doi: 10.1016/j.exger.2010.09.003

Perrot V, Rechler MM. The coactivator p300 directly acetylates the forkhead transcription factor Foxo1 and stimulates Foxo1-induced transcription. Mol Endocrinol. 2005 Sep;19(9):2283-98. doi: 10.1210/me.2004-0292

Philip-Couderc P, Tavares NI, Roatti A, Lerch R, Montessuit C, Baertschi AJ. Forkhead transcription factors coordinate expression of myocardial KATP channel subunits and energy metabolism. Circ Res. 2008 Feb 1;102(2):e20-35. doi: 10.1161/CIRCRESAHA.107.166744

Piper MD, Blanc E, Leitão-Gonçalves R, Yang M, He X, Linford NJ, Hoddinott MP, Hopfen C, Soultoukis GA, Niemeyer C, Kerr F, Pletcher SD, Ribeiro C, Partridge L. A holidic medium for Drosophila melanogaster. Nat Methods. 2014 Jan;11(1):100-5. doi: 10.1038/nmeth.2731. Epub 2013 Nov 17

Piper MDW, Selman C, McElwee JJ, Partridge L. Models of insulin signalling and longevity. Drug Discov. Today Dis. Models 2. 2005, 2(4), pp. 249-256. doi:10.1016/j.ddmod.2005.11.001

Piper MDW, Soultoukis GA, Blanc E, Mesaros A, Herbert SL, Juricic P, He X, Atanassov I, Salmonowicz H, Yang M, Simpson SJ, Ribeiro C, Partridge L. Matching Dietary Amino Acid Balance to the In Silico-Translated Exome Optimizes Growth and Reproduction without Cost to Lifespan. Cell Metab. 2017 Mar 7;25(3):610-621. doi: 10.1016/j.cmet.2017.02.005

Post S, Karashchuk G, Wade JD, Sajid W, De Meyts P, Tatar M. Drosophila Insulin-Like Peptides DILP2 and DILP5 Differentially Stimulate Cell Signaling and Glycogen Phosphorylase to Regulate Longevity. Front Endocrinol (Lausanne). 2018 May 28;9:245. doi: 10.3389/fendo.2018.00245

Postnikoff SD, Malo ME, Wong B, Harkness TA. The yeast forkhead transcription factors fkh1 and fkh2 regulate lifespan and stress response together with the anaphase-promoting complex. PLoS Genet. 2012;8(3):e1002583. doi: 10.1371/journal.pgen.1002583

Povedano JM, Martinez P, Serrano R, Tejera Á, Gómez-López G, Bobadilla M, Flores JM, Bosch F, Blasco MA. Therapeutic effects of telomerase in mice with pulmonary fibrosis induced by damage to the lungs and short telomeres. Elife. 2018 Jan 30;7:e31299. doi: 10.7554/eLife.31299

Puig O, Marr MT, Ruhf ML, Tjian R. Control of cell number by Drosophila FOXO: downstream and feedback regulation of the insulin receptor pathway. Genes Dev. 2003 Aug 15;17(16):2006-20. doi: 10.1101/gad.1098703

Puigserver P, Rhee J, Donovan J, Walkey CJ, Yoon JC, Oriente F, Kitamura Y, Altomonte J, Dong H, Accili D, Spiegelman BM. Insulin-regulated hepatic gluconeogenesis through FOXO1-PGC-1alpha interaction. Nature. 2003 May 29;423(6939):550-5. doi: 10.1038/nature01667

Qiang L, Banks AS, Accili D. Uncoupling of acetylation from phosphorylation regulates FoxO1 function independent of its subcellular localization. J Biol Chem. 2010 Aug 27;285(35):27396-27401. doi: 10.1074/jbc.M110.140228

Ramaswamy S, Nakamura N, Sansal I, Bergeron L, Sellers WR. A novel mechanism of gene regulation and tumor suppression by the transcription factor FKHR. Cancer Cell. 2002 Jul;2(1):81-91. doi: 10.1016/s1535-6108(02)00086-7

Regan JC, Lu YX, Ureña E, Meilenbrock RL, Catterson JH, Kißler D, Fröhlich J, Funk E, Partridge L. Sexual identity of enterocytes regulates autophagy to determine intestinal health, lifespan and responses to rapamycin. Nat Aging 2. 2022 Dec 01; 1145–1158. doi: 10.1038/s43587-022-00308-7

Rena G, Woods YL, Prescott AR, Peggie M, Unterman TG, Williams MR, Cohen P. Two novel phosphorylation sites on FKHR that are critical for its nuclear exclusion. EMBO J. 2002 May 1;21(9):2263-71. doi: 10.1093/emboj/21.9.2263

Richardson NE, Konon EN, Schuster HS, Mitchell AT, Boyle C, Rodgers AC, Finke M, Haider LR, Yu D, Flores V, Pak HH, Ahmad S, Ahmed S, Radcliff A, Wu J, Williams EM, Abdi L, Sherman DS, Hacker T, Lamming DW. Lifelong restriction of dietary branched-chain amino acids has sex-specific benefits for frailty and lifespan in mice. Nat Aging. 2021 Jan;1(1):73-86. doi: 10.1038/s43587-020-00006-2

Riddle DL, Swanson MM, Albert PS. Interacting genes in nematode dauer larva formation. Nature. 1981 Apr 23;290(5808):668-71. doi: 10.1038/290668a0

Riera CE, Merkwirth C, De Magalhaes Filho CD, Dillin A. Signaling Networks Determining Life Span. Annu Rev Biochem. 2016 Jun 2;85:35-64. doi: 10.1146/annurev-biochem-060815-014451

Robida-Stubbs S, Glover-Cutter K, Lamming DW, Mizunuma M, Narasimhan SD, Neumann-Haefelin E, Sabatini DM, Blackwell TK. TOR signaling and rapamycin influence longevity by regulating SKN-1/Nrf and DAF-16/FoxO. Cell Metab. 2012 May 2;15(5):713-24. doi: 10.1016/j.cmet.2012.04.007

Rodan AR. The Drosophila Malpighian tubule as a model for mammalian tubule function. Curr Opin Nephrol Hypertens. 2019 Sep;28(5):455-464. doi: 10.1097/MNH.0000000000000521

Roth TF, Porter KR. Yolk protein uptake in the oocyte of the mosquito Aedes Aegypti. L. J Cell Biol. 1964 Feb;20(2):313-32. doi: 10.1083/jcb.20.2.313

Rudd MD, Gonzalez-Robayna I, Hernandez-Gonzalez I, Weigel NL, Bingman WE 3rd, Richards JS. Constitutively active FOXO1a and a DNA-binding domain mutant exhibit distinct co-regulatory functions to enhance progesterone receptor A activity. J Mol Endocrinol. 2007 Jun;38(6):673-90. doi: 10.1677/JME-07-0017

Sablon A, Bollaert E, Pirson C, Velghe AI, Demoulin JB. FOXO1 forkhead domain mutants in B-cell lymphoma lack transcriptional activity. Sci Rep. 2022 Jan 25;12(1):1309. doi: 10.1038/s41598-022-05334-4

Schmitt-Ney M. The FOXO's Advantages of Being a Family: Considerations on Function and Evolution. Cells. 2020 Mar 24;9(3):787. doi: 10.3390/cells9030787

Schmoll D, Walker KS, Alessi DR, Grempler R, Burchell A, Guo S, Walther R, Unterman TG. Regulation of glucose-6-phosphatase gene expression by protein kinase Balpha and the forkhead transcription factor FKHR. Evidence for insulin response unit-dependent and -independent effects of insulin on promoter activity. J Biol Chem. 2000 Nov 17;275(46):36324-33. doi: 10.1074/jbc.M003616200

Schonbaum CP, Perrino JJ, Mahowald AP. Regulation of the vitellogenin receptor during Drosophila melanogaster oogenesis. Mol Biol Cell. 2000 Feb;11(2):511-21. doi: 10.1091/mbc.11.2.511

Sekiya M, Hiraishi A, Touyama M, Sakamoto K. Oxidative stress induced lipid accumulation via SREBP1c activation in HepG2 cells. Biochem Biophys Res Commun. 2008 Oct 31;375(4):602-7. doi: 10.1016/j.bbrc.2008.08.068

Selman C, Lingard S, Choudhury AI, Batterham RL, Claret M, Clements M, Ramadani F, Okkenhaug K, Schuster E, Blanc E, Piper MD, Al-Qassab H, Speakman JR, Carmignac D, Robinson IC, Thornton JM, Gems D, Partridge L, Withers DJ. Evidence for lifespan extension and delayed age-related biomarkers in insulin receptor substrate 1 null mice. FASEB J. 2008 Mar;22(3):807-18. doi: 10.1096/fj.07-9261com

Senf SM, Sandesara PB, Reed SA, Judge AR. p300 Acetyltransferase activity differentially regulates the localization and activity of the FOXO homologues in skeletal muscle. Am J Physiol Cell Physiol. 2011 Jun;300(6):C1490-501. doi: 10.1152/ajpcell.00255.2010

Shavva VS, Bogomolova AM, Nikitin AA, Dizhe EB, Tanyanskiy DA, Efremov AM, Oleinikova GN, Perevozchikov AP, Orlov SV. Insulin-Mediated Downregulation of Apolipoprotein A-I Gene in Human Hepatoma Cell Line HepG2: The Role of Interaction Between FOXO1 and LXRβ Transcription Factors. J Cell Biochem. 2017 Feb;118(2):382-396. doi: 10.1002/jcb.25651

Shen Y, Xu W, You H, Su D, Xing J, Li M, Li L, Liang X. FoxO1 inhibits transcription and membrane trafficking of epithelial Na+ channel. J Cell Sci. 2015 Oct 1;128(19):3621-30. doi: 10.1242/jcs.171876

Shimokawa I, Komatsu T, Hayashi N, Kim SE, Kawata T, Park S, Hayashi H, Yamaza H, Chiba T, Mori R. The life-extending effect of dietary restriction requires Foxo3 in mice. Aging Cell. 2015 Aug;14(4):707-9. doi: 10.1111/acel.12340

Sieber MH, Spradling AC. Steroid Signaling Establishes a Female Metabolic State and Regulates SREBP to Control Oocyte Lipid Accumulation. Curr Biol. 2015 Apr 20;25(8):993-1004. doi: 10.1016/j.cub.2015.02.019

Singh A, Ye M, Bucur O, Zhu S, Tanya Santos M, Rabinovitz I, Wei W, Gao D, Hahn WC, Khosravi-Far R. Protein phosphatase 2A reactivates FOXO3a through a dynamic interplay with 14-3-3 and AKT. Mol Biol Cell. 2010 Mar 15;21(6):1140-52. doi: 10.1091/mbc.e09-09-0795

Skorupa DA, Dervisefendic A, Zwiener J, Pletcher SD. Dietary composition specifies consumption, obesity, and lifespan in Drosophila melanogaster. Aging Cell. 2008 Aug;7(4):478-90. doi: 10.1111/j.1474-9726.2008.00400.x

Slack C, Giannakou ME, Foley A, Goss M, Partridge L. dFOXO-independent effects of reduced insulin-like signaling in Drosophila. Aging Cell. 2011 Oct;10(5):735-48. doi: 10.1111/j.1474-9726.2011.00707.x

Slade JD, Staveley BE. Enhanced survival of Drosophila Akt1 hypomorphs during amino-acid starvation requires foxo. Genome. 2016 Feb;59(2):87-93. doi: 10.1139/gen-2015-0113

Stein GM, Murphy CT. The Intersection of Aging, Longevity Pathways, and Learning and Memory in C. elegans. Front Genet. 2012 Nov 26;3:259. doi: 10.3389/fgene.2012.00259

Sundaresan NR, Gupta M, Kim G, Rajamohan SB, Isbatan A, Gupta MP. Sirt3 blocks the cardiac hypertrophic response by augmenting Foxo3a-dependent antioxidant defense mechanisms in mice. J Clin Invest. 2009 Sep;119(9):2758-71. doi: 10.1172/JCI39162

Supek F, Bošnjak M, Škunca N, Šmuc T. REVIGO summarizes and visualizes long lists of gene ontology terms. PLoS One. 2011;6(7):e21800. doi: 10.1371/journal.pone.0021800

Tao R, Xiong X, DePinho RA, Deng CX, Dong XC. Hepatic SREBP-2 and cholesterol biosynthesis are regulated by FoxO3 and Sirt6. J Lipid Res. 2013 Oct;54(10):2745-53. doi: 10.1194/jlr.M039339

Tatar M, Kopelman A, Epstein D, Tu MP, Yin CM, Garofalo RS. A mutant Drosophila insulin receptor homolog that extends life-span and impairs neuroendocrine function. Science. 2001 Apr 6;292(5514):107-10. doi: 10.1126/science.1057987

Teleman AA, Chen YW, Cohen SM. Drosophila Melted modulates FOXO and TOR activity. Dev Cell. 2005 Aug;9(2):271-81. doi: 10.1016/j.devcel.2005.07.004

Teleman AA, Hietakangas V, Sayadian AC, Cohen SM. Nutritional control of protein biosynthetic capacity by insulin via Myc in Drosophila. Cell Metab. 2008 Jan;7(1):21-32. doi: 10.1016/j.cmet.2007.11.010

Teleman AA. Molecular mechanisms of metabolic regulation by insulin in Drosophila. Biochem J. 2009 Dec 14;425(1):13-26. doi: 10.1042/BJ20091181

Teumer A, Qi Q, Nethander M, Aschard H, Bandinelli S, Beekman M, Berndt SI, Bidlingmaier M, Broer L; CHARGE Longevity Working Group; et al. Genomewide meta-analysis identifies loci associated with IGF-I and IGFBP-3 levels with impact on age-related traits. Aging Cell. 2016 Oct;15(5):811-24. doi: 10.1111/acel.12490

Tsai KL, Sun YJ, Huang CY, Yang JY, Hung MC, Hsiao CD. Crystal structure of the human FOXO3a-DBD/DNA complex suggests the effects of post-translational modification. Nucleic Acids Res. 2007;35(20):6984-94. doi: 10.1093/nar/gkm703

van der Vos KE, Coffer PJ. The extending network of FOXO transcriptional target genes. Antioxid Redox Signal. 2011 Feb 15;14(4):579-92. doi: 10.1089/ars.2010.3419

van Schaftingen E, Gerin I. The glucose-6-phosphatase system. Biochem J. 2002 Mar 15;362(Pt 3):513-32. doi: 10.1042/0264-6021:3620513

Vereshchagina N, Wilson C. Cytoplasmic activated protein kinase Akt regulates lipid-droplet accumulation in Drosophila nurse cells. Development. 2006 Dec;133(23):4731-5. doi: 10.1242/dev.02659

Vihervaara T, Puig O. dFOXO regulates transcription of a Drosophila acid lipase. J Mol Biol. 2008 Mar 7;376(5):1215-23. doi: 10.1016/j.jmb.2007.12.042

Villa-Cuesta E, Sage BT, Tatar M. A role for Drosophila dFoxO and dFoxO 5'UTR internal ribosomal entry sites during fasting. PLoS One. 2010 Jul 9;5(7):e11521. doi: 10.1371/journal.pone.0011521

Wang B, Moya N, Niessen S, Hoover H, Mihaylova MM, Shaw RJ, Yates JR 3rd, Fischer WH, Thomas JB, Montminy M. A hormone-dependent module regulating energy balance. Cell. 2011 May 13;145(4):596-606. doi: 10.1016/j.cell.2011.04.013

Wang F, Chan CH, Chen K, Guan X, Lin HK, Tong Q. Deacetylation of FOXO3 by SIRT1 or SIRT2 leads to Skp2-mediated FOXO3 ubiquitination and degradation. Oncogene. 2012 Mar 22;31(12):1546-57. doi: 10.1038/onc.2011.347

Wang MC, O'Rourke EJ, Ruvkun G. Fat metabolism links germline stem cells and longevity in C. elegans. Science. 2008 Nov 7;322(5903):957-60. doi: 10.1126/science.1162011

Wang Q, Wang N, Dong M, Chen F, Li Z, Chen Y. GdCl3 reduces hyperglycaemia through Akt/FoxO1-induced suppression of hepatic gluconeogenesis in Type 2 diabetic mice. Clin Sci (Lond). 2014 Jul;127(2):91-100. doi: 10.1042/CS20130670

Wang X, Hu S, Liu L. Phosphorylation and acetylation modifications of FOXO3a: Independently or synergistically? Oncol Lett. 2017 May;13(5):2867-2872. doi: 10.3892/ol.2017.5851

Wang YQ, Cao Q, Wang F, Huang LY, Sang TT, Liu F, Chen SY. SIRT1 Protects Against Oxidative Stress-Induced Endothelial Progenitor Cells Apoptosis by Inhibiting FOXO3a via FOXO3a Ubiquitination and Degradation. J Cell Physiol. 2015 Sep;230(9):2098-107. doi: 10.1002/jcp.24938

Wang Z, Yu T, Huang P. Post-translational modifications of FOXO family proteins (Review). Mol Med Rep. 2016 Dec;14(6):4931-4941. doi: 10.3892/mmr.2016.5867

Webb AE, Brunet A. FOXO transcription factors: key regulators of cellular quality control. Trends Biochem Sci. 2014 Apr;39(4):159-69. doi: 10.1016/j.tibs.2014.02.003

Webb AE, Kundaje A, Brunet A. Characterization of the direct targets of FOXO transcription factors throughout evolution. Aging Cell. 2016 Aug;15(4):673-85. doi: 10.1111/acel.12479

Weigel D, Jürgens G, Küttner F, Seifert E, Jäckle H. The homeotic gene fork head encodes a nuclear protein and is expressed in the terminal regions of the Drosophila embryo. Cell. 1989 May 19;57(4):645-58. doi: 10.1016/0092-8674(89)90133-5

WHO, Ageing and health, https://www.who.int/news-room/fact-sheets/detail/ageing-and-health.

Willcox BJ, Tranah GJ, Chen R, Morris BJ, Masaki KH, He Q, Willcox DC, Allsopp RC, Moisyadi S, Poon LW, Rodriguez B, Newman AB, Harris TB, Cummings SR, Liu Y, Parimi N, Evans DS, Davy P, Gerschenson M, Donlon TA. The FoxO3 gene and cause-specific mortality. Aging Cell. 2016 Aug;15(4):617-24. doi: 10.1111/acel.12452

Wu Z, Isik M, Moroz N, Steinbaugh MJ, Zhang P, Blackwell TK. Dietary Restriction Extends Lifespan through Metabolic Regulation of Innate Immunity. Cell Metab. 2019 May 7;29(5):1192-1205.e8. doi: 10.1016/j.cmet.2019.02.013

Wullschleger S, Loewith R, Hall MN. TOR signaling in growth and metabolism. Cell. 2006 Feb 10;124(3):471-84. doi: 10.1016/j.cell.2006.01.016

Xiong S, Salazar G, Patrushev N, Alexander RW. FoxO1 mediates an autofeedback loop regulating SIRT1 expression. J Biol Chem. 2011 Feb 18;286(7):5289-99. doi: 10.1074/jbc.M110.163667

Xiong X, Tao R, DePinho RA, Dong XC. Deletion of hepatic FoxO1/3/4 genes in mice significantly impacts on glucose metabolism through downregulation of gluconeogenesis and upregulation of glycolysis. PLoS One. 2013 Aug 28;8(8):e74340. doi: 10.1371/journal.pone.0074340

Xiong X, Tao R, DePinho RA, Dong XC. The autophagy-related gene 14 (Atg14) is regulated by forkhead box O transcription factors and circadian rhythms and plays a critical role in hepatic autophagy and lipid metabolism. J Biol Chem. 2012 Nov 9;287(46):39107-14. doi: 10.1074/jbc.M112.412569

Xu M, Pirtskhalava T, Farr JN, Weigand BM, Palmer AK, Weivoda MM, Inman CL, Ogrodnik MB, Hachfeld CM, Fraser DG, Onken JL, Johnson KO, Verzosa GC, Langhi LGP, Weigl M, Giorgadze N, LeBrasseur NK, Miller JD, Jurk D, Singh RJ, Allison DB, Ejima K, Hubbard GB, Ikeno Y, Cubro H, Garovic VD, Hou X, Weroha SJ, Robbins PD, Niedernhofer LJ, Khosla S, Tchkonia T, Kirkland JL.

Senolytics improve physical function and increase lifespan in old age. Nat Med. 2018 Aug;24(8):1246-1256. doi: 10.1038/s41591-018-0092-9

Yamada T, Habara O, Kubo H, Nishimura T. Fat body glycogen serves as a metabolic safeguard for the maintenance of sugar levels in Drosophila. Development. 2018 Mar 14;145(6):dev158865. doi: 10.1242/dev.158865

Yamamura Y, Lee WL, Inoue K, Ida H, Ito Y. RUNX3 cooperates with FoxO3a to induce apoptosis in gastric cancer cells. J Biol Chem. 2006 Feb 24;281(8):5267-76. doi: 10.1074/jbc.M512151200

Yamaza H, Komatsu T, Wakita S, Kijogi C, Park S, Hayashi H, Chiba T, Mori R, Furuyama T, Mori N, Shimokawa I. FoxO1 is involved in the antineoplastic effect of calorie restriction. Aging Cell. 2010 Jun;9(3):372-82. doi: 10.1111/j.1474-9726.2010.00563.x

Yan L, Lavin VA, Moser LR, Cui Q, Kanies C, Yang E. PP2A regulates the pro-apoptotic activity of FOXO1. J Biol Chem. 2008 Mar 21;283(12):7411-20. doi: 10.1074/jbc.M708083200

Yang Y, Li W, Liu Y, Sun Y, Li Y, Yao Q, Li J, Zhang Q, Gao Y, Gao L, Zhao J. Alpha-lipoic acid improves high-fat diet-induced hepatic steatosis by modulating the transcription factors SREBP-1, FoxO1 and Nrf2 via the SIRT1/LKB1/AMPK pathway. J Nutr Biochem. 2014 Nov;25(11):1207-1217. doi: 10.1016/j.jnutbio.2014.06.001

Yeagley D, Guo S, Unterman T, Quinn PG. Gene- and activation-specific mechanisms for insulin inhibition of basal and glucocorticoid-induced insulin-like growth factor binding protein-1 and phosphoenolpyruvate carboxykinase transcription. Roles of forkhead and insulin response sequences. J Biol Chem. 2001 Sep 7;276(36):33705-10. doi: 10.1074/jbc.M101215200

Yu H, Fellows A, Foote K, Yang Z, Figg N, Littlewood T, Bennett M. FOXO3a (Forkhead Transcription Factor O Subfamily Member 3a) Links Vascular Smooth Muscle Cell Apoptosis, Matrix Breakdown, Atherosclerosis, and Vascular Remodeling Through a Novel Pathway Involving MMP13 (Matrix Metalloproteinase 13). Arterioscler Thromb Vasc Biol. 2018 Mar;38(3):555-565. doi: 10.1161/ATVBAHA.117.310502

Yuan Z, Becker EB, Merlo P, Yamada T, DiBacco S, Konishi Y, Schaefer EM, Bonni A. Activation of FOXO1 by Cdk1 in cycling cells and postmitotic neurons. Science. 2008 Mar 21;319(5870):1665-8. doi: 10.1126/science.1152337

Zhang J, Li X, Yu H, Larre I, Dube PR, Kennedy DJ, Tang WHW, Westfall K, Pierre SV, Xie Z, Chen Y. Regulation of Na/K-ATPase expression by cholesterol: isoform specificity and the molecular mechanism. Am J Physiol Cell Physiol. 2020 Dec 1;319(6):C1107-C1119. doi: 10.1152/ajpcell.00083.2020

Zhang W, Patil S, Chauhan B, Guo S, Powell DR, Le J, Klotsas A, Matika R, Xiao X, Franks R, Heidenreich KA, Sajan MP, Farese RV, Stolz DB, Tso P, Koo SH, Montminy M, Unterman TG. FoxO1 regulates multiple metabolic pathways in the liver: effects on gluconeogenic, glycolytic, and lipogenic gene expression. J Biol Chem. 2006 Apr 14;281(15):10105-17. doi: 10.1074/jbc.M600272200

Zhang WB, Aleksic S, Gao T, Weiss EF, Demetriou E, Verghese J, Holtzer R, Barzilai N, Milman S. Insulin-like Growth Factor-1 and IGF Binding Proteins Predict All-Cause Mortality and Morbidity in Older Adults. Cells. 2020 Jun 1;9(6):1368. doi: 10.3390/cells9061368

Zhang X, Gan L, Pan H, Guo S, He X, Olson ST, Mesecar A, Adam S, Unterman TG. Phosphorylation of serine 256 suppresses transactivation by FKHR (FOXO1) by multiple mechanisms. Direct and indirect effects on nuclear/cytoplasmic shuttling and DNA binding. J Biol Chem. 2002 Nov 22;277(47):45276-84. doi: 10.1074/jbc.M208063200

Zhao P, Huang P, Xu T, Xiang X, Sun Y, Liu J, Yan C, Wang L, Gao J, Cui S, Wang X, Zhan L, Song H, Liu J, Song W, Liu Y. Fat body Ire1 regulates lipid homeostasis through the Xbp1s-FoxO axis in Drosophila. iScience. 2021 Jul 7;24(8):102819. doi: 10.1016/j.isci.2021.102819

Zhao X, Gan L, Pan H, Kan D, Majeski M, Adam SA, Unterman TG. Multiple elements regulate nuclear/cytoplasmic shuttling of FOXO1: characterization of phosphorylation- and 14-3-3-dependent and -independent mechanisms. Biochem J. 2004 Mar 15;378(Pt 3):839-49. doi: 10.1042/BJ20031450

Zhao Y, Yang J, Liao W, Liu X, Zhang H, Wang S, Wang D, Feng J, Yu L, Zhu WG. Cytosolic FoxO1 is essential for the induction of autophagy and tumour suppressor activity. Nat Cell Biol. 2010 Jul;12(7):665-75. doi: 10.1038/ncb2069

Zhu Y, Tchkonia T, Pirtskhalava T, Gower AC, Ding H, Giorgadze N, Palmer AK, Ikeno Y, Hubbard GB, Lenburg M, O'Hara SP, LaRusso NF, Miller JD, Roos CM, Verzosa GC, LeBrasseur NK, Wren JD, Farr JN, Khosla S, Stout MB, McGowan SJ, Fuhrmann-Stroissnigg H, Gurkar AU, Zhao J, Colangelo D, Dorronsoro A, Ling YY, Barghouthy AS, Navarro DC, Sano T, Robbins PD, Niedernhofer LJ, Kirkland JL. The Achilles' heel of senescent cells: from transcriptome to senolytic drugs. Aging Cell. 2015 Aug;14(4):644-58. doi: 10.1111/acel.12344

Zimmermann R, Strauss JG, Haemmerle G, Schoiswohl G, Birner-Gruenberger R, Riederer M, Lass A, Neuberger G, Eisenhaber F, Hermetter A, Zechner R. Fat mobilization in adipose tissue is promoted by adipose triglyceride lipase. Science. 2004 Nov 19;306(5700):1383-6. doi: 10.1126/science.1100747

Contributions

Genomic engineering of the *dfoxo* locus and generation of dFOXO mutant lines (*foxo-null,* FLAG, DBD2, 5KQ, 5KR) was done by Dr. Victor Bustos. Partial mutant flies dFOXO-2KQ, -3KQ and -3KQII were generated by microinjection by Jacqueline Eßer. Mass spectrometry was performed in the Proteomics Facility of the Max Planck Institute for Biology of Ageing and the analysis was carried out by Dr. Ilian Atanassov. Research presented here was performed under supervision of Prof. Dr. Linda Partridge and Dr. Sebastian Grönke.

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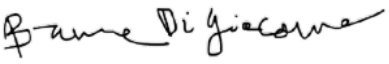
16.03.2023, Bruna Di Giacomo, 

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