Functional analysis of the role of FOXO in ageing and metabolism in *Drosophila melanogaster*

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Victor Bustos
You cannot avoid making mistakes, so you better learn from them.

ABBREVIATIONS

Δ	null mutants
4E-BP	4 Eukaryotic initiantion factor Binding Protein
4xFRE	4xFOXO responsive element
AA	Amino acid
AcCoAS	Acetyl Coenzyme A synthase
AMPK	AMP-activated protein kinase
atg	Autophagy related gene
ATGL	Adipose Triglyceride lipase
BLRP	Biotin ligase recognition peptide
bmm	brummer
CBP	CREB binding protein
Cdk1	Cyclin-dependent kinase 1
ChIP	Chromatin Immunoprecipitation
colt	congested-like trachea
CREB	cAMP-response element-binding protein
da	daugtherless
Dah	Dahomey
DBD	DNA binding domain
dHR96	Hormone receptor-like in 96
DILP	Drosophila ILP
DNA	Deoxyribonucleic acid
FB	Fat body
FFA	Free fatty acids
FLP	Flippase
FOXO	Forkhead Box O
FRT	Flippase recognition target
G6Pase	Glucose-6-phosphatase
GcK	Glucose carboxykinase
HDAC	Histone deacetylase
hid	head involution defective
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
IRES	Internal ribosomal entry site
IRS	Insulin receptor substrate
JNK	Jun-N-terminal kinase
Lip3	Lipase 3
Lip4	Lipase 4
Lipa	Lysosomal acid lipase
min	minute
mTORC1	mammalian TOR complex 1

NES	Nuclear exclusion signal
NHR	Nuclear hormone receptor
NID	NHR interacting domain
NLS	Nuclear localization signal
O-GlcNac	O-linked-N-acetylglucosamine
OGT	O-GlcNac transferase
P	Phosphate / phosphorylated residue
PDK1	Phosphoinositide-dependent kinase 1
pepck	Phosphoenolpyruvate carboxykinase
PH	Pleckstrin homology
PI3K	Phosphatidylinositol 3-OH kinase
PIP2	Phosphatidylinositol 3-phosphate
PIP3	Phosphatidylinositol 3-phosphate
PKB	Protein kinase B
PolyQ	Polyglutamine
PTM	Post-translational modification
qRT-PCR	quantitative Real Time Plymerase Chain Reaction
SGK1	Serum- and glucocorticoid-inducible kinase 1
Sirt	Sirtuin
TAG	Triacylglycerol
TNPO1	Transportin 1
TOR	Target of Rapamycin
Ups	Ultraspiricle
w	white
yip2	yippee interacting protein 2

ZUSAMMENFASSUNG

Eine verringerte Aktivität des evolutionär hochkonservierten Insulin/Insulin-ähnlichem Wachstumsfaktor-Signalweges (IIS) verlängert die Lebenszeit von Nematoden, Fruchtfliegen und Mäusen. In Nematoden und Fruchtfliegen ist der Forkhead Box-O (FOXO) Transkriptionsfaktor notwendig für den lebensverlängernden Effekt durch verringerten IIS, und auch erhöhte FOXO Aktivität alleine verlängert das Leben. FOXO Transkriptionsfaktoren sind jedoch nicht nur am Alterungsprozess beteiligt, sondern auch an der Entwicklung und dem Stoffwechsel eines Organismus. Daher ist es essentiell zu verstehen, durch welche molekularen Mechanismen der FOXO Transkriptionsfaktor diese Prozesse steuert.

In meiner Doktorarbeit habe ich mithilfe der Fruchtfliege *Drosophila melanogaster* untersucht, wie die Aktivität von FOXO reguliert wird und welche unterschiedlichen Funktionen dFOXO hat. Ich habe neue *dfoxo-null* Mutanten generiert, mit denen ich unterschiedliche Modifikationen von *dfoxo* in das Genom inserieren konnte. Insertion des Wildtyp-dFOXOs oder von dFOXO mit FLAG-Tag am C-Terminus hatte keinen Effekt auf die Funktion von dFOXO und wurde verwendet, um die *in vivo* Funktion von dFOXO zu charakterisieren. Außerdem habe ich *dfoxo* Allele generiert, die das Binden von dFOXO an DNA *in vivo* verhindern. Diese Mutanten erlaubten die Separierung von dFOXO Funktionen, die abhängig (Fertilität, Resistenz gegen oxidativen Stress und Lebensspanne) und unabhängig (Gewicht und Lipid-Verbrauch während Hungerperioden) von der Interaktion von dFOXO mit der DNA sind. Unsere Ergebnisse weisen darauf hin, dass dFOXO die Genexpression und Autophagie in Hungerperioden unabhängig von der Fähigkeit an DNA zu binden moduliert, vermutlich durch die Interaktion mit einem anderen Transkriptionsfaktor.

Zudem habe ich *dfoxo* Mutanten erzeugt, die Acetylierung an einem konservierten Lysin-Rest imitieren oder verhindern. Unsere Experimente lassen vermuten, dass diese posttranslationalen Modifikationen auch den Phosphorylierungs-Status von dFOXO beeinflussen. Außerdem scheint dFOXO-Acetylierung eine wichtige Rolle in der Antwort auf den Entzug von Aminosäuren zu spielen, jedoch müssen weitere Experimente den verantwortlichen Mechanismus identifizieren.

Zusammenfassend beschreibt diese Doktorarbeit ein neues Genome Editing-Werkzeug zur Modifikation des *dfoxo* locus und separiert wichtige Funktionen des FOXO Transkriptionsfaktors in *Drosophila melanogaster.*

SUMMARY

Reduced activity of the highly evolutionarily conserved insulin/insulin-like growth factor signalling pathway (IIS) has been shown to increase lifespan in nematode worms, fruit flies and mice. In both worms and flies the single *Forkhead Box-O* (FOXO) transcription factor is required for increased lifespan from reduced IIS, and increased FOXO activity itself lengthens life. However, FOXO transcription factors are involved in the regulation of diverse cellular and organismal processes besides ageing, including development and metabolism. It is therefore of crucial importance to understand the molecular mechanisms by which this transcription factor acts to regulate those processes.

During my PhD, I used the fruit fly *Drosophila melanogaster* to investigate how FOXO activity is regulated and tried to dissect the different functions associated with dFOXO. I have generated novel *dfoxo-null* mutant lines that allow me to reintroduce modified versions of the *dfoxo* gene. Wild type and C-terminal tagged reinsertion lines seem to have no effect on normal dFOXO function and, hence, were used to better characterize dFOXO regulation *in vivo*. Next, I generated *dfoxo* mutant alleles that abolished DNA binding *in vivo*. dFOXO-DBD mutant flies permitted the separation of dFOXO-functions that are dependent (fecundity, redox stress resistance, and lifespan) or independent (body weight and lipid usage under starvation) of dFOXO-DNA binding. Our results suggest that dFOXO modulates gene expression and autophagy under starvation conditions independent of DNA binding, probably through the interaction with another transcription factor.

On the other hand, I generated *dfoxo* alleles that either mimic or abolish acetylation within conserved lysine residues. Our results indicate that this post-translational modification regulates dFOXO-phosphorylation state. Moreover, dFOXO-acetylation seems to play a crucial role in the response associated with amino acid starvation. However, further studies should identify the mechanisms at play.

In conclusion, this PhD thesis describes a gene-editing tool for the *dfoxo* locus and separates some of the important functions associated with FOXO transcription factors in *Drosophila*.

INTRODUCTION

1.1 AGEING AND THE INSULIN SIGNALLING PATHWAY

The idea of immortality has intrigued humans for thousands of years. Whether it was alchemists trying to create the philosophers' stone, explorers trying to find the fountain of youth, or the promise of an afterlife by most religions, the idea of eternal live has shaped humanity. For a long time ageing has been regarded as an immutable process due to damage accumulation over time that leads to functional decline and finally death. However, this view was challenged by the identification of a mutation in a single gene called *daf-2* in the roundworm *Caenorhabditis elegans* (*C. elegans*), which resulted in worms that lived twice as long as their wild type counterparts (Kenyon et al., 1993). This observation changed our entire perspective of the ageing 'process' and led to the conclusion that longevity is amenable to genetic interventions, just like other biological processes.

Daf-2 encodes the *C. elegans* homolog of the insulin/insulin-like growth factor (IGF) receptor (Kimura et al., 1997), an upstream component of the insulin/IGF-signalling (IIS) pathway. Similar to the worm *daf-2* mutants, reduced IIS can increase lifespan in other animal models, including fruit flies and mammals. In *Drosophila*, mutations of the insulin-like receptor (InR) extend lifespan (Tatar et al., 2001). Similarly, removal of InR in adipose tissue or full-body heterozygous mutants of the IGF-1 receptor (IGF-1R) in mice is able to extend lifespan when compared to their respective controls (Bluher, 2003; Holzenberger et al., 2003). Moreover, removal of the insulin receptor substrate in flies (*chico*) or mice (IRS1) extends lifespan and, at least in mice, it seems to cause a delay in ageing (Clancy et al., 2001; Selman et al., 2008; Tu et al., 2002). These observations make the IIS pathway a prominent target of ageing research. Importantly, the invertebrate models *C. elegans* and *Drosophila* are ideal to the study the relationship between IIS and ageing due to their short lifespan and the diverse molecular/genetic tools available (Piper et al., 2005).

The IIS pathway is an evolutionarily conserved nutrient-sensing network that plays key roles in diverse cellular and organismal processes, including growth, metabolism, stress response, reproduction and lifespan (Piper et al., 2005). IIS is activated by insulin-like peptides (ILPs), hormonal signals secreted, for example, in response to nutrient availability. Mammalian genomes code for at least eight ILPs (Wallis, 2009), including insulin and insulin-like growth factor-1 (IGF-1). In worms, 40 ILPs have been identified,

whereas flies express at least eight peptides (Lau and Chalasani, 2014). Interestingly, IIS manipulation at the ligand level can also extend lifespan in flies, as mutation of the *Drosophila* ILP (DILP) 2, or the triple *dilp2-3,5* mutation, produces longer lived flies (Grönke et al., 2010). Unlike worms and flies, where all ILPs seem to transduce the signal through the single insulin-like receptor (the aforementioned Daf-2 and InR), mammals have multiple IIS-related receptors that, in most cases, bind a particular ILP with higher affinity than the rest (Fernandez and Torres-Alemán, 2012). Despite the differences, insulin or ILPs regulation by a favourable nutritional status has similar consequences in different organisms, inducing growth by stimulating protein synthesis, and promoting energy storage in the form of glycogen and fat (Piper et al., 2005).

Under nutrient availability, circulating insulin interacts with the insulin receptor, which activates a complex phosphorylation cascade (Figure 1.1) that leads to, among others, glucose uptake by the muscle and fat body, while at the same time inhibiting glucose production by the liver (Boucher et al., 2014; Saltiel and Kahn, 2001). In simplified terms, insulin promotes a conformational change in the InR that allows auto-phosphorylation and phosphorylation of at least nine different targets, including a group of four proteins known as insulin receptor substrates (IRS1-4) (Patti and Kahn, 1998). In *Drosophila*, there is only one homolog protein of IRS (*chico*) (Clancy et al., 2001). When phosphorylated, IRS1 functions as a scaffold and allows the interaction with several regulatory proteins, most importantly, Phosphatidylinositol 3-OH kinase (PI3K). Activated PI3K in turn catalyzes the conversion of phosphatidylinositol 2-phosphate (PIP2) to phosphatidylinositol 3-phosphate (PIP3) (Vadas et al., 2011).

Proteins baring a pleckstrin homology (PH) domain can interact with PIP3 and hence be recruited to the cell membrane. This translocation to the membrane activates a series of kinases, of which Phosphoinositide-dependent kinase 1 (PDK1) is best characterized. The interaction between PIP3 and PDK1 leads to the phosphorylation and therefore activation of another fundamental kinase called protein kinase B (PKB or AKT). Importantly, AKT also contains a PH domain, which facilitates its localization to the membrane and therefore activation by PDK1 upon insulin signaling. Among its many targets, AKT phosphorylates the family of transcription factors Forkhead Box O (FOXO). The AKT dependent phosphorylation of FOXO proteins on three highly conserved residues induces their nuclear exclusion, in part by facilitating the interaction with scaffold protein 14-3-3,

and hence the down regulation of FOXO-dependent target genes (Piper et al., 2005). In contrast, under reduced nutrient availability, IIS is reduced, repression of FOXO factors is released and a plethora of target genes are therefore activated (Figure 1.1). While mammalian genomes encode for four different FOXO genes (*FOXO1*, *3*, *4* and *6*), invertebrate organisms like *C. elegans* and *Drosophila* contain only one homolog, termed *daf16* and *dfoxo,* respectively (Jünger et al., 2003; Lin et al., 1997; Ogg et al., 1997; Puig et al., 2003).

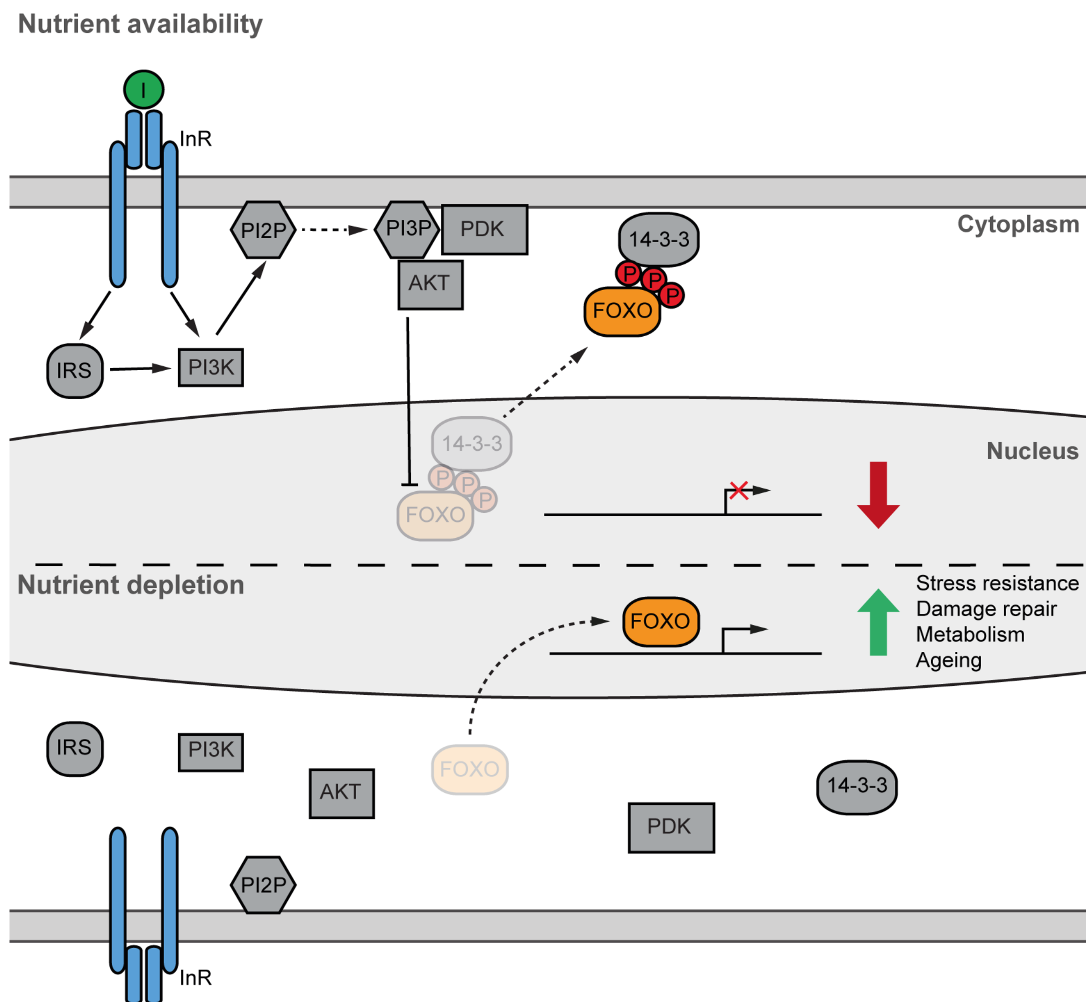

Figure 1.1 Simplified representation of the highly conserved Insulin/Insulin-like growth factor signalling (IIS) pathway. Under nutrient availability (fed state), secreted insulin initiates a phosphorylation cascade that culminates in the repression of FOXO transcription factors by nuclear exclusion. On the other hand, under reduced nutrients (starved state), FOXO repression is released and transcription of multiple target genes can be initiated.

1.2. FOXO TRANSCRIPTION FACTORS

FOXO coding genes were first identified as chromosomal translocations associated with different kinds of cancer, i.e. the genomic fusions between PAX3 and FOXO1 was described in alveolar rhabdomyosarcomas (Galili et al., 1993). Multiple studies have now proven that FOXO proteins are in fact powerful tumour suppressors (Fu and Tindall, 2008).

FOXO proteins belong to the Forkhead family of transcription factors characterized by their evolutionary conserved ~100-amino-acid monomeric DNA binding domain (DBD) (Burgering, 2008) (Figure 1.2A). The DBD, also called *winged helix* domain, is a variant of the helix-turn-helix motif that consists of three α-helices and two loops or ‘butterfly like wings’ (Brent et al., 2008; Weigelt et al., 2001) (Figure 1.2B). Over 100 proteins belong to the Forkhead family and are classified according to the sequence similarity of their DBDs, into sub-categories denoted by a capital letter (Kaestner et al., 2000), e.g. *Forkhead box subgroup O* → *FOXO*.

FOXO proteins also have a characteristic lysine-rich nuclear localization signal (NLS) embedded at the 5’ end of their DBD (Brownawell et al., 2001; Zhang et al., 2002). Of note, a key residue phosphorylated by AKT is part of the NLS and it mediates the interaction with 14-3-3 (Obsilova et al., 2005) (Figure 1.2A). This phosphorylation-dependent interaction masks the NLS sequence from the nuclear import machinery and allows FOXO shuffling to the cytoplasm.

In addition to the NLS, mammalian FOXO1, 3 and 4 have a leucine-rich nuclear exclusion signal (NES) that, just like the AKT-phosphorylation/interaction with 14-3-3, is required to mediate FOXO translocation to the cytoplasm under high nutrient availability (Brunet et al., 2002). Interestingly, neither DAF-16 nor dFOXO seem to have a conserved NES, suggesting that FOXO shuttling out of the nucleus is NES-independent in those species. Similarly, mammalian FOXOs contain a conserved LXXLL motif that mediates interaction with Sirt1 (Nakae et al., 2006) and, possibly, nuclear hormone receptors (NHRs), however, this motif seems to be absent in flies and worms (van der Vos and Coffer, 2008).

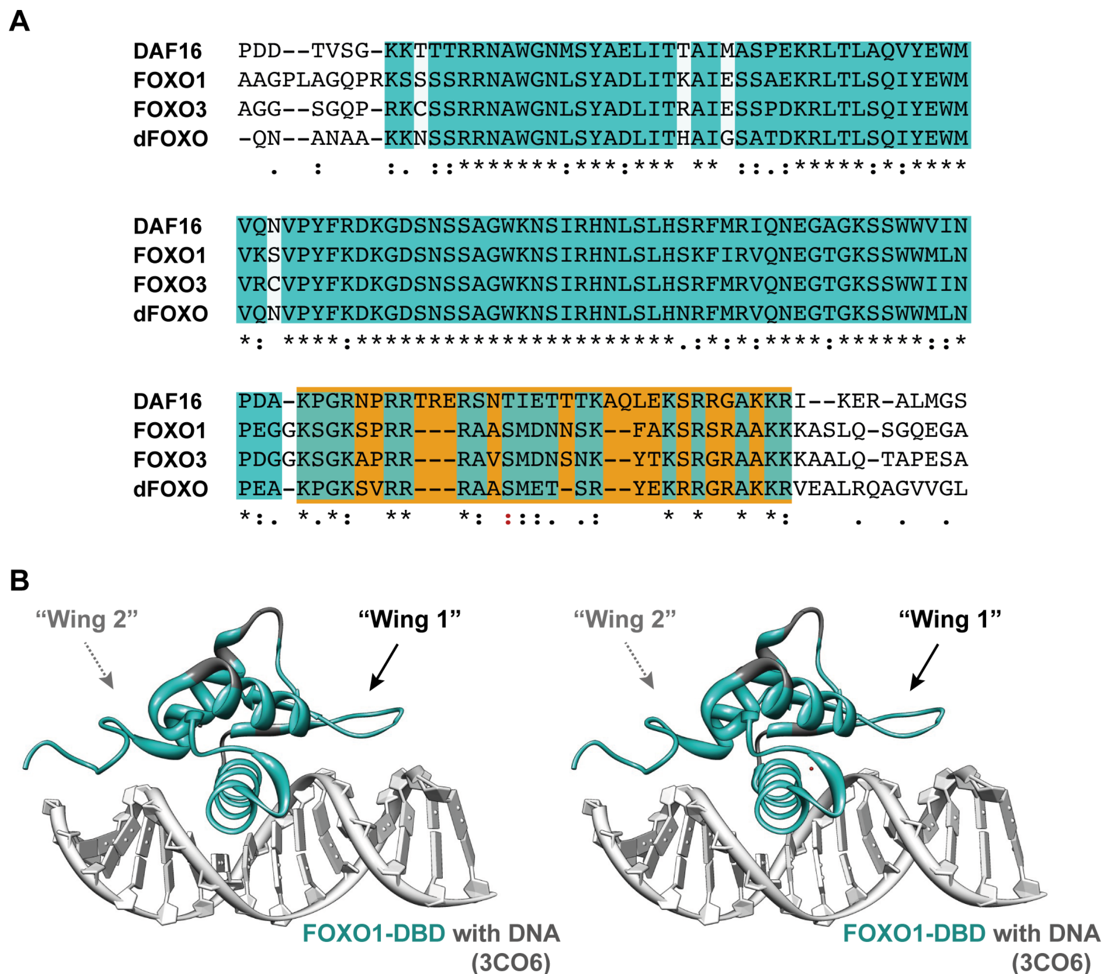

Figure 1.2 The FOXO DNA binding domain (DBD) is conserved in evolution.
(A) Amino acid alignment of the DBD of four different FOXO proteins using *MUSCLE* (Edgar, 2004). Residues highlighted in blue show some degree of conservation between all species. Orange residues represent the partially conserved nuclear localization signal (NLS). Consensus symbols indicate the degree of conservation: an * (asterisk) indicates perfect conservation whereas a : (colon) or a . (Period) indicate conservation of stronger or weaker similar properties. Within the NLS, the red colon (:) highlights the conserved residue phosphorylated by AKT. **(B)** Crystal structure of FOXO1-DBD in contact with DNA. Blue structure covers evolutionarily conserved residues, as in the alignment. "Wing 2" points at the position wing 2 would be, since it was not part of the crystal structure. Displayed as stereo pair – PDB code 3CO6 (Brent et al., 2008).

Unlike other FOXO paralogs, *Drosophila* FOXO (dFOXO) has *polyglutamine* (polyQ) *repeats* within its C-terminal domain. PolyQ regions have been well studied in the context of human neurodegenerative diseases (Orr and Zoghbi, 2007). However, we now know that eukaryotic transcription factors are rich in polyQ repeats (Gemayel et al., 2010) and that these repeats modulate the transcription factor activity by tuning its solubility and interactions (Gemayel et al., 2015). These polyQ regions can mediate interaction with other polyQ regions, facilitating co-regulation between transcription factors (Atanesyan et

al., 2012). How the polyQ regions within dFOXO modulate its activity or interactions is still unknown.

In mammals, FOXO proteins are expressed throughout the body, regulate different tissue-specific functions (Salih and Brunet, 2008) and several cellular processes such as apoptosis, stress response and cell cycle (Burgering, 2008). For example, FOXO1 is a key component of energy metabolism and is predominantly expressed in white adipose tissue, liver and muscle (Gross et al., 2008; Kousteni, 2012; Matsumoto et al., 2007). Additionally, FOXO proteins modulate immune function (Kim et al., 2013; Ouyang et al., 2012) and play important roles in stem cell maintenance in diverse tissues including muscle, neurons and the hematopoietic stem cell pool (Gopinath et al., 2014; Miyamoto et al., 2007; Paik et al., 2009). How exactly FOXO proteins coordinate such a vast range of processes is still largely unknown.

Due to the high degree of conservation, FOXO paralogs virtually share identical DBDs, allowing them to bind the same DNA motifs and therefore have, at least partially, redundant functions. A recent meta-analysis of FOXO-target-genes across tissues found that, even though specific FOXO proteins have tissue specific targets, there is a core set of genes regulated across tissues, and even species (Webb et al., 2016). These conserved target genes are involved in metabolism, proteostasis, stress resistance and growth factor signalling (Webb et al., 2016). This observation indicates that regulation of those core processes, which are fundamental for ageing modulation, is the main function of FOXO factors across evolution.

The presence of only one FOXO orthologue makes *C. elegans* and *Drosophila* ideal model systems to study FOXO function independent of redundancy between FOXO paralogues. In both organisms, FOXO factors are mediators of IIS action, and the removal of FOXO function suppresses lifespan-extension upon reduced IIS (Kenyon et al., 1993; Slack et al., 2011; Yamamoto and Tatar, 2011). Moreover, *foxo-null* mutants are short-lived in worms and flies, indicating that FOXO proteins are required for normal ageing (Lin et al., 2001; Slack et al., 2011). In addition, DAF-16 over-expression is sufficient to extend lifespan in the worm (S T Henderson and Johnson, 2001). Similarly, muscle- or fat-specific over-expression of dFOXO increases longevity in flies (Demontis and Perrimon, 2010; Giannakou et al., 2004; Hwangbo et al., 2004), suggesting that tissue-

specific functions of dFOXO are important for regulating organismal features, including ageing. These observations are consistent with studies in *C. elegans* that highlight the gut, which also functions as adipose tissue, as a key factor for modulating ageing (Libina et al., 2003). Even though many of the downstream gene targets of FOXO proteins have been identified in multiple organisms (Webb et al., 2016) , the exact mechanisms by which FOXO factors modulate lifespan are unclear.

Both DAF-16 and dFOXO have different isoforms. In *C. elegans* there are three isoforms with distinct tissue specificity and impact on ageing (Kwon et al., 2010). In contrast, *Drosophila* has four isoforms that differ in their 5'UTR, which contains internal ribosomal entry sites (IRES) which are important to mediate translation of *dfoxo* under low nutritional conditions (Villa-Cuesta et al., 2010). Two of those four *dfoxo* transcripts produce a 613aa protein (the preferred version for transgene generation), whereas the other two produce a 10aa longer version. Whether these isoforms are preferentially expressed in specific tissues or the amino acid difference is biologically relevant is still unknown.

In summary, FOXO proteins are evolutionarily conserved factors that modulate the organismal response to different kinds of environmental stress. Indeed, FOXO proteins are now seen as fundamental mediators of homeostasis maintenance (Eijkelenboom and Burgering, 2013), even though the mechanisms by which these proteins regulate so many processes are still unclear.

1.3 FOXO activity is regulated by post-translational modifications

FOXO proteins are metabolic nodes where multiple pathways converge. Hence, it is perhaps not surprising that these proteins are regulated by a great number of post-translational modifications (PTMs) (Figure 1.3). In 2008, Calnan and Brunet proposed that different PTM combinations on FOXO could work as a 'code' to elicit a specific transcriptional output in response to diverse stimuli (Calnan and Brunet, 2008). FOXO PTMs include phosphorylation, methylation, acetylation, mono- and poly-ubiquitination, O-glycosylation and poly-ADPribosylation (Daitoku et al., 2011; Klotz et al., 2015; Zhao et al., 2011). However, it is not clear to what extent these different PTMs contribute to FOXO-dependent regulation of lifespan. Nevertheless, this *FOXO* code modulates target genes involved, in grand terms, in three kinds of biological processes: metabolism, stress response and cell proliferation/apoptosis (Calnan and Brunet, 2008).

1.3.1 FOXO phosphorylation

AKT-dependent phosphorylation of FOXO proteins takes place at three evolutionary conserved residues that are part of a motif recognized by this kinase (Figure 1.3). Even though AKT phosphorylation is the best characterized FOXO-PTM, FOXO factors are phosphorylated by a panoply of kinases (Klotz et al., 2015). For example, AKT works in concert with serum- and glucocorticoid-inducible kinase 1 (SGK1), a kinase also activated by PI3K, to phosphorylate at the three conserved residues and inactivate FOXO3a (Brunet et al., 2001) (Figure 1.3A). While AKT prefers to phosphorylate the FOXO3 residue S253, SGK1 prefers S315, suggesting that the combination of both enzymes is what allows full FOXO phosphorylation under high nutrient conditions (Brunet et al., 2001).

AKT/SGK1 phosphorylations do not only reduce DNA binding affinity but also induce the interaction with the scaffold protein 14-3-3. T32 and S253 phosphorylation mediates the 14-3-3 interaction, which changes and probably masks the NLS sequence within FOXO, since S253 is buried within the NLS (Brunet et al., 1999; Obsilova et al., 2005) (Figure 1.3A). Nuclear exclusion of FOXO1 by IGFR1 signalling seems to be 14-3-3 independent, suggesting that at least one additional mechanism can shuttle FOXO1 into the cytoplasm (Rena et al., 2001). On the other hand, *Drosophila* has an additional scaffold protein that modulates the AKT-phosphorylation of dFOXO, called Melted, which seems to recruit dFOXO to the cell membrane and hence allow dFOXO and AKT to be in close proximity under activated IIS (Teleman et al., 2005). Nevertheless, once in

the cytoplasm, phosphorylated FOXO can undergo ubiquitination followed by proteosomal degradation (Aoki et al., 2004; Huang et al., 2005; Matsuzaki et al., 2003; Plas and Thompson, 2003). What exactly leads to FOXO degradation or cytoplasmic retention is currently unclear.

Phosphorylation of FOXO proteins can, however, also induce nuclear localization and/or activation (Figure 1.3). For example, cyclin-dependent kinase 1 (Cdk1) phosphorylation induces FOXO1-dependent transcription and causes cell death in neurons (Yuan et al., 2008). In *Drosophila* cell culture, multiple kinases were shown to modulate dFOXO transcriptional activity (Mattila et al., 2008). However, it is currently unclear whether these phosphorylations actually take place *in vivo* and whether they have a physiological consequence. Additionally, FOXO proteins are phosphorylated by AMPK (AMP-activated protein kinase) and JNK (Jun-N-terminal kinase) in response to nutritional or oxidative stress respectively (Essers et al., 2004; E. L. Greer et al., 2007; Eric L. Greer et al., 2007). The control that these two kinases exert over FOXO seems to be evolutionarily conserved. Moreover, both AMPK and JNK contribute to the modulation of longevity in worms and flies, however, the exact mechanism behind this function are not fully understood (Biteau et al., 2011; Burkewitz et al., 2014).

1.3.2 FOXO acetylation

FOXO activity can also be regulated by acetylation and deacetylation of specific lysine residues. In fact, acetylation was shown to both activate (Motta et al., 2004; Perrot and Rechler, 2005) and repress (Frescas et al., 2005; Fukuoka et al., 2003; Jing et al., 2007; Matsuzaki et al., 2005; Mihaylova et al., 2011; Wang et al., 2011, 2007) FOXO proteins. The reasons for such discrepancies are currently under investigation, but one possible explanation is that activating-acetylation seems to occur at the carboxy-terminal region of the protein, whereas repressive-acetylation takes place close to and within the NLS. Of note, *Drosophila* does not have any lysine residues in its carboxy-terminal region, suggesting that this acetylation is not evolutionarily conserved. Of the two, repressive-acetylation is better understood. This process can reduce FOXO DNA-binding ability and increase its sensitivity to phosphorylation by AKT, which in turn results in reduced transcriptional activity (Brent et al., 2008; Brunet et al., 2004; Matsuzaki et al., 2005; Qiang et al., 2010).

In mammals, FOXO proteins are acetylated by the co-activators p300 and cAMP response element-binding protein (CREB)-binding protein (CBP) and deacetylated by Sirt1 (Matsuzaki et al., 2005). Additionally, class II histone deacetylases (HDACs) were shown to modulate FOXO acetylation status in both mice and flies in response to nutritional stress (Mihaylova et al., 2011; Wang et al., 2011).

A mouse knock-in study revealed a fundamental role of FOXO1 acetylation in the regulation of glucose and lipid metabolism. An acetylation-mimicking-FOXO1 allele is lethal during early development, whereas the acetylation-null allele has a distinct metabolic phenotype, where mice seem to relay mostly on lipids as an energy source (Banks et al., 2011). The putatively acetylated lysine residues are mostly localized within the NLS of FOXO factors and are evolutionarily conserved. Deacetylation of these conserved residues by HDAC4 was recently suggested to allow dFOXO transcriptional regulation of the starvation response (Wang et al., 2011). Furthermore, dFOXO and FOXO1 seem to be promptly acetylated upon re-feeding of the flies and mice respectively (Banks et al., 2011; Wang et al., 2011). On the other hand, the transcriptional co-factor KDM5 (also known as Lid) seems to also interact with HDAC4 to facilitate FOXO deacetylation in order to elicit a transcriptional response under oxidative stress (Liu et al., 2014). On the other hand, cell culture and xenograft experiments indicate that acetylated FOXO1 is able to interact with ATG7 (autophagy related gene 7) in the cytoplasm to induce autophagy under serum starvation or oxidative stress (Zhao et al., 2010). These observations suggest acetylation may regulate multiple functions of FOXO proteins. However, the precise role of FOXO regulation by acetylation, and its effect on lifespan, is currently unclear.

In summary, FOXO proteins are regulated by multiple PTMs, however, it is not clear how these modifications regulate each other *in vivo* nor the biological consequences of their interaction at the cell, tissue or organism level. Thus, it would be important to develop a tool that would allow the *in vivo* identification and characterization of FOXO PTMs.

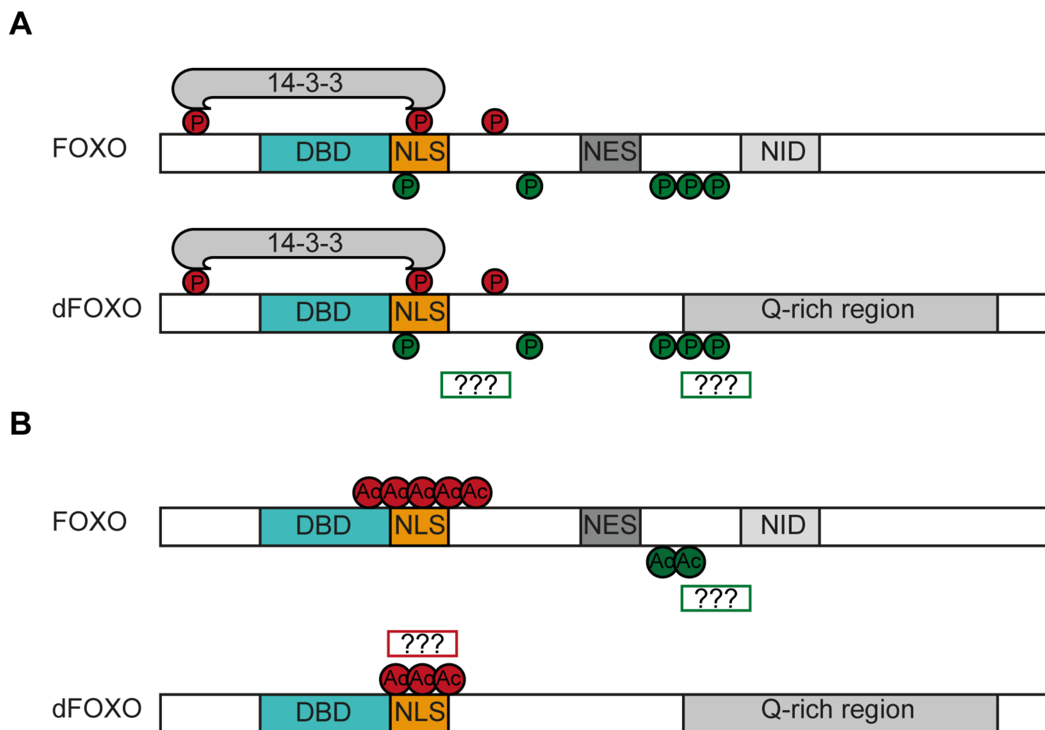

Figure 1.3 Phosphorylation and acetylation on mammalian FOXO compared to *Drosophila* dFOXO.

(A) Repressive-phosphorylation (red P) by AKT takes place at three evolutionarily conserved residues in both mammals and flies. Two of these residues mediate the interaction with scaffold protein 14-3-3. In contrast, many kinases are known to activate (green P) FOXO factors in mammals. Some of these kinases, including AMPK and JNK, can also activate *Drosophila* FOXO. However, the exact residues where the modifications take place are unknown. **(B)** Repressive acetylation of mammalian FOXO proteins was shown to take place at different lysine residues within the NLS. Since some of these residues are evolutionarily conserved, it was proposed that this kind of acetylation also happens in the fly. Conversely, activating-acetylation of FOXO proteins has only been reported in mammals. DBD-DNA binding domain; NLS-Nuclear localization signal; NES-Nuclear exclusion signal; NID-NHR interacting domain; Q-Glutamine rich region.

1.4 FOXO is regulated by protein-protein interactions

The best-characterised FOXO interactor is the scaffold protein 14-3-3. Under high insulin levels, AKT-dependent phosphorylated FOXO interacts with 14-3-3 and is therefore excluded from the nucleus (Figure 1.4A) (Brunet et al., 2002). However, multiple interaction partners are known to regulate FOXO function, while at the same time FOXO proteins are able to function as co-regulators and modulate the role of other transcription factors (Daitoku et al., 2011; van der Vos and Coffer, 2008).

In mammals, FOXO proteins have been shown to interact with a wide range of nuclear hormone receptors (NHRs), including the androgen, progesterone, glucocorticoid, retinoic acid, peroxisome and thyroid hormone receptors (van der Vos and Coffer, 2008). Interaction of FOXO with non-steroid receptors leads to co-activation, while biding to steroid NHRs leads to the opposite effect, co-repressing target genes (Zhao et al., 2001). This kind of interaction can lead to expression alteration of both NHR and/or FOXO target genes (Figure 1.4B). For example, binding of the androgen receptor can suppress FOXO1 transcriptional activity in prostate cancer cells (Li et al., 2003). Moreover, FOXO1 interacts with Hepatocyte Nuclear Factor-4 (HNF4) (Hirota et al., 2003). However, the consequences of this interaction seem to be complex. Under fasting conditions, FOXO binds to HNF4 and represses certain HNF4-target genes while at the same time it has a synergistic effect on HNF4 and FOXO1 shared target genes (Hirota et al., 2008). However, whether the repressive and/or synergistic interaction is evolutionarily conserved and the exact mechanism by which this kind of regulation takes place is currently unknown.

FOXO factors translocate to the nucleus and are hyper-activated under stressful conditions, such as starvation or oxidative stress (S T Henderson and Johnson, 2001). For example, it was recently reported in mammalian cell culture that FOXO4 is able to interact with a transporter called Transportin1 specifically under Redox stress (Putker et al., 2013). Of note, this interaction seems to be evolutionarily conserved since DAF-16 is able to interact with Transportin1, and its worm homolog IMB-2, to regulate this translocation (Putker et al., 2013) (Figure 1.4C). On the other hand, a study on a human cancer cell line showed that, upon oxidative stress or serum starvation, cytosolic FOXO1 lost the interaction with NAD-dependent histone deacetylase*,* SIRT2. This release led to FOXO1 acetylation, which in turn was then able to induce autophagy by interacting with ATG7

(Zhao et al., 2010) (Figure 1.4D). Nevertheless, whether any of these stress-induced interactions has a role in the regulation of ageing through FOXO factors is still unknown.

1.4.1 FOXO functions independent of DNA binding

Besides their prominent role as transcriptional regulators, FOXO proteins also have functions that are independent of the genes they regulate, making the understanding of their functions ever more complex. For example, FOXO1 over-expression in mammalian cell culture inhibits cell cycle progression by down-regulating *D-type cyclins* and inducing apoptosis. At the same time, inhibition of cell cycle progression is independent of the ability of FOXO1 to bind to DNA. Over-expression of a FOXO1 DNA binding mutant blocked cell cycle, but it did not affect apoptosis, suggesting that some functions of FOXO1 may not require DNA binding ability (Ramaswamy et al., 2002). FOXO1 also regulates progesterone receptor A activity independently of DNA binding (Rudd et al., 2007) and cytoplasmic FOXO1 was shown to mediate autophagy in a human cancer cell line (Zhao et al., 2010) (Figure 1.4D). These results suggest that FOXO proteins may act as transcriptional co-regulators and that DNA binding is not required to fulfil these functions. Interestingly, some of the FOXO1 effects on energy metabolism seem to be DNA-binding-independent, as seen in cell culture (Matsumoto et al., 2006), and liver-specific mouse mutants (Cook et al., 2015). The liver-specific study further suggests that FOXO1 may partially regulate liver lipogenesis, acting as a co-activator of another transcription factor (Cook et al., 2015). The putative interaction partner mediating these effects is still unidentified. Whether any of the dFOXO DNA-binding-independent functions are evolutionarily conserved, and their effect on ageing, is currently unknown.

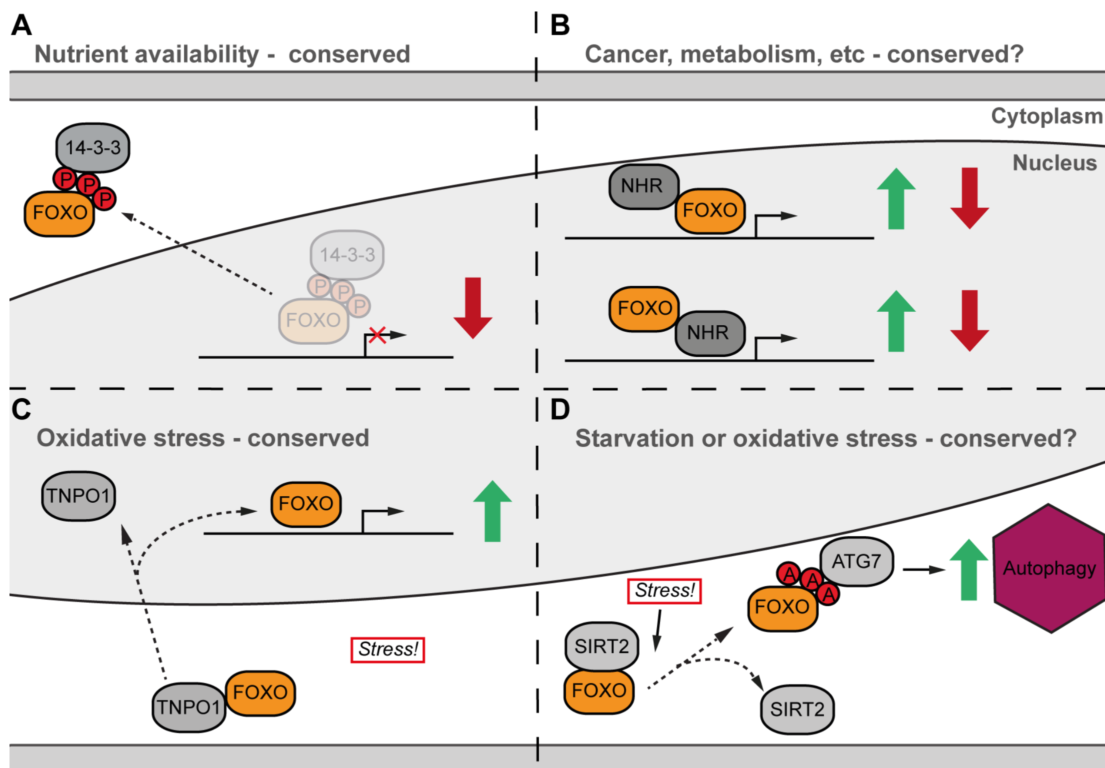

Figure 1.4 Simplified representation of different FOXO interactions
(A) AKT-dependent phosphorylation of FOXO factors mediates the interaction with scaffold protein 14-3-3, which in turn induces nuclear exclusion. This mode of FOXO regulation is evolutionarily conserved. **(B)** Nuclear FOXO can interact with multiple NHRs. This interaction can lead to expression or repression of FOXO and/or NHR target genes. **(C)** Redox stress can induce the interaction of FOXO with TNPO1, which leads to nuclear translocation followed by up-regulation of genes involved in redox stress response. This type of interaction seems to be evolutionarily conserved between worms and mice (see text). **(D)** Cytosolic FOXO loses the interaction with SIRT1 under starvation or redox stress, which leads to acetylated FOXO accumulation, interaction with ATG7 and induction of autophagy. NHR-Nuclear hormone receptor; TNPO1-Transportin; ATG7-Autophagy related gene 7.

1.5 Metabolism regulation and FOXO factors

Nutrient availability modulates the behaviour and metabolism of all organisms. The IIS pathway plays a fundamental role integrating nutrient sensing and energy homeostasis, for example, by repressing FOXO factors under nutrient abundance and, conversely, releasing said repression upon starvation periods (Saltiel and Kahn, 2001). FOXO proteins play critical roles in metabolism homeostasis; however, it is currently not fully understood how these transcription factors modulate the diverse processes involved in glucose and lipid metabolism.

Among mammalian FOXO proteins, FOXO1 is portrayed as the key regulator of energy metabolism (Kousteni, 2012). This protein is prominently expressed in tissues relevant for glucose homeostasis, such as liver, pancreas and adipose tissue. Moreover, FOXO1 mediates the organismal response to reduced nutrients (low insulin), stimulating hepatic glucose production and inhibiting adipogenesis (Matsumoto et al., 2007; Nakae et al., 2002; Qiao and Shao, 2006). For example, hepatic glucose production is induced by up-regulating *pepck*, which codes for the phosphoenolpyruvate carboxykinase, the limiting enzyme for gluconeogenesis. In addition, FOXO1 induces the Adipose triacylglycerol lipase (ATGL) expression in both adipose tissue and liver (Chakrabarti and Kandror, 2009; Zhang et al., 2016). ATGL is the limiting rate enzyme regulating lipolysis and its expression can therefore stimulate triacylglycerol (TAG) usage (Zimmermann, 2004). Moreover, starvation induces FOXO-dependent expression of eukaryotic initiation factor 4E binding protein (4EBP), which in turn dampens translation.

TAG reservoirs are found in lipid droplets, conserved cellular structures that are present across all organisms and have acquired multiple regulatory roles, such as lipid homeostasis, throughout evolution (Murphy, 2012). Consistently, lipid droplets are found across *Drosophila* tissues and serve mainly as TAG stores (Kuhnlein, 2012). Under fasting conditions, TAG stores are hydrolyzed in the cytosol by lipases, such as ATGL, into free fatty acids (FFA), which in turn are transported into the mitochondria to undergo β-oxidation and serve as an energy source. Fasting also induces autophagy, a process in which a double membrane vesicle, termed autophagosome, grows and engulfs organelles or cytoplasmic entities to later fuse with the lysosome and break its cargo down for energy production (Russell et al., 2014). The autophagy machinery can uptake TAGs and, with

the help of specialized lysosomal-associated-lipases, hydrolyze them to release FFA in a process termed lipophagy (Singh et al., 2009).

FOXO factors promote autophagy in different cell types, such as neurons and hepatocytes, by up-regulating multiple autophagy-related genes and have therefore another layer of control over the starvation response (Webb and Brunet, 2014). A recent study implicated FOXO1 in the regulation of ATG14, a protein that mediates the autophagosome fusion with lysosomes, and hence is fundamental for proper autophagy (Diao et al., 2015; Xiong et al., 2012). While ATG14 knockdown induced lipid accumulation in the liver, its over-expression protected the liver from fat accumulation under high fat diet, suggesting ATG14 is in fact a critical regulator of lipid homeostasis (Xiong et al., 2012). Furthermore, FOXO1 mediates the expression of lysosomal acid lipase (Lipa) in adipose tissue (Lettieri Barbato et al., 2013). In this study, nutrient restriction stimulated Lipa expression in a FOXO1-dependent manner, and Lipa expression was required for lipophagy induction (Lettieri Barbato et al., 2013). In accordance with these phenotypes, *lipa* mutant mice are unable to properly mobilize TAGs in the liver (Du et al., 2001). These observations implicate FOXO factors as key modulators of the starvation response at multiple levels.

1.5.1 dFOXO and metabolism regulation in *Drosophila*

During the last 15 years, *Drosophila* has been increasingly used as a powerful model to study the evolutionarily conserved mechanisms of energy homeostasis. The fly has functionally analogous tissues to those found in mammals that mediate energy storage in the form of glycogen and lipids when conditions are favourable (Baker and Thummel, 2007; Kuhnlein, 2012). For example, the fly fat tissue acts both as liver and adipose tissue in mammals, storing energy and modulating its usage under nutrient depravation. Under nutritional stress, these energy stores are mobilized to provide energy for the cells (Baker and Thummel, 2007).

A great number of the proteins involved in the response to energy deprivation have a clear homolog in the fly. For example, the *Drosophila* homolog of *4ebp* (termed *thor*) is one of the best characterized dFOXO target genes (Puig et al., 2003). Under nutritional stress, dFOXO induces *4ebp* expression, which in turn dampens general translation, ensuring careful allocation of energy recourses. Moreover, *Drosophila brummer* (*bmm* – the

homolog of mammalian ATGL) is also the rate limiting enzyme during lipolysis (Grönke et al., 2005). *bmm* was recently characterised as a dFOXO target gene by showing transcriptional induction under starvation in a dFOXO-dependent manner (Wang et al., 2011). These results highlight FOXO as a key mediator of metabolism in flies and mammals. Indeed, it was recently suggested that metabolic regulation by FOXO factors is evolutionarily conserved, at least based on common target genes across species (Webb et al., 2016).

In addition to *bmm* transcriptional regulation under starvation, dFOXO also stimulates expression of *lip4* (*lipase 4* – a homolog of mammalian Lipa) by direct binding to its promoter (Vihervaara and Puig, 2008). Moreover, dFOXO regulates the expression of *atg8* in the muscle, a key protein involved in autophagy induction (Bai et al., 2013). These results suggest that dFOXO, just like its mammalian counterparts, may in fact be able to regulate autophagy and lipophagy in *Drosophila*. However, this hypothesis awaits experimental testing.

1.6 Aim of the thesis

FOXO transcription factors are involved in several cellular and physiological processes, such as development, metabolism and ageing. In order to exert control over such diverse functions among different tissues, these proteins are regulated by multiple PTMs and protein-protein interactions. The multiple functions and levels of regulation make the study of FOXO factors complex. Moreover, mammalian genomes encode four FOXO paralogs that are able to target common genes, making it harder to determine the role of FOXO proteins in diverse processes. *Drosophila* only has one *dfoxo* gene, making it a simpler model to understand the regulation and functions of FOXO transcription factors. Hence, I aimed to generate a genetic tool that would allow me to modify the endogenous *dfoxo gene.* With this, I would be able to dissect the different dFOXO-associated functions by generating *in locus* mutant alleles of specific regions.

Genomic engineering of the *dfoxo locus*

Until now, full *dfoxo* removal or over-expression have been used to characterize the functions related to this transcription factor in *Drosophila*. However, many of these studies neglect the presence of endogenous dFOXO and overlook the secondary effects of over-expressing proteins in an organism. Therefore, I aimed to generate a genetic tool that would allow me, and others in the future, to modify the endogenous *dfoxo* gene. This tool would enable us to study *in vivo* any *dfoxo*-mutant allele.

Generation and evaluation of dFOXO DNA binding mutants

Preliminary studies suggest FOXO transcription factors may have functions independent of DNA binding. Hence, I aimed to determine which processes could be regulated by FOXO in the absence of DNA binding ability. For this, I planned to generate two *dfoxo* mutant alleles, using the newly generated gene-editing tool, which would abolish the protein-DNA interaction.

Generation and evaluation of dFOXO lysine acetylation mutants

How acetylation regulates FOXO functions is not fully understood. Thus, I aimed to generate *dfoxo* mutant alleles to either mimic or abolish acetylation in conserved residues. Using these mutants, I intend to determine the *in vivo* roles of dFOXO acetylation.

Material & methods

2.1 Generation, maintenance and characterization of transgenic fly lines

2.1.1 Genomic engineering of the *dfoxo locus* (see also, Results 2.2)

The new *dfoxo*Δ*V1*, Δ*V2* and Δ*V3* knockout founder lines were generated by genomic engineering as previously reported (Huang et al., 2009). In a first step, each of the *dfoxo* target regions, V1, V2, or V3 (Figure 2.3), were substituted by a $white^{hs}$ marker gene and an *attP*-site using ends-out homologous recombination. For this, ~4Kb flanking sequences of each target region were cloned into a pBlueScript II SK(+) vector, using ET recombineering (Muyrers et al., 1999; Zhang et al., 1998) and the respective primers SOL572-579 Supplementary Table S1). As template for the ET recombineering, a BAC clone that contains the *dfoxo locus* (CH321-24|13, BACPAC resource center, Oakland, California) was used. After sequence verification using sequencing primers SOL580-607 and SOL628-635 (Supplementary Table S1), homologous arms were brought into the pGX-attP targeting vector (Huang et al., 2009). To target the V1 region, we cloned a pGXattP vector containing homologous arms 1 and 2; for the V2 region, arms 1 and 4; and for the V3 region arms 3 and 4 (Figure 2.3). P-element-mediated transformation was done by the BestGene Drosophila embryo injection service (Chino Hills, USA) to generate transgenic flies carrying the pGXattP donor constructs for targeting the V1, V2 and V3 region.

Crosses for ends-out homologous recombination were set for direct targeting as described before (Huang et al., 2008) (Figure 2.4). Subsequently, the $white^{hs}$ marker gene was mapped to the third chromosome using a *TM3 Sb* balancer chromosome. Homozygous flies carrying the $white^{hs}$ marker on the third chromosome were screened by PCR for the absence of the corresponding region of the *dfoxo* gene using primers SOL665/666 for V1, SOL667/668 for V2 and SOL669/670 for V3 (Supplementary Table S1). We obtained one knockout founder (KO) line for V1, V2 and V3. Subsequently, KO flies were crossed with *cre-recombinase* expressing flies to remove the $white^{hs}$ marker gene (Groth et al., 2004). The generated *w[-]* lines, denoted *dfoxo*Δ*V1w[-]*, Δ*V2w[-]* and Δ*V3w[-]*, were brought into a fly line expressing the ΦC31-integrase (Groth et al., 2004), and used as parental lines for any future reinsertion within the *dfoxo locus*.

To generate the *dfoxo* gene replacement constructs, the genomic regions V2 and V3 were cloned in the pBlueScript II SK(+) vector by ET recombineering using primers SOL681/682 and SOL683/682, respectively (Supplementary Table S1). Inserts were

sequence verified using primers SOL690/700 (Supplementary Table S1). Subsequently, reinsertion inserts were transferred to the pGEattBGMR vector (Huang et al., 2009) by restriction enzyme cloning using NheI and AscI to cut vector and insert. Ligation was carried out overnight at 18ºC according to standard T4-ligase protocols (NEB). The V1 region was PCR-cloned in the same vector using the In-Fusion system (Clontech) with pBS-V2 as template and primers SOL795/796 (Table 2.1 and Supplementary Table S1). Mutations on the pBS-V3-3xFLAG construct were introduced using QuickChange II XL site-directed mutagenesis (Agilent Technologies) and the V3-*mutant*-3xFLAG sequence was subsequently In-Fusion (Clonetech) cloned in the pGEattBGMR vector using the respective primers (Table 2.1 and Supplementary Table S1). pGEattBGMR gene replacement constructs were injected into embryos of the respective KO-parental lines ΔV1w[-] or ΔV3w[-]. Microinjections were done by Jacqueline Dols of the transgenic fly core facility of the Max-Planck Institute for Biology of Ageing.

Table 2.1. Transgenic *dfoxo* alleles. Transgenic *dfoxo* flies generated by embryo microinjection. Injected vector was always pGEattBGMR. InFusion templates labeled V1short, V3short or V3-MAD-3xFLAG were synthesized by Eurofins. Primer sequences are summarized in supplementary Table S1.

<table>
<tr><th>dfoxo knock-in lines</th><th>Mutagenesis primers on pBS-V3</th><th>InFusion primers</th><th>InFusion PCR template</th><th>Seq primers</th></tr>
<tr><td>V3</td><td rowspan="4"></td><td>SOL813-817</td><td>pBS-V3</td><td rowspan="3">SOL692-700</td></tr>
<tr><td>V3-3xFLAG</td><td rowspan="3">SOL728-729</td><td>V3short-3xFLAG</td></tr>
<tr><td>V3-Tev-BLRP</td><td>V3short-Tev-BLRP</td></tr>
<tr><td>V3-mCherry</td><td>V3short-mCherry</td><td>SOL692-700 and SOL717-721</td></tr>
<tr><td>V3-DBD1-3xFLAG</td><td>SOL809-810</td><td rowspan="4">SOL813-824</td><td rowspan="4">pBS-V3 with corresponding mutation</td><td rowspan="4">SOL692-700</td></tr>
<tr><td>V3-DBD2-3xFLAG</td><td>SOL811-812</td></tr>
<tr><td>V3-5KR-3xFLAG</td><td>SOL393-394, SOL397-398 and SOL563-564</td></tr>
<tr><td>V3-5KQ-3xFLAG</td><td>SOL514-515, SOL516-517 and SOL561-562</td></tr>
<tr><td>V3-MAD-3xFLAG</td><td rowspan="3"></td><td>SOL813-814</td><td>V3-MAD-3xFLAG</td><td>SOL692</td></tr>
<tr><td>V1</td><td>SOL795-796</td><td>pBS-V2</td><td>SOL690-691</td></tr>
<tr><td>3xFLAG-V1</td><td>SOL713-714</td><td>3xFLAG-V1short</td><td>SOL690-691 and SOL717-718</td></tr>
</table>

2.1.2 Fly maintenance

Fly stocks were maintained and experiments were conducted at 25ºC on a 12:12 h light:dark cycle at 65% humidity. To generate experimental flies, larvae were reared at controlled densities by transferring 20µl of eggs ("egg squirts") into a fly stock bottle containing 1SYA food (5% w/v sucrose, 10% w/v brewer's yeast, 1,5% w/v agar) (Bass et al., 2007). Freshly eclosed adult flies were allowed to mate for 48h before being sorted according to gender. All fly lines used for experiments were backcrossed for at least 6 generations into the outbred, wild-type *white Dahomey* (w^{DahT}) strain (Grönke et al., 2010). This line was previously treated with tetracycline and does not contain the endosymbiotic bacterium *Wolbachia*. All fly lines used in this PhD thesis are summarized in Table 2.2.

2.1.3 Fly lines used in this study

Table 2.2. Drosophila stocks used in this study. Balancer and experimental flies were backcrossed into the wDahT background for at least 6 generations. G.E = Genomic Engineering; VB = Victor Bustos.

Fly line	Chr.	Designed by/obtained from	Function
w;;TM3Sb/+	3	Bloomington	Balancer
w;;TM6B/+	3	Bloomington	Balancer
w;;MKRSSb/+	3	Bloomington	Balancer
w;CyO/+	2	Bloomington	Balancer
w;UAS-Lip3RNAi	2	VDRC (108639)	Experiments
w;;UAS-Lip3-3xHA	3	FlyOrf (F002854)	Experiments
w;S1106GS-Gal4	2	Bloomington	Experiments
w;;FB-Gal4	2	Bloomington	Experiments
w;;GS-Gal4	3	Bloomington	Experiments
w;6934-hid		(Huang *et al.*, 2009)	G.E.
w;6938		(Huang *et al.*, 2009)	G.E.
w,pGXattP-V1 Donor	1	VB	G.E.
w;pGXattP-V2 Donor	2	VB	G.E.
w;pGXattP-V3 Donor	2	VB	G.E.
w,Cre;Sco/CyO		(Huang *et al.*, 2009)	G.E.
w,vas-int;;TM6B/MKRSSb		(Huang *et al.*, 2009)	G.E.
w,vas-int;dfoxoΔV1w[-]	3	VB	Microinjection
w,vas-int;dfoxoΔV2w[-]	3	VB	Microinjection
w,vas-int;dfoxoΔV3w[-]	3	VB	Microinjection
w;;dfoxoΔ94	3	(Slack *et al.*, 2011)	Experiments
w;;dfoxoΔV1	3	VB	Experiments
w;;dfoxoΔV2	3	VB	Experiments
w;;dfoxoΔV3	3	VB	Experiments

w;;dfoxo-V3	3	VB	Experiments
w;;dfoxo-V3-3xFLAG	3	VB	Experiments
w;;dfoxo-V3-Tev-BLRP	3	VB	Experiments
w;;dfoxo-V3-mCherry	3	VB	Experiments
w;;dfoxo-V3-DBD1-3xFLAG	3	VB	Experiments
w;;dfoxo-V3-DBD2-3xFLAG	3	VB	Experiments
w;;dfoxo-V3-MAD-3xFLAG	3	VB	Experiments
w;;dfoxo-V3-5KR-3xFLAG	3	VB	Experiments
w;;dfoxo-V3-5KQ-3xFLAG	3	VB	Experiments
w;;dilp2-3,5	3	(Grönke et al., 2010)	Experiments
w[DhaT]		(Grönke et al., 2010)	Experiments
w;;dilp2-3,5, dfoxoΔV3	3	VB	Experiments
w;;dilp2-3,5, dfoxo-V3-3xFLAG	3	VB	Experiments
w;;dilp2-3,5, dfoxo-V3-DBD1-3xFLAG	3	VB	Experiments
w;;dilp2-3,5, dfoxo-V3-DBD2-3xFLAG	3	VB	Experiments
w;S1106GS-Gal4;dfoxoΔV3	3	VB	Experiments
w;;UAS-Lip3-3xHA, dfoxoΔV3	3	VB	Experiments

2.1.4 Lifespan and fecundity assays

For lifespan assays, 48h mated flies were sorted by sex at a density of 10 flies per small glass vial and 10 vials per genotype (n=100). Flies were tipped to fresh food 3 times per week and dead flies were scored. For fecundity assays, in parallel to the lifespan, 10 vials per genotype with 3 flies per vial were used. During the first 3 weeks, eggs laid per vial were counted after egg-laying periods of ~20h.

2.1.5 Stress assays and fly experiments

For stress assays, flies were sorted at 20 flies per wide plastic vial, 5 vials per genotype (n=100), and kept on 1SYA food for 7 days before starting the stress unless otherwise specified. Starvation food contained 1% w/v agarose, food for oxidative stress assays contained 5% w/v sucrose, 1,5% w/v agarose and 5% v/v H_2O_2 (Grönke et al., 2010). Dead flies were scored three times per day. In the case of yeast or sugar starvation, the respective component was omitted during normal 1SYA preparation and dead flies were scored every 2-3 days while tipping flies into fresh food vials.

For over-expression or RNAi-mediated knock down of gene expression the corresponding UAS-lines were mated with Gal4 driver lines (see Table 2.2) for induction of constitutive expression or with GS-driver lines (Roman et al., 2001) for inducible expression. When using an inducible GS-driver line, food was supplemented with RU486 (Sigma) at a concentration of 200µM in 1SYA or at 50µM in starvation food. The respective controls contained equivalent volumes of the drug carrier ethanol.

2.1.6 Fly developmental time and body weight

Fly development and body weight were measured similarly to protocols described previously (Grönke et al., 2010). Briefly, for developmental timing, eggs laid over 3h on grape juice plates were collected and transferred to 1SYA food at a density of 50 eggs per vial and 10 vials per genotype. Upon eclosion of the first flies, their numbers were counted at regular intervals.

For body weight determination, batches of 5 flies were flash frozen in liquid nitrogen and weighted on a ME235S analysis balance (Sartorius Mechatronics). A total of 50 flies per genotype was measured.

2.2 Biochemistry and molecular biology methods

2.2.1 DNA extraction for genotyping

DNA from single whole flies was extracted as previously described (Gloor et al., 1993). Briefly, individual flies were homogenized in 50µl of squishing buffer (10mM Tris, 25mM NaCl, 1mM EDTA and freshly added proteinase K to 200µg/ml) and incubated for 30min at 37ºC. This was followed by proteinase K inactivation at 95ºC for 2min. PCR was done according to manufacturer instructions for the HotStar Taq Plus polymerase (Qiagen) using 1µl of DNA template.

For genotyping of live flies, a single leg was removed from the fly and incubated in 50µl of lysis buffer (10mM Tris pH 8, 50mM KCl, 0.5% tween and freshly added proteinase K to 200µg/ml) at 65ºC for 1h. Proteinase K was then inactivated for 10min at 95ºC. HotStar Taq Plus (Qiagen) was used as polymerase following manufacturer instructions. All PCR products were analyzed on 0.5-2% agarose gels according to the expected band size. Flies identified as positive by PCR were subsequently further mated.

2.2.2 RNA extraction and qPCR Analysis

Total RNA from five 7-day-old female flies (control) or 7-day-old plus 48h starved female flies, per biological replicate, was extracted using standard Trizol (Invitrogen) protocols and treated with DNAse I (Ambion). Reverse transcription of 1µg total RNA was done using the SuperScript VILO kit (Invitrogen). Quantitative real-time PCR was done using Taqman probes (Applied Biosystems) (Table 2.3) in a 7900HT real-time PCR machine (Applied Biosystems). Relative gene expression was determined by the ΔΔCT method (Livak and Schmittgen, 2001) and normalized to *actin* (*act5C*) and/or *RNA polymerase II* (*RpII*). Four independent biological replicates per genotype were analyzed. A representative graph is shown and data are presented as fold change relative to controls. Control and starved flies from the same genotype were compared by unpaired student t-test method and significance is indicated in graphs as: ns = $p>0.05$; * $p<0.05$; ** $p<0.01$; *** $p<0.005$.

Table 2.3. Taqman probes for relative mRNA quantification by qPCR

Gene	Taqman probe	Gene	Taqman probe
acc	Dm01811986_g1	***fasn-1***	Dm01801118_g1
act5C	Dm02361909_s1	***InR***	Dm02136224_g1
atg14	Dm02145973_g1	***lip3***	Dm02135029_g1
atg5	Dm01833509_g1	***lip4***	Dm01810401_g1
atg8	Dm01825265_g1	***npc2b***	Dm02140219_g1
bmm	Dm01805237_g1	***pepck***	Dm01816546_s1
dfoxo-V1	Dm02140211_g1	***rpll***	Dm01813383_m1
dfoxo-V3	Dm02140205_g1	***thor***	Dm02136224_g1

2.2.3 Chromatin preparation, immunoprecipitation and qPCR

Chromatin extraction and immunoprecipitation were done according to a previous protocol (Alic et al., 2011) and slightly modified to adjust to a smaller scale. Briefly, ~100 7-day-old flies per biological replicate were homogenized in 1ml PBS supplemented with Protease inhibitors and 0.5% formaldehyde in a small dounce homogenizer with a loose pestle (20 strokes). Three biological replicates were used per genotype. Samples were then transferred to a 2ml tube, allowed to crosslink for 10min in total and the reaction was subsequently quenched with 250µl of 2.5M glycine. Chromatin was then centrifuged at 12000rpm for 20min at 4ºC, washed twice with FA/SDS buffer (50mM Hepes-KOH pH7.5, 150mM NaCl, 1mM EDTA, 0.1% Na Deoxycholate, 0.1% SDS, 1% Triton-X 100 and freshly added 1mM PMSF), resuspended in the same buffer and rotated for 1h at 4ºC. After centrifugation (12000rpm for 20min at 4ºC), chromatin was resuspended in 1ml FA/SDS buffer and sheared in a *Covaris M220 Focused-ultrasonicator*™ with 1 ml millitubes (AFA fiber), to a ~500bp average size.

Chromatin immunoprecipitation was done using 600µl of resuspended chromatin and 25µl of anti-DYKDDDDK (FLAG) micro beads (Miltenyi). Beads were later eluted in 100µl of pronase buffer (25mM Tris pH7.5, 5mM EDTA, 0.5%SDS) and incubated at 65ºC for 20min to elute chromatin. After removal of the beads, eluted chromatin samples were treated with 6.25µl of pronase (Sigma) for 1h at 37ºC followed by overnight incubation at 65ºC to induce decrosslinking. Samples were then treated with 3.3µl of 1mg/ml RNAse A (Qiagen) for 1h at 37ºC. Chromatin was purified with a PCR purification kit (Qiagen). For ChIP-qPCR detection of the *4ebp* promoter region, triplicates from 3 biological replicates

of each genotype were analyzed by the ΔΔCT method. Values are expressed as percentage input and the primers used were reported elsewhere (SOL988/989) (Liu et al., 2014).

2.2.4 Lipid assays

Triglyceride storage quantification was performed as previously described (Grönke et al., 2003). Briefly, four flies per biological replicate and five biological replicates per genotype were homogenized in 1ml PBS, 0.05% Tween using a Fastprep-24 system (MP Biomedicals™). The homogenate was then inactivated at 70ºC for 5minutes and centrifuged. 50µl aliquots of the supernatants were incubated with 200µl of pre-warmed Infinity™ Triglycerides reagent (Thermo Scientific). Absolute values were determined using a Triglyceride standard (Cayman Chemical). Triglyceride values were normalized to protein content measured by BCA assay (Pierce).

2.2.5 Immunoprecipitation

At least 60 flies were homogenized with a dounce homogenizer (20 strokes) in 1ml cold RIPA buffer (Pierce) supplemented with EDTA-free protease and phosphatase inhibitors (Roche). Lysate was then centrifuged at 15000 rpm for 15min at 4ºC to remove debris, followed by a filtration step using VDR 0.45µm centrifuge filters to remove residual debris and fat. A part of the lysate (50µl) was kept as input sample and the rest (~700µl) was incubated with 50µl anti-DYKDDDDK micro beads (Miltenyi) for 30-45min at RT. The mixture was then put on a pre-equilibrated magnetic column and non-bound proteins were separated by gravity flow. Washing was done twice with 1ml RIPA buffer and once with 500µl wash buffer (20mM Tris pH 7.5). Elution was carried out by boiling the beads with sample buffer (50mM Tris pH 6.8, 2%SDS, 10%glycerol, 1% -mercaptoethanol, 12.5mM EDTA, 0.02% bromophenol blue) or by incubating the beads with 3xFLAG peptide (100µg/ml in TBS - Sigma) for 2h at room temperature. IP Samples (5-15µl) were run on 7.5% SDS polyacrylamide gels (SDS-PAGE) (BioRad) for western blot analysis.

2.2.6 Western Blot

At least five flies were homogenized on ice in 200µl RIPA buffer (50 mM Tris-HCl, pH 7.4, 1% NP-40, 0.5% Na-deoxycholate, 0.1% SDS, 150 mM NaCl, 2 mM EDTA, 50 mM NaF, protease and phosphatase inhibitors (Roche)) and incubated for 20-30min. Subsequently, samples were centrifuged for 15 min at 15.000 rpm at 4ºC and protein content was quantified with BCA (Pierce). Proteins were separated on a 7.5% SDS-PAGE (BioRad). Proteins were then transferred to nitrocellulose membranes (GE healthcare) by wet transfer at 100V for 30min. After membrane blocking with 5% Milk TBS-T buffer, primary antibody incubation was done overnight at 4ºC. Membranes were then incubated with secondary antibodies coupled with HRP for 1h at RT. Antibodies were diluted in 5% Milk TBS-T buffer (Table 2.4). Protein detection was done by incubating membranes with ECL Prime (Amersham) for 5min, followed by detection using a Chemidoc station (BioRad) at high-resolution mode. Western blot bands quantification was done using *Image J* (Scion Software).

Table 2.4. Antibodies used in this study.

Antibody	Dilution	Source
dFOXO	1:5000	Dr. Nazif Alic (Giannakou et al., 2007)
dHNF4	1:1000	Prof. Thummel (Palanker et al., 2009)
FLAG (M2)	1:5000	Sigma (F1804)
GADPH	1:1000	Santa Cruz Biotechnology (sc-25778)
H3	1:5000	Sigma (H0164)
HA (HA-7)	1:5000	Sigma (H9658)
P-AKT-substrate	1:1000	Cell Signaling (9614)
α-Tubulin	1:10000	Sigma (T9026)

2.2.7 Mass spectrometry

To identify post-translational modifications of the dFOXO protein we used mass spectrometry on dFOXO^{FLAG} protein purified by immunoprecipitation (compare 2.2.5). Mass spectrometry was done at the Mass Spectrometry core facility of the Max-Planck Institute for Biology of Ageing. Purified dFOXO^{FLAG} protein (~50µg) was diluted in digestion buffer (100mM Tris, 6M Guanidinium chloride, 5mM TCEP, 10mM CAA) and digested with trypsin (1µg - Promega) at 37ºC or 1h treatment with Chemotrypsin (1µg - Promega). Samples were then acidified with 2µl of 50% formic acid (FA) to stop digestion, and peptides were purified on a C18 stage tip (Hubner et al., 2010). Peptides were eluted in 100µl of elution buffer (60% Acetonitril (ACN), 0.1% FA) and dried in a Speed-Vac for 45min at 45ºC, then resuspended in 20µl of 0.1% FA and used for liquid chromatography coupled Mass spectrometry (LC-MS) on a Q Exactive Hybrid-Quadrupol-Orbitrap Mass Spectometer (ThermoFischer). Peptide analysis and post-translational modification identification was performed by the Mass Spectrometry core facility.

2.3 *Drosophila* cell culture methods

2.3.1 Cloning of cell culture plasmids

The *dfoxo* ORF was cloned into a pUbiP-EGFP-rfA vector, kindly donated by Alf Herzig, using the Gateway system (ThermoFischer). Mutations in the *dfoxo* ORF were introduced using QuickChange II XL site-directed mutagenesis (Agilent Technologies) using the same mutagenesis primers as for *dfoxo-V3* constructs (Table 2.1 and Supplementary Table S1). The dHNF4 ORF was PCR amplified using cDNA clone RE09535 (Berkeley Drosophila Genome Project) as template and primers SOL1160/1162 and Phusion High Fidelity Master Mix (ThermoFischer). The SOL1160 primer introduces an N-terminal 3xHA tag to the dHNF4 protein. The 3xHA the dHNF4 PCR product was then cloned in the pUbiP, kindly donated by Alf Herzig, by InFusion cloning and sequence verified with primers SOL1160-1171.

The pACT-renilla construct was kindly donated by Prof. Michael Hoch. The pGL-InR luciferase and pGL-4xFRE (4xFOXO Responsive Element) luciferase constructs were reported elsewhere (Puig et al., 2003). Lip3-short and lip3-long promoter regions were PCR amplified from w^{DahT} DNA using primers SOL 1093/1094 and SOL1092/1094 respectively (Supplementary Table S1). Amplified sequences were then cloned in the

pGL3 vector (Promega) backbone by InFusion cloning and sequenced verified using primers SOL1092-1094 and SOL1139-1140. Supplementary Table S1).

2.3.2 Cell culture maintenance, luciferase assay and cell imaging

Drosophila S2-R+ (Schneider's line 2 receptor plus) cells (Schneider, 1972)were grown at 25ºC on Schneider's Medium (Gibco™) supplemented with 10% FCS, penicillin and streptomycin. Cells were maintained through serial passage.

For luciferase assay, cells were grown on 96-well plates and transfection of 1,25µg of total DNA (1:10 ration for luciferase:renilla constructs) was done using the Qiagen-Effectene® transfection reagent according to the manufactures instructions (Qiagen). Measurement of firefly and renilla luciferase was done with Dual-Glo kit according to manufacturer's instructions (Promega) 24h after transfection in a Infinity200 multimode reader (Techan). Data is presented as average fold change of firefly/renilla values ± standard error of the mean (SEM).

For imaging of EGFP-dFOXO subcellular localization, cells were grown on a *µ-slide* (Ibidi) and transfected with pUbi-EGFP constructs. Transfection was carried out with 1.25µg of total DNA as described before with Effectene® transfection reagent (Qiagen). 24h after transfection, cells were starved on serum-free medium (Schneider's medium) for 2h and nuclei were stained with *Hoechst 33342* (Sigma). Imaging was performed in a fluorescent microscope at 64x magnification (Leica DMI4000B). After the initial imaging, cells were then treated with 10µg/ml insulin (Sigma) in serum-free medium for 10min and imaged again.

2.4 Statistical Analysis

For lifespan and stress assays statistical analysis was performed in Excel (Microsoft) using log-rank test. All other data, presented as average ± standard error of the mean (SEM), were analyzed in Graphpad Prism®. Differences were calculated either by two-tailed unpaired Student's t-test or one-way ANOVA followed by Dunnett's test to compare to a control sample (specified in each experiment). ns – $p>0.05$; * $p<0.05$; ** $p<0.01$; *** $p<0.005$.

GENOMIC ENGINEERING AND FOXO DNA BINDING

3.1 INTRODUCTION

FOXO transcription factors are key regulators of multiple organismal processes such as development, metabolism and ageing. Therefore, FOXO proteins are under tight regulatory control of several upstream signalling networks including the IIS, TOR and AMPK pathways. These pathways regulate FOXO activity by PTMs, and direct protein-protein interactions. However, the exact mechanisms by which FOXO proteins are regulated are not well understood. Moreover, preliminary cell culture studies suggest FOXO proteins may also have regulatory roles independent of DNA binding (Matsumoto et al., 2006; Ramaswamy et al., 2002; Rudd et al., 2007), adding an extra level of complexity. Thus, in order to separate the different functions associated with FOXO proteins, it is critical to better understand how these transcription factors are regulated *in vivo*. Here, I used genomic engineering in the fruit fly *Drosophila melanogaster* to establish a tool that allows *dfoxo* gene editing and analysis of dFOXO function *in vivo*. This new model permits quick and easy generation of any desired *dfoxo* allele in the fly. As a proof of principle, I used this knock-in platform to generate two novel *dfoxo* alleles containing mutations within the DBD. Furthermore, I used these mutants to separate the physiological functions that are dependent and independent of dFOXO DNA-binding.

3.2 RESULTS

3.2.1 Genomic Engineering of the *Drosophila foxo locus*

The first step in the genomic engineering protocol (Huang et al., 2009) is the replacement of the endogenous *dfoxo* gene by an attP site using ends-out homologous recombination. AttP is a small DNA sequence, used as a target site of the φC31-Integrase, that can be used to re-introduce mutated versions of the *dfoxo* gene by site-directed integration (Huang et al., 2009) (Figure 3.1A-D).

3.2.1.1 Generation of dfoxo parental knock-out lines

Since homologous recombination and site-directed integration are more efficient with shorter deletions and shorter DNA fragments respectively (Huang et al., 2009), we designed three different donor constructs to cover the whole coding sequence of the *dfoxo* gene. The donor constructs had homologous arms flanking one of the exon-clusters of interest within *dfoxo*, arbitrarily denoted V1, V2 and V3, respectively (Figure 3.1A-B): V1 is a small ~1 kb region (exons coding for amino acids 1-145), V3 represents a ~3 kb sequence (exons coding for amino acids 146-613) and V2 is a large (16kb) sequence

encompassing the whole protein coding part of the *dfoxo* gene. This strategy allows re-introduction of any desired mutation into the *dfoxo* open reading frame (ORF) once the 'parental knock-out lines' are generated.

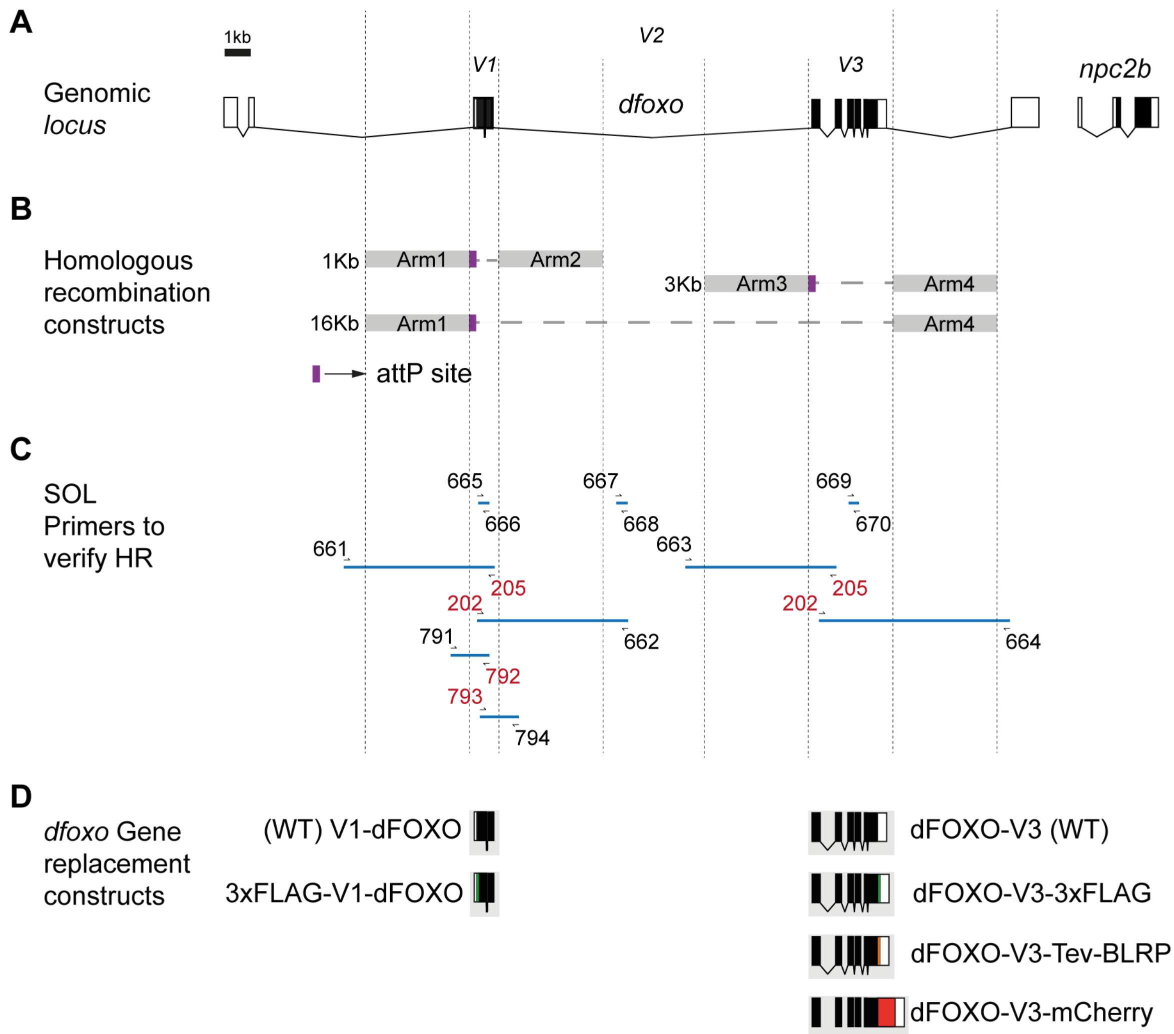

Figure 3.1. Genomic engineering of the *dfoxo* locus.
(A) Schematic view of the *dfoxo* gene locus. Boxes represent UTRs (white boxes) and the CDS (black boxes) within the exons. Regions to be deleted were arbitrarily denoted V1 (1Kb), V3 (3Kb) and V2 (16Kb – which includes V1 and V3). **(B)** Representation of donor constructs used for ends-out homologous recombination. Three different donor constructs were created, covering three different regions of the *dfoxo* gene (V1, V2and V3). Donor constructs were cloned into the pGXattP vector, which contained the denoted homologous arms flanking an attP site, to allow targeted reinsertion, and a *mini-white*$^{+}$ marker gene to facilitate detection of successful reinsertion events. **(C)** Primer combinations used to screen (SOL665-670) and validate (all other SOLs) correct recombination events in the newly generated *dfoxo-null* lines (*dfoxo*$^{\Delta V1}$, *dfoxo*$^{\Delta V2}$ and *dfoxo*$^{\Delta V3}$). Primers in red bind inside the pGE vector and only amplify bands if correct recombination took place. **(D)** *dfoxo* replacement constructs coding for wild type or N-/C-terminal tagged versions of the gene. WT = wild type.

To generate the homologous recombination constructs (Figure 3.1B), we cloned the relevant homologous arms into the pGX-attP vector (Huang et al., 2009). This vector has an attP-50 cassette between the homologous arms along with a mini-*white* marker gene to allow easy detection of transgenic flies. Homologous recombination donor constructs, targeting each of the desired *dfoxo* regions (V1, V2 and V3), were introduced into the *Drosophila* genome by P-element mediated germline transformation.

Ends out homologous recombination crosses using the transgenic *dfoxo* donor lines were carried out as described before (Huang et al., 2008) (Figure 3.2A). Homologous recombination screens for the V2 and V3 region were done as part of this PhD project, whereas the homologous recombination screen for the V1 region was done by Dr Sebastian Grönke. First, *dfoxo* donor males were crossed with females of the fly line *6934-hid.* Offspring embryos of this cross were heat shocked to induce expression of Flipase (FLP) and the homing endonuclease I-SceI. These enzymes cut specific DNA sequences within the donor construct and release the targeting cassette including the homologous arms and attP replacement cassette as a linear DNA fragment (Figure 3.2A). This linearized targeting cassette is able to move within the nucleus and find the *dfoxo* flanking arm regions to undergo homologous recombination. The heat shock also induced expression of the pro-apoptotic gene *head involution defective* (*hid)*, which induced apoptosis specifically in males, since the corresponding transgene is integrated on the *Y* chromosome. Expression of *hid* killed developing males and therefore assured that all females remained virgins, which facilitated the collection of white/mosaic eyed females.

In order to identify homologous recombination events I employed the rapid targeting scheme (Rong and Golic, 2001). Therefore, females from the first crossing round were mated to flies expressing FLP (*6938*). In case of a successful homologous recombination event, only one FRT target sites will be present at the endogenous *dfoxo* locus and the FLP cannot cut out the targeting cassette. This will result in flies that have red eyes without mosaicism. In contrast, improper targeting events where the construct was not mobilized will cause mosaic eyes because both FRT sites are still present in the targeting construct. Flies with red eyes were collected and individually mated to TM3 Sb balancer flies to test whether the *white* marker gene was inserted on the third chromosome, where the *dfoxo* gene locus is located. Flies were made homozygous for the targeting event and

screened by PCR with primers within the excised regions (Figure 3.1A-C and Figure 3.2B) to identify targeted homologous recombination events. Numbers of flies used in the different crossing rounds are indicated in Figure 3.2A. Although several fly lines were established in which the white marker gene did move to the third chromosome, 30 for V2 and 18 for V3, only one fly line per targeted region was identified as a true homologous recombination event for the *dfoxo* gene locus (Figure 3.2).

In order to verify correct homologous recombination, we performed long-range PCR analysis using primers inside the recombination cassette and outside the homologous arms (Figure 3.1C and 3.2C). Long-range PCR confirmed the correct recombination events for the newly generated *dfoxo*$^{\Delta V2}$ and *dfoxo*$^{\Delta V3}$ lines. In contrast, the long range PCR covering *arm1* in the line *dfoxo*$^{\Delta V1}$ did not look as expected. However, after removal of the white marker gene (see below), PCR with new primers (SOL791-794) showed the expected banding pattern, suggesting proper homologous recombination (Figure 3.1C and 3.2C).

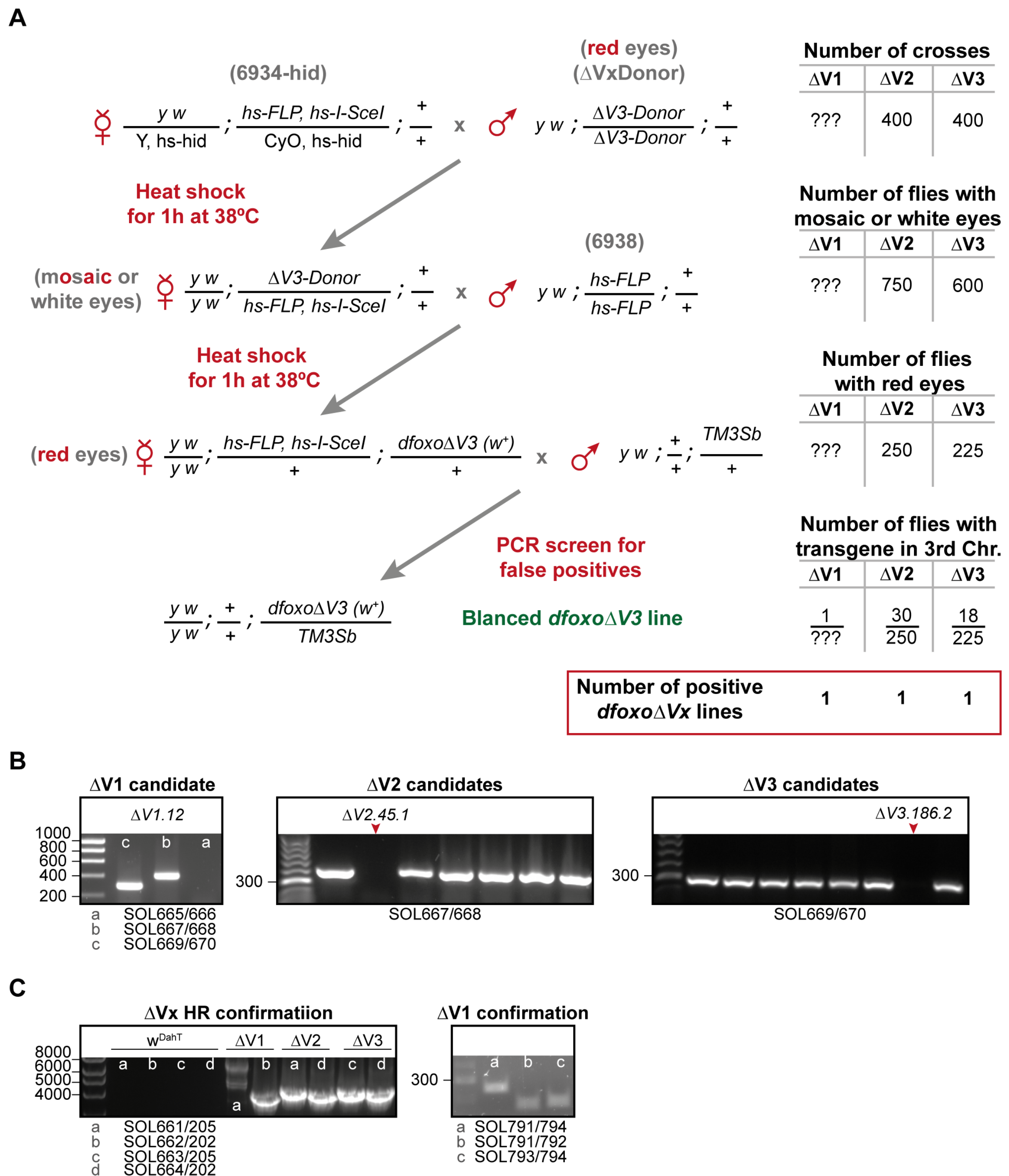

Figure 3.2. Generation of new *dfoxo* alleles by ends-out homologous recombination.
(A) Crossing scheme to generate novel *dfoxo-null* (Δ) lines by ends-out homologous recombination (Δ*V1**, Δ*V2* and Δ*V3*, where ΔV3 is used as a representative example) accompanied by the fly numbers used for each cross. y-yellow; w-white; hs-heat shock; hid-head involution defective,; Flipase (FLP)-recombinase that recognizes FRT (flipase recognition sites) in the ΔV3-Donor sequence; I-SceI – endonuclease that recognizes I-SecI sites in the ΔV3-Donor sequence; CyO and TM3Sb- balancer chromosomes for Chr.2 and 3, respectively. **(B)** PCR screening identified novel dfoxo-null (Δ) alleles using allele specific primer combination (see also Figure 3.3 and supplemental Table S1). **(C)** Validation of the correct homologous recombination by long range PCR followed by normal PCR on the ΔV1 line to confirm correct reinsertion after removal of the *white* gene (refer to text). *Dr. Sebastian Grönke performed crossings for the ΔV1 allele.

3.2.1.2 Generation of *dfoxo* replacement lines

In order to use the attP site within the knockout parental lines *dfoxo*$^{\Delta V1}$, *dfoxo*$^{\Delta V2}$ and *dfoxo*$^{\Delta V3}$ as a landing site for exogenous DNA sequences, it was necessary to remove the mini-*white* gene marker and to introduce a vasa-ΦC31-integrase expressing construct. Therefore, two more crosses were carried out (Figure 3.3A). In the first cross, *dfoxo* homologous recombination event males were mated to females expressing a Cre-recombinase. The white marker gene is flanked by two loxP sites and was therefore removed by the Cre expression. Resulting flies had white eyes and were then crossed to flies carrying a vasa-ΦC31-integrase construct (Figure 3.3A), which allows for germ-line specific-expression of the ΦC31-integrase (Huang et al., 2009).

To verify that we could introduce modified *dfoxo* sequences into the attP site at the endogenous *dfoxo* locus, we used the newly generated *dfoxo*$^{\Delta V3}$-attP fly line. The *dfoxo*$^{\Delta V3}$-attP fly line was chosen because the V3-exon cluster covers the majority of the *dfoxo* ORF. We first introduced a wild type *dfoxo* gene (control) and a Carboxy-terminally FLAG-tagged *dfoxo* gene, as well as *Biotin ligase recognition peptide* (BLRP) and mCherry tags, respectively (Figure 3.1D and 3.3B). We chose the FLAG-tag because it allows easy detection on western blot and immunoprecipitation to enrich dFOXO protein. In contrast to the specific dFOXO antibody, the anti-FLAG antibody is not limiting and is available in unlimited amounts, one of the big advantages of the newly generated dFOXO line. The mCherry-tagged protein was chosen mainly to monitor the subcellular localization of dFOXO *in vivo*, since shuttling between nucleus and cytosol is a key regulatory mechanism of FOXO transcription factors. The introduction of the small BLRP tag will permit the study of dFOXO in a tissue specific manner (Beckett et al., 1999). dFOXO-BLRP flies could be combined with lines expressing BirA in certain tissues. BirA adds a biotin moiety to the BLRP tag and allows subsequent purification of biotinylated FOXO. This would allow us to evaluate tissue-specific, post-translational modifications of dFOXO or tissue-specifically target genes by ChIP-chip. After generation, the replacement lines were verified by PCR and subsequent sequencing. In addition, we have also successfully generated re-insertion fly lines for the *dfoxo*$^{\Delta V1}$-attP site (data not shown). These lines were verified by sequencing but remain to be fully characterized.

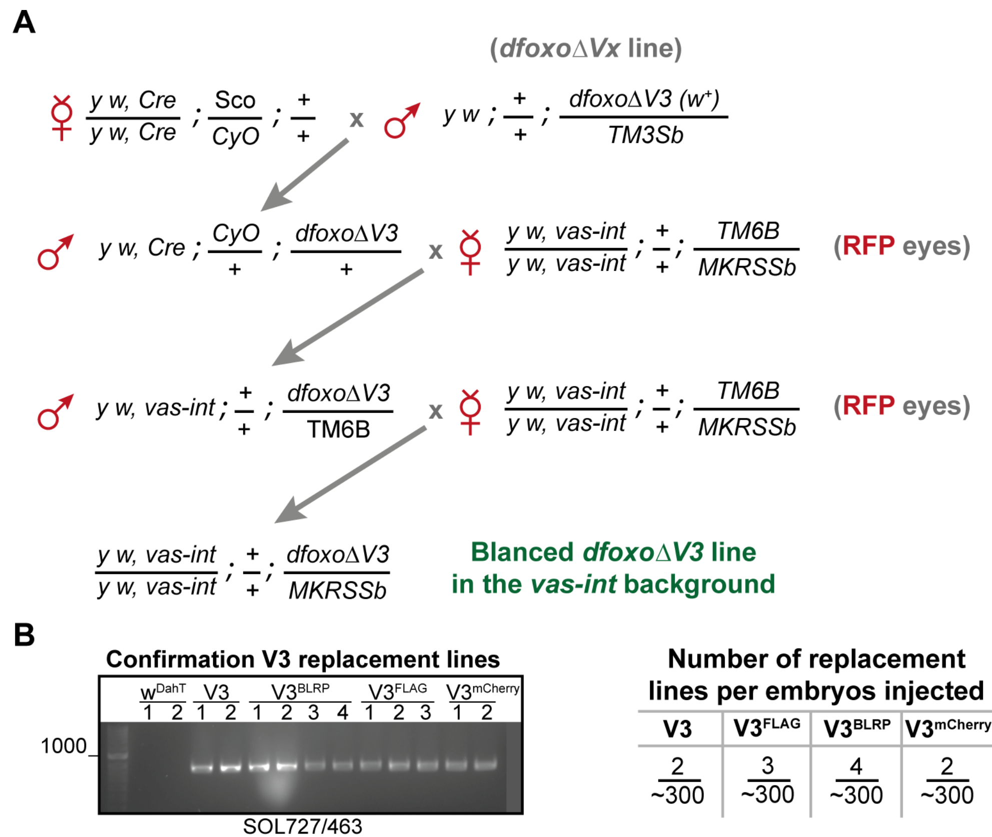

Figure 3.3. Crossing scheme and PCR verification for gene replacements at the *dfoxo V3* locus. (**A**) Crossing scheme to bring *dfoxoΔV3* into the phage integrase ΦC31 (vas-int) background. y-yellow; w-white; cre-recombinase to remove additional DNA containing the vector backbone and the white marker gene located between two loxP sites; Balancer chromosomes for chr. 2 (CyO) and chr. 3 (TM6B and MKRSSb). (**B**) PCR analysis confirmed the correct reinsertion of V3 (wild-type or tagged) gene replacement constructs. Table to the right summarizes the number of lines obtained per number of injected embryos. Fw primer binds to the reintroduced fragment whereas Rv primer binds to arm4 outside of the reinsertion region.

In order to validate the newly generated *dfoxo* mutants and to test whether the gene replacement constructs restored dFOXO function, we first checked the expression of *dfoxo* mRNA by quantitative Real-Time (qRT)-PCR (Figure 3.4A). qRT-PCR confirmed the lack of mRNA expression of the corresponding sequence in the *dfoxo* mutant lines *(Δ lines)* and showed wild type-like expression levels in the *dfoxo* gene replacement lines (Figure 3.4A). As a positive control for the null alleles, we used a previously generated *dfoxo-null* line (*dfoxo*^{Δ94}) (Slack et al., 2011). Expression of *npc2b*, a gene directly downstream of *dfoxo* was not changed in the *dfoxo* mutant or re-insertion fly lines, demonstrating that the introduced modification is specific to the *dfoxo* gene and does not affect the neighbour gene (Figure 3.4B). dFOXO protein was not detected in the *dfoxo* mutants, but was restored in the gene replacement lines, consistent with the qRT-PCR results (Figure 3.4C). In addition, dFOXO-V3^{FLAG} was specifically detected at the

expected size using an anti-FLAG antibody, indicating proper expression of the tagged dFOXOs. Furthermore, subcellular localization of dFOXO-V3^{mCherry} was observed *in vivo* and it was localized in the nucleus in the larval epidermis under the tested conditions (Figure 3.4D). This line was used by Dr. Parisa Kakanj to study the relationship between the IIS pathway and wound healing (Kakanj et al. in press).

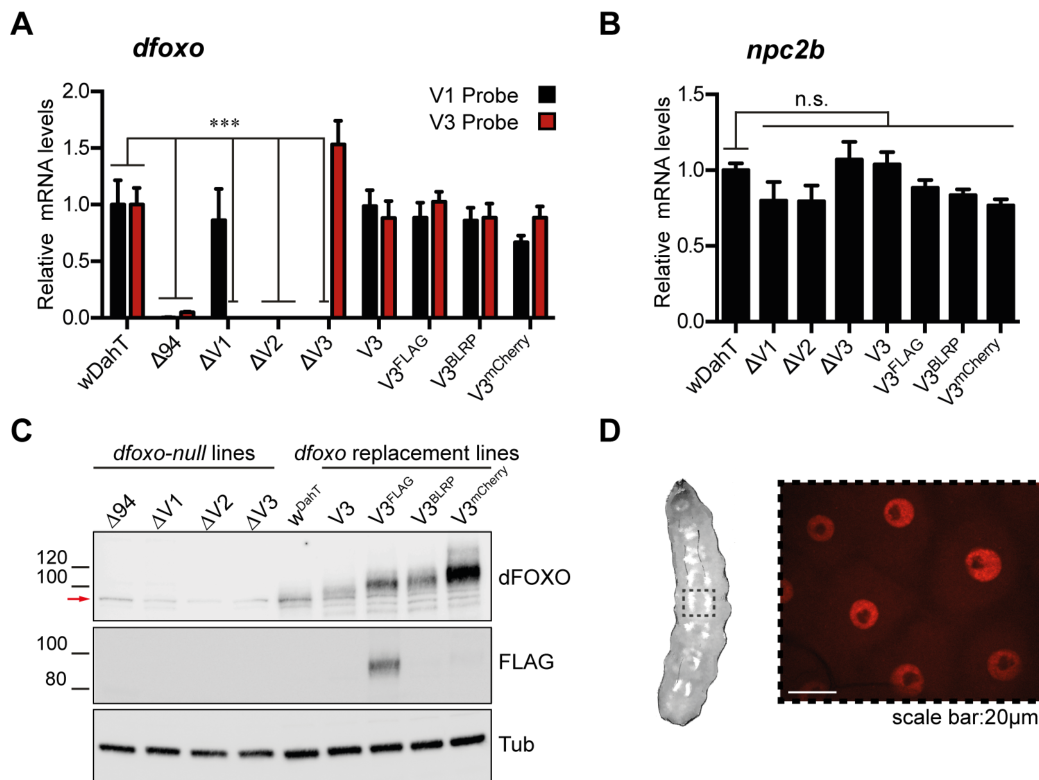

Figure 3.4. Molecular validation of novel *dfoxo-null* mutants and V3 gene replacement lines. (A) Quantitative PCR analysis of relative mRNA levels of *dfoxo* transcript using probes against the *V1* region (black) or the *V3* region (red) in the *dfoxo*-null Δ*94*, (Slack et al., 2011), Δ*V1,* Δ*V2* and Δ*V3*, wild type (w^{DahT}) or gene replacement lines V3, V3^{FLAG}, V3^{BLRP} and V3^{mCherry}. Pairwise comparison between control (w^{DahT}) and all other genotypes tested with the same probe (***p<0.001, Students t-test). **(B)** Relative mRNA levels of *npc2b*, a gene located directly downstream of the *dfoxo* locus (see also Figure 3.5), were unaffected by *dfoxo* mutations or gene replacements. Pairwise comparison between control (w^{DahT}) and all other genotypes tested with the same probe (n.s. p>0.05, Students t-test). **(C)** Western blot on whole-body protein extracts from female flies confirmed dFOXO expression in all gene replacement lines and FLAG tagged-FOXO specifically in dFOXO-V3^{FLAG} line. Red arrow shows unspecific band **(D)** Live imaging of larval epidermis confirms the expression and nuclear localization of dFOXO-V3^{mCherry}. Larvae drawing by Joana Gonçalves and imaging performed by Dr. Parisa Kakanj. (Kakanj et al, in press).

To study physiological phenotypes of the newly generated *dfoxo* alleles, all mutant fly lines were backcrossed into a common genetic background, the outbred w^{DahT} fly strain (Grönke et al., 2010). Removal of the *dfoxo* gene yields smaller flies with a slight delay in

development (Jünger et al., 2003; Slack et al., 2011). Consistent with these phenotypes, the newly generated *dfoxo-null* alleles (ΔV1, ΔV2 and ΔV3) had reduced body weight and were developmentally delayed when compared to the wild type control (w^{DahT}) (Figure 3.5A-C). These phenotypes were fully rescued in the replacement lines, independent of the presence of a tag. Moreover, in replacement lines V3 and $V3^{FLAG}$ the fecundity observed in *dfoxo-null* (ΔV3) flies was rescued (Figure 3.5D). *dfoxo-null* mutants are sensitive to starvation and are short-lived (Slack et al., 2011). This was also the case for the *dfoxo-null* alleles (ΔV1, ΔV2 and ΔV3), whereas the *dfoxo* replacement constructs were able to fully rescue these phenotypes in both male and female flies (Figure 3.6A-D).

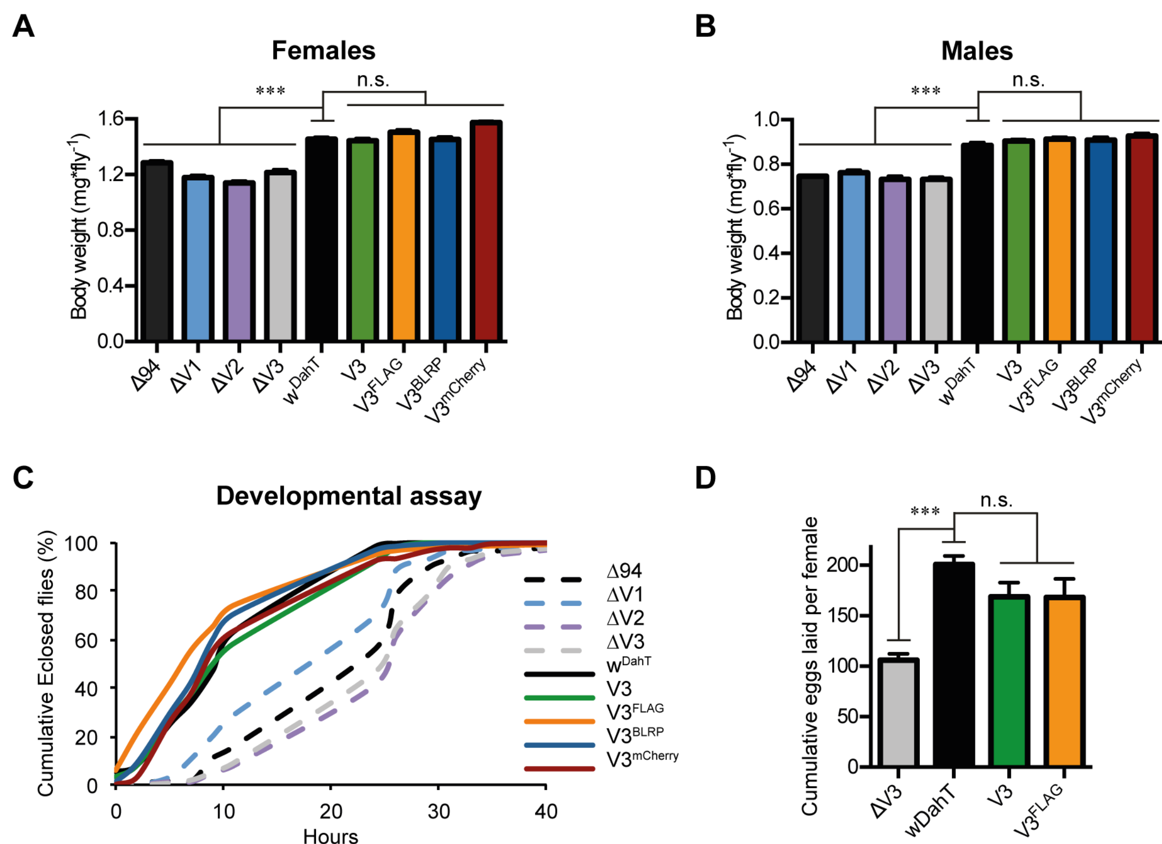

Figure 3.5. Body weight and development time of the novel *dfoxo-null* mutant and V3 gene replacement flies. (A) Female and **(B)** male *dfoxo-null* (Δ) flies had reduced body weight when compared to wild type (w^{DahT}). This reduction in body weight was fully reversed in all V3 replacement lines (n=50/genotype and gender). n.s. p>0.05, ***p<0.001, one-way ANOVA with post-hoc Dunnett's comparison test with control (w^{DahT}). **(C)** All *dfoxo-null* (Δ) mutant flies were developmentally delayed with respect to wild type control and gene replacement flies. n=500 eggs per genotype. **(D)** *dfoxo-null* line ΔV3 showed reduced cumulative eggs laid than wild type flies, as reported before (Slack et al., 2011). The gene replacement lines V3 and $V3^{FLAG}$ showed wild type-like fecundity.

In summary, I have successfully generated three novel *dfoxo-null* mutants (ΔV1, ΔV2 and ΔV3) and shown that the *dfoxo*$^{\Delta V3}$ line can be used to reinsert modified alleles of the *dfoxo* gene to study its *in vivo* function. The C-terminal insertion of the tag did not seem to interfere with dFOXO function.

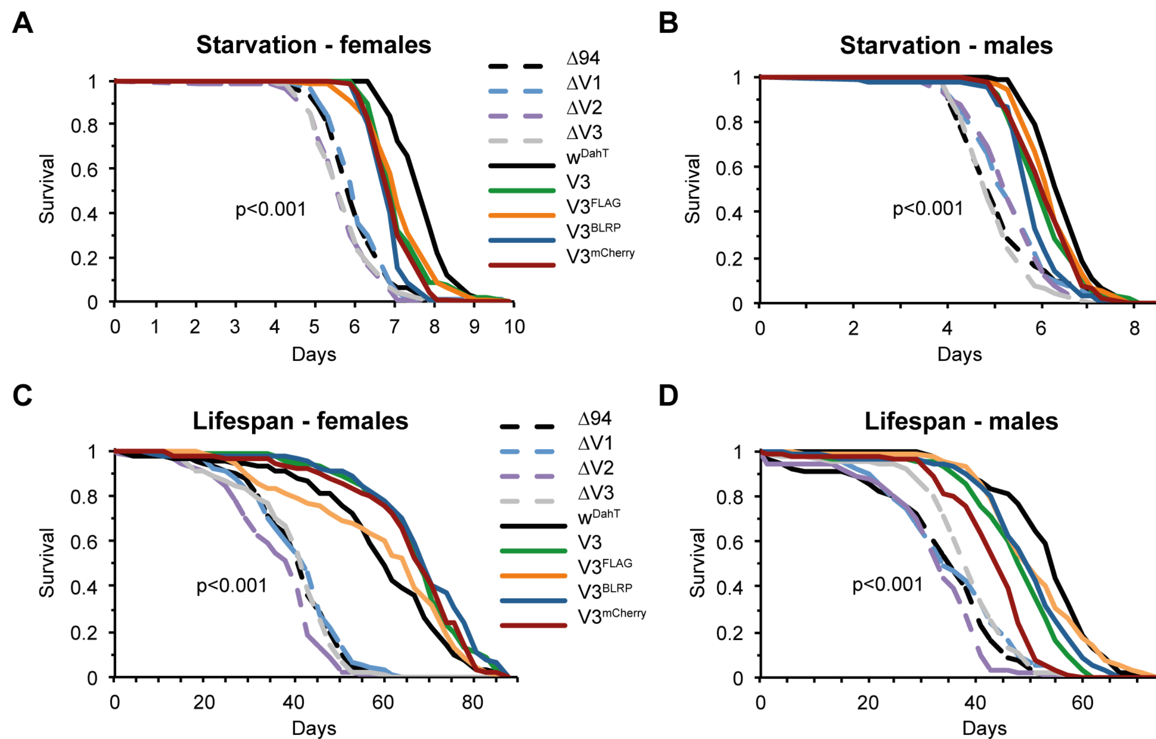

Figure 3.6. Starvation and lifespan of *dfoxo-null* mutants and V3 gene replacement flies. Starvation assay for females **(A)** and males **(B)** showed that all *dfoxo-null* mutants (Δ) were starvation-sensitive, whereas the gene replacement lines ($V3^x$) rescued this sensitivity. Flies of each genotype were placed on starvation medium after 2 days on 1xSYA food (n=100/genotype). ***p<0.001 log-rank test for comparison of *dfoxo-nulls* (Δ) vs control (w^{DahT}) flies. **(C)** Female and **(D)** male lifespan assays showed *dfoxo-null* lines were shorter-lived than wild type control (w^{DahT}), whereas $V3^x$ replacement lines rescued this shortening. Flies of each genotype were placed on 1xSYA food and followed throughout life (n=100/genotype). ***p<0.01 log-rank test for comparison of *dfoxo-nulls* (Δ) vs control (w^{DahT}) flies.

3.2.2 Dissection of dFOXO functions independent of DNA binding

Mammalian cell culture studies suggest that FOXO transcription factors may have functions independent of DNA binding (Matsumoto et al., 2006; Ramaswamy et al., 2002; Zhao et al., 2010). More recently, a study in mice further suggests that FOXO1 can modulate lipid biosynthesis in the liver, probably working as a transcriptional co-regulator, independent of DNA binding (Cook et al., 2015). However, it is currently unknown whether this DNA-binding-independent function of FOXO proteins is restricted to lipid metabolism or is also involved in regulation of other physiological processes like development or lifespan. Furthermore, it is unknown if FOXO's DNA-binding-independent functions are evolutionary conserved or originated recently in the mammalian homologs. To determine whether any of the dFOXO-associated functions are DNA binding-independent in *Drosophila*, I decided to generate endogenous DNA-binding mutants.

3.2.2.1 Identification of DNA binding deficient dFOXO

FOXO proteins share a highly evolutionarily conserved DNA binding domain (DBD) (Figure 3.7A). Therefore, I generated eGFP-tagged dFOXO constructs and mutated one (H150A) or two residues (H150A and N146A) within the DBD to obtain two independent mutants (DBD1 and DBD2, respectively). Both these conserved residues are in direct contact with DNA according to the crystal structure of FOXO1 (Brent et al., 2008) (Figure 3.7B) and mutation of the H150 homolog residue alone is sufficient to abolish DNA binding of mammalian FOXO1 (Ramaswamy et al., 2002; Tang et al., 1999). Unlike wild type dFOXO, DBD mutants are unable to drive luciferase expression under two different promoters, 4xFOXO responsive elements (4xFRE) and Insulin receptor (InR) (Figure 3.7C). This suggests that single mutations within the DBD are sufficient to abolish the ability of dFOXO to induce transactivation of target genes. Remarkably, mutation of these residues does not interfere with the protein's ability to translocate between cytoplasm and nucleus in response to nutritional cues (starvation vs insulin) (Figure 3.7D). These experiments suggest that mutations H150A and N146A interfere with the DNA binding ability of dFOXO.

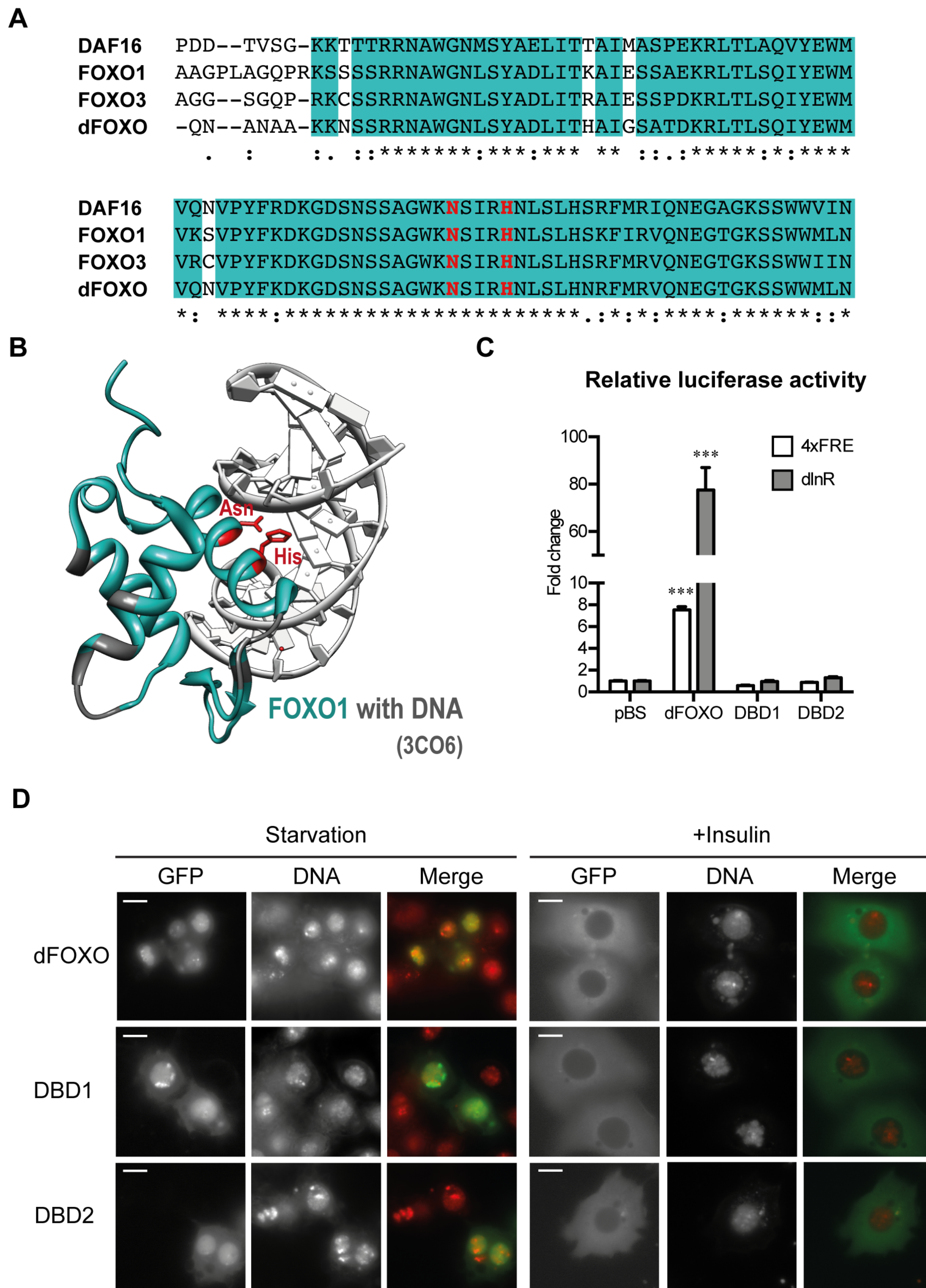

Figure 3.7. The residues mediating dFOXO–DNA interaction are evolutionary conserved. **(A)** Protein alignment of the FOXO DNA binding domain (DBD) between mouse (FOXO1, FOXO3), worm (DAF16) and fly (dFOXO) showed a high degree of conservation. Blue background highlights identical (*), strongly similar (:) or weakly similar (.) residues between sequences. Red residues are conserved mediators of DNA interaction. **(B)** Crystal structure of FOXO1 DBD bound to DNA (3CO6) (Brent et al., 2008) showed the critical location of residues histidine and asparagine that allows them to mediate DNA interaction. Blue resides are identical or similar to dFOXO as shown in the sequence alignment. Red residues are conserved mediators of

DNA interaction. **(C)** Luciferase reporter assay showed mutation of one or both residues is sufficient to abolish dFOXO transactivation activity. S2-R+ cells were transfected with pBluescript (pBS), EGFP tagged dFOXO, -DBD1 or -DBD2 along with reporter luciferase constructs 4xFRE-Luc or InR-Luc. n.s. $p>0.05$, *$p<0.05$, **$p<0.01$, ***$p<0.001$, one-way ANOVA with post-hoc Dunnett's comparison with pBS. **(D)** All dFOXO proteins were able to respond to nutritional cues in *Drosophila* cell culture, as shown by representative fluorescent microscopy images. S2-R+ cells transfected with EGFP tagged dFOXO, -DBD1 and -DBD2, maintained in serum-free medium (2h) and later treated with insulin (10mM) for 10mins. Scale bar = 5µm.

3.2.2.2 Generation and validation of *in locus dfoxo-DBD* mutants

Taking advantage of the already validated *dfoxo*-editing tool and the ΔV3-attP line, we reintroduced two $V3^{FLAG}$ constructs with mutations for DBD1 (H150A) and DBD2 (H150A and N146A) (Figure 3.8A). Reinsertion of these two constructs restored normal dFOXO mRNA and protein levels (Figure 3.8B-C). To verify that mutation of those residues also interfered with DNA binding *in vivo*, we performed a chromatin immunoprecipitation (ChIP) followed by qPCR on mock (V3), replacement ($V3^{FLAG}$) and mutant ($V3^{FLAG}$-DBD2, from here on called DBD2) flies. We evaluated enrichment of proteins on the promoter regions of the known dFOXO target *thor* (homolog of mammalian *4E-BP*). As expected, we found dFOXO, and not DBD2, on the promoter of *thor* (Figure 3.8D), consistent with the hypothesis that the DBD2 mutations interfere with DNA binding of dFOXO *in vivo*.

To evaluate which of the dFOXO functions were affected by the DBD mutations, we tested phenotypes associated with lack of *dfoxo* (Slack et al., 2011). Both DBD mutants have reduced daily and cumulative levels of female fecundity compared to the $V3^{FLAG}$ flies (Figure 3.9A-B). Furthermore, DBD mutants have a reduced lifespan and reduced hydrogen peroxide resistance in both males and females (Figure 3.9C-F). These results indicate that mutation of the DBD yields a dFOXO protein unable to fulfill many of the functions associated with this transcription factor. Even more, it suggests that normal fecundity, lifespan and oxidative stress resistance require a transcriptional output that is dFOXO-dependent.

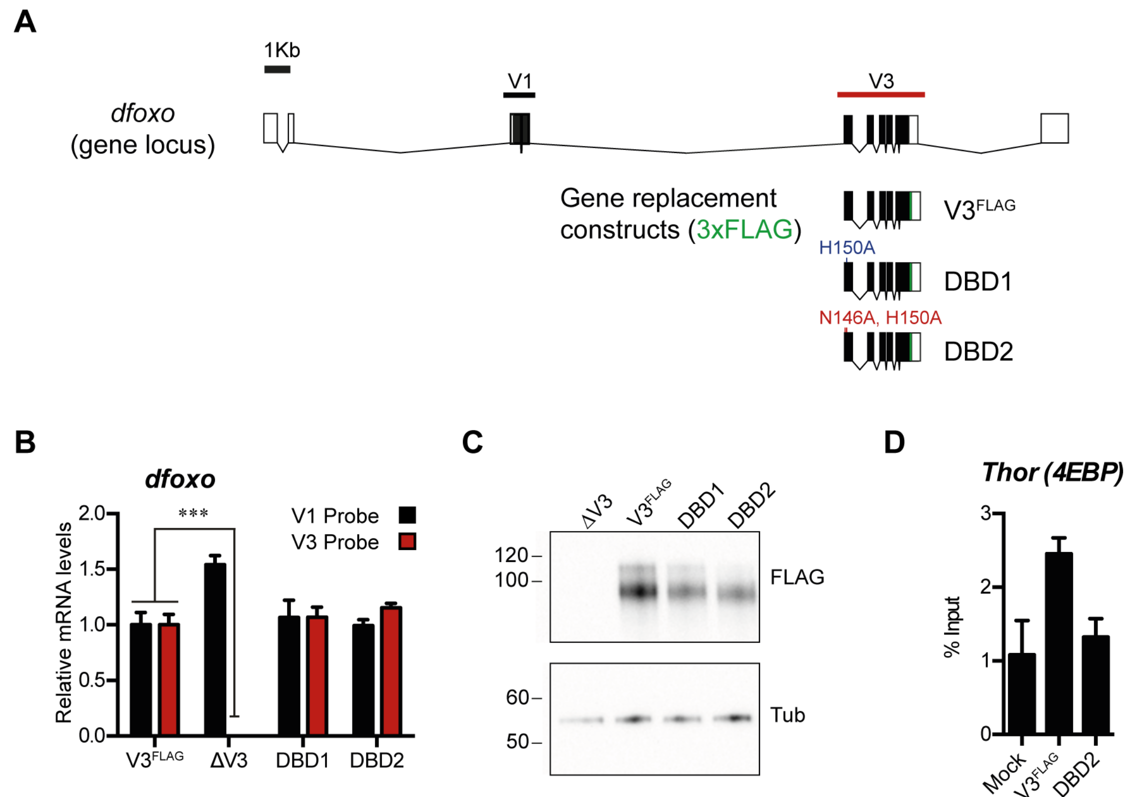

Figure 3.8. Generation and molecular validation of dFOXO-DBD mutants *in vivo.*
(A) Schematic view of the *dfoxo* gene *locus* and the gene replacement constructs used to generate the DBD mutant lines. Boxes represent UTRs (white boxes) and the CDS (black boxes) within the exons. Regions arbitrarily denoted V1 (1Kb) and V3 (3Kb). **(B)** Quantitative PCR analysis of relative mRNA levels of *dfoxo* showed transcript levels restored to normal in DBD gene replacement lines. Probes against the V1 region (black) or the V3 region (red) were used in *dfoxo-null* (ΔV3) and gene replacement lines $V3^{FLAG}$, DBD1 ($V3\text{-}DBD1^{FLAG}$) and DBD2 ($V3\text{-}DBD2^{FLAG}$). **(C)** Western blot on whole-body protein extracts from female flies confirmed proper $dFOXO^{FLAG}$ expression in all gene replacement lines. **(D)** Chromatin Immunoprecipitation (ChIP) experiment showed dFOXO ($V3^{FLAG}$), but not DBD2 nor a mock control (V3 without FLAG), can be found at the promoter region of the know dFOXO-target gene *4ebp*. Chromatin was evaluated by qPCR using primers SOL988-989, previously reported in (Liu et al., 2014).

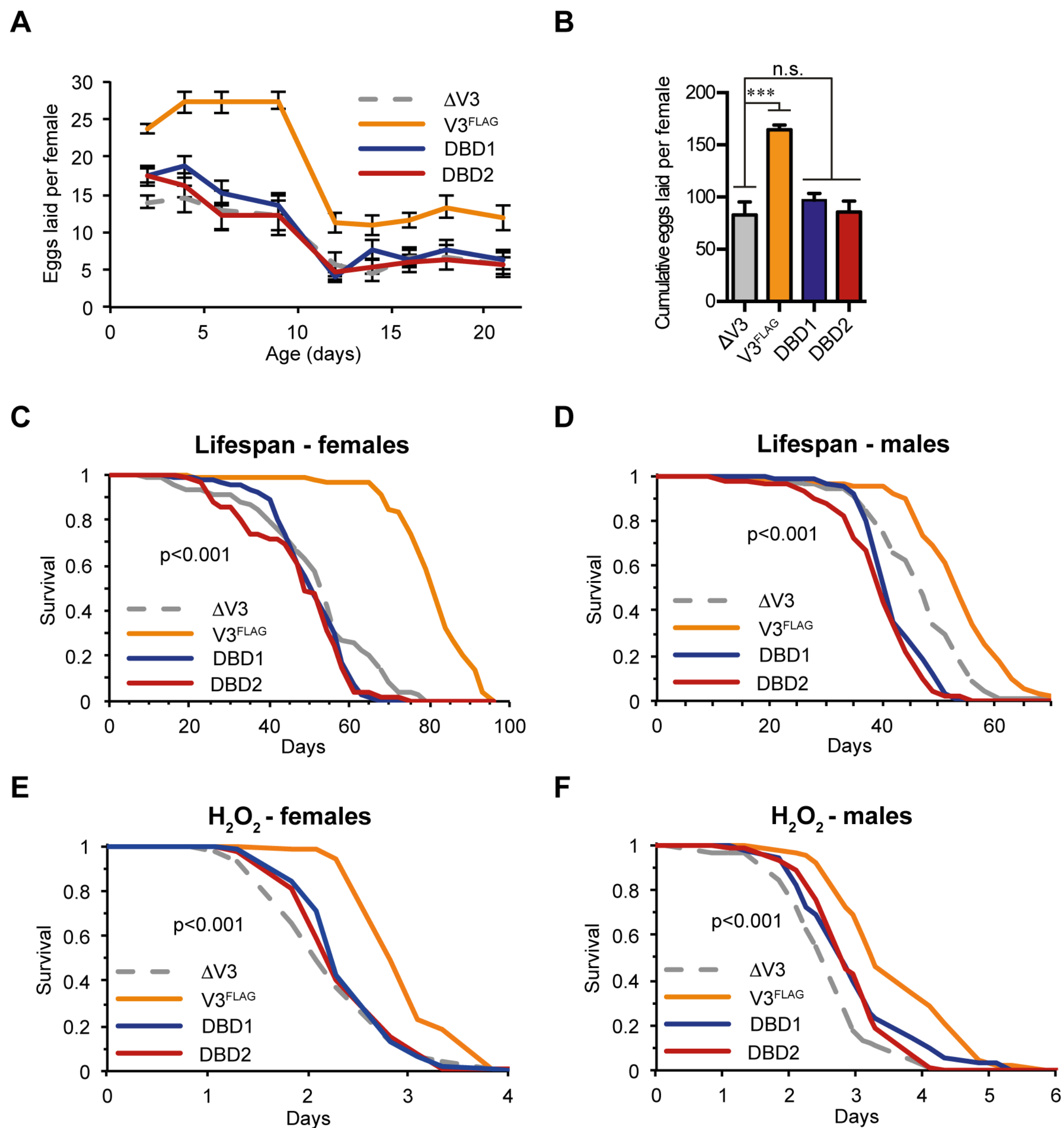

Figure 3.9. DBD mutations interfere with normal dFOXO functions *in vivo.*

(A) Daily and **(B)** cumulative fecundity was equally reduced in *dfoxo-null* (ΔV3) and DBD mutants when compared to V3^{FLAG} flies. n.s. p>0.05, ***p<0.001, one-way ANOVA with post-hoc Dunnett's comparison with ΔV3 flies **(C)** Female and **(D)** male lifespan assays showed DBD mutants were, just like ΔV3, shorter lived than V3^{FLAG} flies. ***p<0.001 log-rank test for comparison of *dfoxo-null* (ΔV3) or DBD mutants vs control (V3^{FLAG}). Flies of each genotype were placed on 1xSYA food and followed throughout life (n=100/genotype). **(E)** Female and **(F)** male ΔV3 and DBD flies were sensitive to oxidative stress when compared to V3^{FLAG}. ***p<0.001 log-rank test for comparison of *dfoxo-null* (ΔV3) or DBD mutants vs control (V3^{FLAG}). Flies of each genotype were placed on H_2O_2 media after seven days on 1xSYA food (n=100/genotype).

3.2.2.3 dFOXO regulates body size independent of DNA binding

Lack of *dfoxo* causes delayed development and smaller body size (Slack et al., 2011). Therefore, we evaluated whether DBD mutations would interfere with fly development. DBD mutants were developmentally delayed (Figure 3.10A), even though the delay was not as strong as the one observed for the *dfoxo-null* mutant allele *dfoxo*$^{\Delta V3}$. Surprisingly, DBD mutants showed normal hatching rates and bodyweights for both males and females (Figure 3.10B-D), suggesting that these phenotypes are regulated independently of DNA binding.

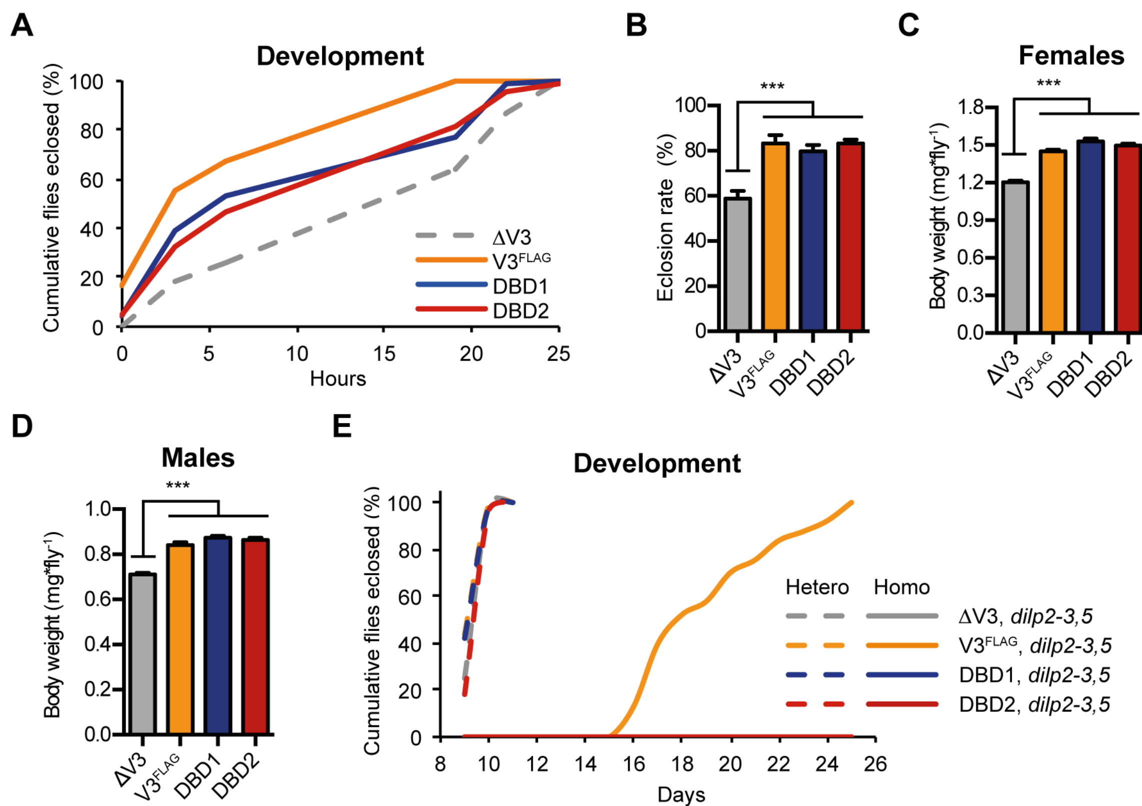

Figure 3.10. dFOXO regulates some developmental features independent of DNA binding. **(A)** DBD mutants showed a small developmental delay with respect to V3^{FLAG} although not as drastic as the one in *dfoxo*$^{\Delta V3}$ flies. **(B)** The characteristic reduced eclosion rate of *dfoxo-null* flies was fully rescued by the DBD mutants. (n=500; eggs per genotype were picked on 1xSYA food and followed at 25°C). **(C)** Female and **(D)** male DBD mutants restore normal body weight (n=50/genotype and gender). ***p<0.001, one-way ANOVA with post-hoc Dunnett's comparison with ΔV3 flies. **(E)** *dfoxo*$^{\Delta V3}$ and DBD mutant flies were unviable when placed in a *dilp2-3,5* background, a model of reduced insulin signalling (Grönke et al., 2010). n=~600 eggs per genotype were squirted on 1xSYA food and followed at 25°C.

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When we introduced the DBD mutants in a model of reduced IIS, the *dilp2-3,5* mutant background (Grönke et al., 2010), this combination caused lethality, similar to the effect with the *dfoxo-null* (ΔV3) allele (Figure 3.10E). Similarly, over-expression of dFOXO and the DBD mutants using a constitutive driver, *da*-Gal4 caused lethality during development (data not shown). These observations suggest that some functions of dFOXO during development are independent of DNA binding and, hence, further studies are required to understand the mechanism by which dFOXO regulates them.

3.2.2.4 dFOXO promotes lipid usage independent of DNA binding

FOXO transcription factors, especially FOXO1, have important functions in carbohydrate and lipid metabolism in response to insulin (Kousteni, 2012). Moreover, dFOXO is a mediator of the starvation response in *Drosophila* (Chatterjee et al., 2014; Teleman et al., 2008). Several studies in mammalian cell culture and a recent *in vivo* study in mice suggest that the ability of FOXO1 to regulate lipid metabolism in the liver is, at least partially, independent of its DNA binding ability (Cook et al., 2015; Matsumoto et al., 2006). However, the mechanism by which this is achieved is still unclear.

To test whether lipid metabolism was altered in the DBD mutants, we first evaluated starvation sensitivity as a proxy for lipid storage. Surprisingly, reintroduction of the DBD mutants was able to fully rescue, in males and females, the starvation sensitivity observed in *dfoxo-null* (ΔV3) flies (Figure 3.11A-B). This sensitivity to starvation in the dFOXO$^{\Delta V3}$ flies compared to the other genotypes could be due to either reduced lipid synthesis or impaired mobilization of fat stores. Thus, we evaluated the lipid content of pre-starvation and starved-to-death flies. We observed no significant differences between genotypes pre-starvation (Figure 3.11C-D), suggesting that fat synthesis and storage is not affected by lack of dFOXO or in the DBD mutants. In fact, mRNA levels of two key enzymes for TAG biosynthesis, *fasn-1* and *acc*, were not changed under normal conditions in any of the tested genotypes (Figure 3.12A). Moreover, feeding of a high sugar or high fat diet lead to an increase of lipid accumulation in all the genotypes (Figure 3.12B), suggesting that lack of dFOXO function does not affect fatty acid synthesis.

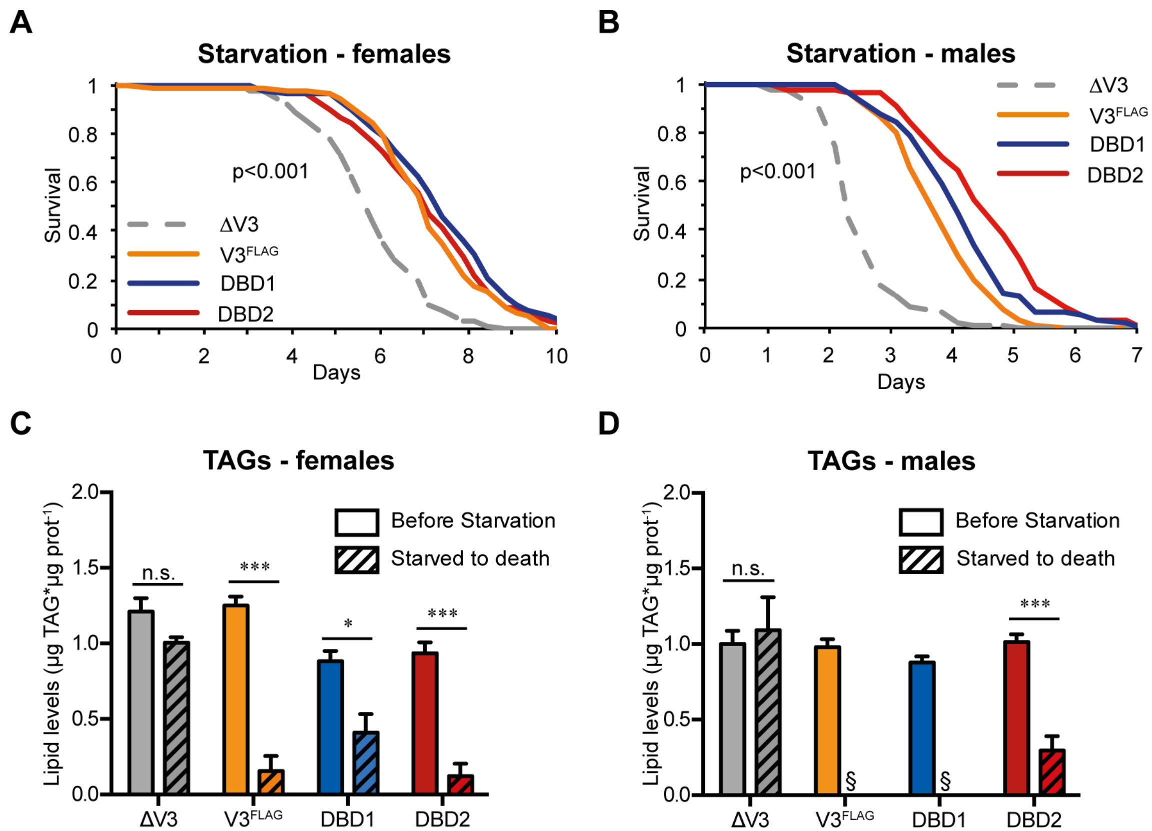

Figure 3.11. dFOXO regulates lipid usage independent of DNA binding.
(A) Starvation assay for females and **(B)** males showed DBD mutants, unlike ΔV3 flies, were as resistant to starvation as $V3^{FLAG}$ flies. ***p<0.001 log-rank test for comparison of *dfoxo-null* (ΔV3) and $V3^{FLAG}$ or DBD mutant flies. Flies of each genotype were placed on starvation medium after 7 days on 1xSYA food (n=100/genotype). **(C)** TAG content of female or **(D)** male flies before starvation or post-mortem revealed *dfoxo-null* (ΔV3) flies had problems mobilizing fat stores, whereas DBD mutants rescued this phenotype. Five biological replicates per assay were used, each of them with n=4 flies/genotype and gender. §-marked samples had no detectable TAG content.

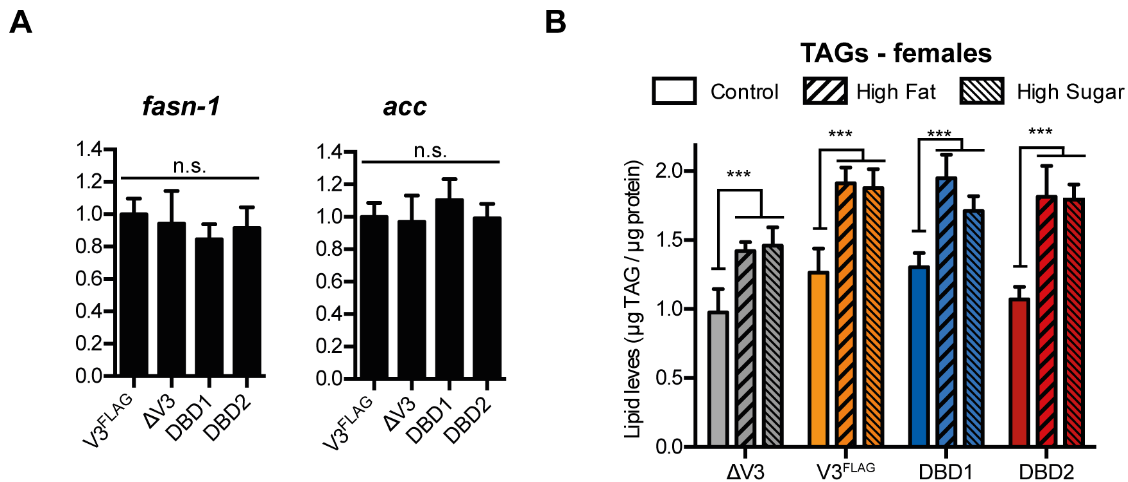

Figure 3.12. dFOXO does not seem to be required for fat synthesis.
(A) Key enzymes involved in TAG biogenesis (*fasn-1* and *acc*) were expressed at comparable levels as assessed by quantitative PCR of relative mRNA levels **(B)** TAG content of female flies kept for seven days on control (1xSYA), high fat (5% soy oil) and high sugar (4xSugar on 1xSYA) food showed all genotypes were able to built extra fat. Five biological replicates per assay were used, each of them with n=4 flies/genotype and gender.

dfoxo$^{\Delta V3}$ null mutants flies had increased lipid levels post mortem compared to *dfoxo* replacement (V3^{FLAG}) and DBD mutants flies. There was no significant difference between pre-starvation and post-mortem lipid levels in the *dfoxo*$^{\Delta V3}$ null mutants, suggesting that dFOXO function is required for lipid mobilization under starvation. In contrast, dFOXO-V3^{FLAG} and -DBD mutant flies mobilized most of their lipid stores under starvation, suggesting that the presence of dFOXO, but not its DNA binding ability, is required to fully mobilize fat under starvation (Figure 3.11C-D). Upon starvation dFOXO migrates into the nucleus in cell culture (Figure 3.7D). Likewise, starvation induces dFOXO nuclear localization *in vivo* in the fat body of adult flies (Chatterjee et al., 2014). This observation suggests that dFOXO could be required in the nucleus, perhaps as a co-factor, to regulate expression of genes involved in the starvation-response independent of DNA binding.

Starvation induces the transcription of multiple genes (Chatterjee et al., 2014; Zinke et al., 2002). To test how starvation-induced genes were regulated in the DBD mutants, we evaluated their mRNA levels under control and starvation conditions (Figure 3.13A-F). Expression of the insulin receptor (*InR*), a known dFOXO target, was increased by ~2 fold upon starvation in all genotypes. This indicates that, although a dFOXO target (Alic et al., 2011; Puig et al., 2003), *InR* expression under starvation is largely independent of this

transcription factor (Figure 3.13A). In contrast, expression of *thor* was fully dependent on dFOXO's ability to bind DNA, since neither *dfoxo*$^{\Delta V3}$ nor the DBD mutants showed increased expression of *thor* under starvation (Figure 3.13B). Phosphoenolpyruvate carboxykinase (*pepck*), the rate-limiting enzyme of gluconeogenesis, was induced by starvation in all genotypes, indicating that dFOXO is not required for its transcription (Figure 3.13C). These results suggest that starvation-induced *thor*-repression of translation, but not gluconeogenesis, is dependent on dFOXO and its ability to bind DNA.

Among the multiple starvation-induced genes, lipases required for triglyceride (TAG) hydrolysis are of prime importance for survival under starvation. *Brummer* (*bmm*), the *Drosophila* homolog of mammalian *adipose triglyceride lipase* (ATGL), is the rate limiting enzyme for cytosolic TAG hydrolysis (Grönke et al., 2005). Bmm was recently shown to be a dFOXO target under starvation conditions (Wang et al., 2011). Consistently, *dfoxo-null* (ΔV3) and -DBD mutants were unable to properly up-regulate *bmm* under starvation (Figure 3.13D). This observation indicates that, even though dFOXO enhances *bmm* expression under starvation, this is not fully required for proper lipid mobilization, since the DBD mutants are able to survive fasting similar to wild type flies. Similarly, lipase 4 (*lip4*), a lipase associated with the lysosome and presumably involved in TAG hydrolysis by the lipophagosome, was shown to be a dFOXO target (Vihervaara and Puig, 2008). However, *lip4* is greatly induced in the presence or absence of dFOXO (Figure 3.13E), suggesting that other factors may also regulate its expression under starvation.

Lipase 3 (*lip3*), another putative lysosome associated lipase, is greatly induced in wild type larvae under starvation, similar to *lip4* (Zinke et al., 2002, 1999). *Lip3* expression seems to be fully dependent on the starvation stimuli, since its mRNA is almost undetectable under control conditions in all tissues of *larvae* or adult flies according to FlyAtlas (Chintapalli et al., 2007). Consistently, *lip3* expression was strongly induced in V3^{FLAG}, but not in *dfoxo*$^{\Delta V3}$, flies (Figure 3.13F). Surprisingly, DBD mutants were able to, at least partially, rescue *lip3* expression under starvation conditions (Figure 3.13F). This observation indicates that *lip3* starvation-induced expression is dependent on dFOXO but not on its DNA binding ability, suggesting dFOXO indirectly regulates the expression of *lip3*.

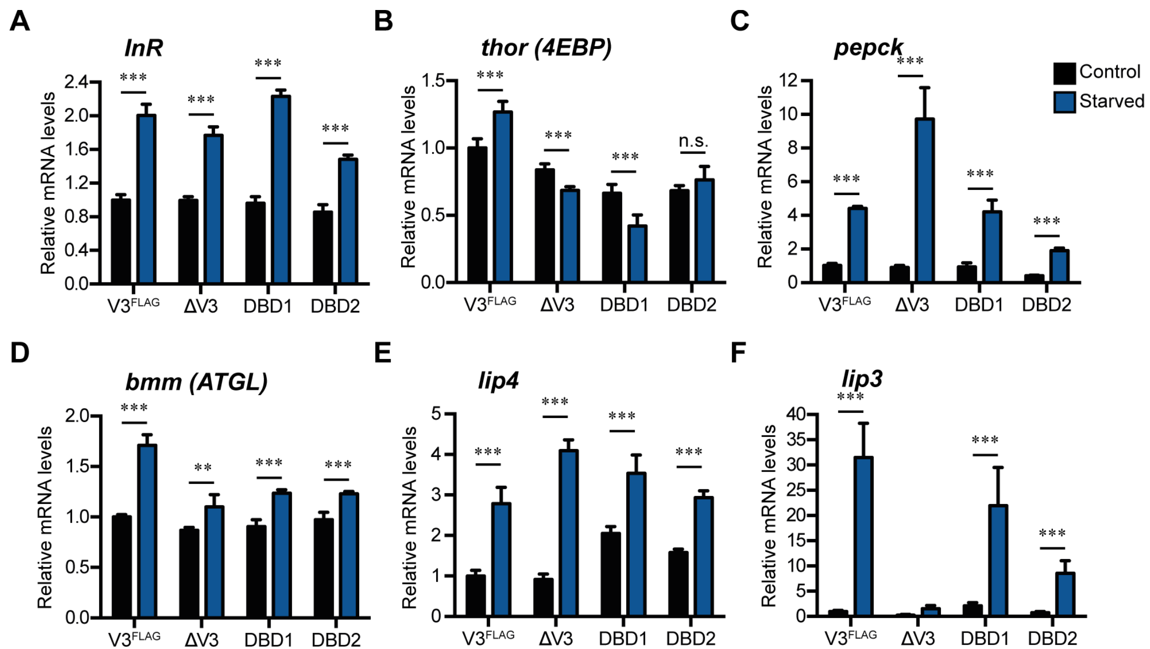

Figure 3.13. dFOXO regulates multiple genes involved in the starvation response.
(A-F) Relative mRNA levels of starvation-inducible genes (*InR, thor, pepck, bmm, lip3* and *lip4*) under control (black) or starved (48h – blue) conditions assessed by qPCR. n.s. $p>0.05$, *$p<0.05$, **$p<0.01$, ***$p<0.001$, Students t-test.

In summary, dFOXO promotes lipid usage, partly in a DNA binding-independent manner. One possible explanation for this is that, under starvation, dFOXO may act as a co-activator of different transcription factors to facilitate the expression of fasting-inducible genes, such as *lip3*. Therefore, we decided to evaluate which transcription factor(s) could regulate *lip3* expression and test whether *lip3* is in fact a limiting factor for lipid usage under starvation.

3.2.2.5 dHNF4 seems to regulate *lip3* expression in an indirect manner.

In order to better understand how *lip3* is regulated under starvation, we looked at the promoter region of this gene for binding motifs of different transcription factors. To do this, we used the *motif scanning* program FIMO (Grant et al., 2011) to compare the promoter sequence to several databases containing characterized motifs of different transcription factors in diverse model organisms. Among the many suggested motifs, two seemed very relevant for this study, a FOXO motif ~250bp upstream of the start codon and a HNF4 motif ~1380bp upstream. The same HNF4 motif was discovered using NHRscan tool (Sandelin and Wasserman, 2005). *Drosophila* HNF4 (dHNF4) is a known mediator of the starvation response and, when mutated, *lip3* is no longer induced under fasting conditions (Palanker et al., 2009). The presence of a FOXO and a HNF4 motif

opened the possibility of direct regulation of *lip3* by these transcription factors. To test whether dHNF4 regulates *lip3* transcription we generated two luciferase-reporter constructs: a long (~1600 bp) *lip3* promoter region, containing both the HNF4 and FOXO motifs, and a short (~600 bp) *lip3* promoter region, which contains only the FOXO motif (Figure 3.14A). Unexpectedly, over-expression of dHNF4 did not enhance, but rather repressed, the expression of both *lip3*-promoter constructs (Figure 3.14B-C). This repression was observed even in the presence of a known dHNF4 activating ligand, palmitic acid (Palanker et al., 2009). This observation suggests that, at least *in vitro*, *lip3* may not be a dHNF4 direct target.

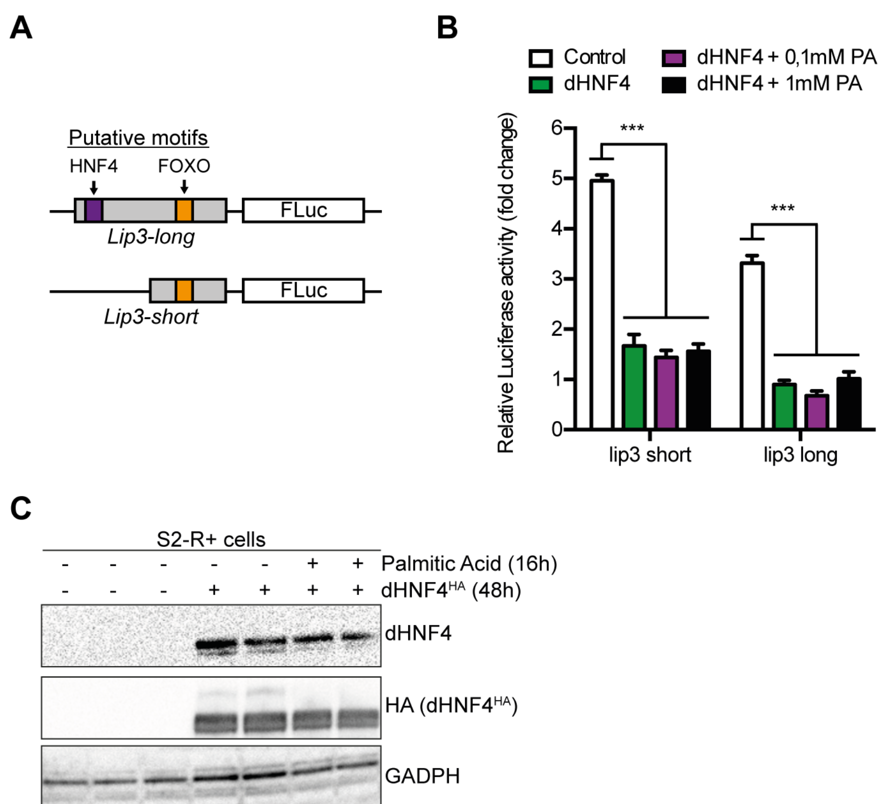

Figure 3.14. dHNF4 does not induce *lip3* expression in cell culture.
(A) Schematic representation of the reporter luciferase constructs generated to drive luciferase expression under the lip3 long (1.3Kb) or short (0.6Kb) promoter regions. Bioinformatic analysis revealed the presence of, among many others, putative binding sites for HNF4 and FOXO factors in the lip3 promoter – Analysis performed by the Bioinformatic core facility. **(B)** Luciferase reporter assay showed dHNF4 represses gene expression under the lip3 promoter, even in the presence of its reported activator (Palmitic acid – PA) (Palanker et al., 2009). S2-R+ cells were transfected with pBluescript (pBS) or dHNF4^{HA} along with reporter luciferase constructs lip3-long-Luc or lip3-short-Luc. Luciferase assay performed by Ralf Meilenbrock. **(C)** Western blot of independently transfected cell confirmed the correct overexpression of dHNF4 under the tested conditions.

A mammalian cell culture study previously reported that FOXO1 and HNF4 are able to synergistically up-regulate *Glucose-6-phosphatase* under fasting conditions (Hirota et al., 2008). Moreover, dHNF4 mediates the expression of genes involved at different levels of starvation response (Palanker et al., 2009). Hence, we decided to test whether the expression of some of these putative, highly expressed, dHNF4 targets are affected by *dfoxo* mutation. The selected genes are involved in free fatty acid (FFA) transport into mitochondria (*AcCoAS*, *CG2107*, *colt*), and the β-oxidation cascade (*CG9577*, *scully*, *yip2*). Even though the expression patterns are somewhat variable, none of the tested genes seemed to depend on dFOXO for normal expression under control or starved conditions (Figure 3.15A-F). This suggests that, at least for these genes, there is no synergy between dFOXO and dHNF4.

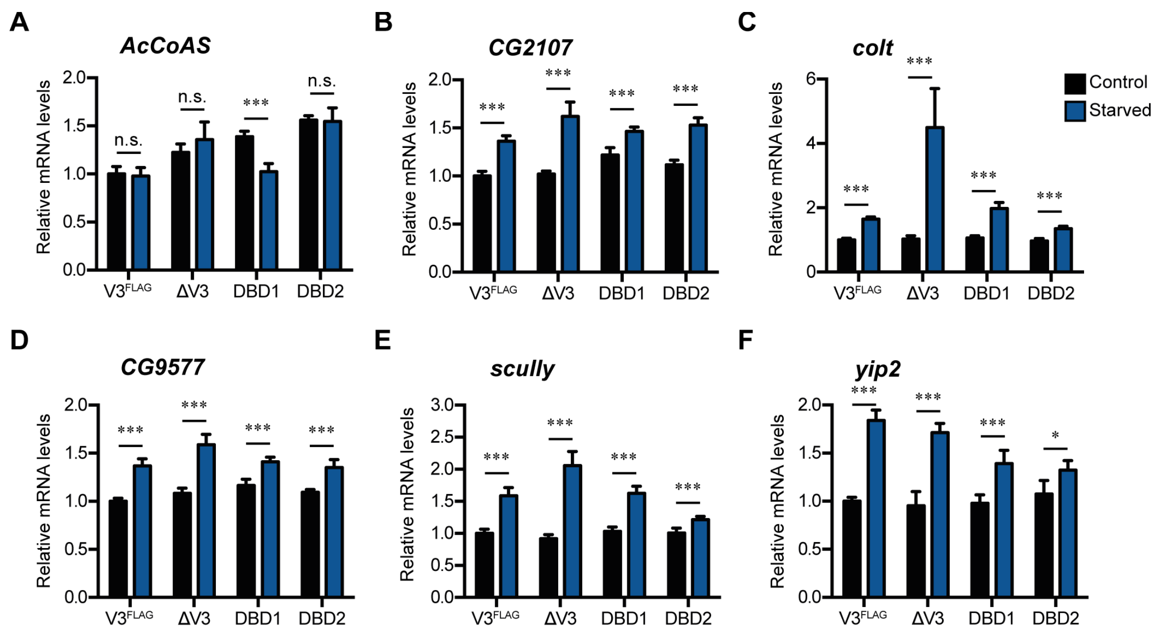

Figure 3.15. Relative expression of dHNF4-target genes (involved in the starvation response) is dFOXO independent. **(A-F)** Relative mRNA levels of dHNF4-regulated genes, involved in free fatty acid transport into mitochondria (*AcCoAS*, *CG2107*, *colt*), and β-oxidation (*CG9577*, *scully*, *yip2*), under control (black) or starved (48h – blue) conditions was unaffected by the presence of dFOXO. n.s. p>0.05, *p<0.05, **p<0.01, ***p<0.001, Students t-test.

In summary, even though dHNF4 is a fundamental mediator of the starvation response in *Drosophila*, the regulation it exerts on target genes associated with starvation seems to be independent of dFOXO. Future studies should address whether dHNF4 and dFOXO can physically or genetically interact under different circumstances.

3.2.2.6 Is *lip3* fundamental for lipid mobilization under starvation?

lip3 expression is strongly up-regulated upon starvation in larvae (Zinke et al., 2002, 1999). Therefore, we considered that the lack of *lip3* induction in the *dfoxoΔV3* flies could be limiting for lipid mobilization under starvation. To test if reduced *lip3* induction can cause starvation sensitivity, we constitutively overexpressed Lip3^{RNAi}, in fat tissue (*FB-* and *GS-Gal4*) or whole body (*da-Gal4*), and evaluated the starvation sensitivity of these flies. When compared to the respective driver controls, these flies were slightly more sensitive to starvation (Figure 3.16A). However, the *UAS* control line (Lip3^{RNAi}/+) also showed a mild detrimental effect, suggesting the observed effects could be due to leaky expression of Lip3^{RNAi}. We also checked *lip3* expression levels while overexpressing Lip3^{RNAi} in the whole fly and found a ~60% reduction of *lip3* mRNA levels upon starvation (Figure 3.16B). This reduction, although significant, may not be sufficient to completely block *lip3* function in the starvation response. In the future, it would be interesting to generate a full *lip3* knockout to assess whether lip3 function is essential for lipid mobilization under starvation conditions.

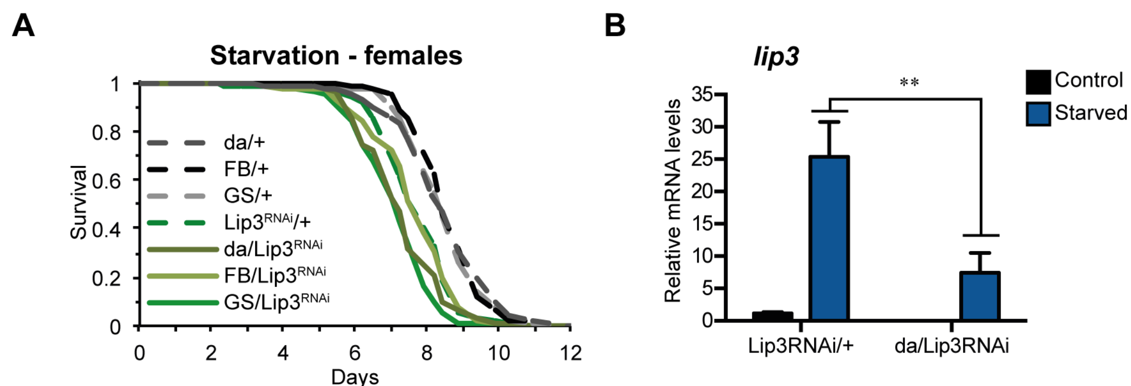

Figure 3.16. Partial *lip3* repression by RNAi is insufficient to mimic the starvation sensitivity *from dfoxo-null* mutation. **(A)** Constitutive lip3^{RNAi} over-expression showed a small increase in starvation-sensitivity compared to the driver controls, but was unaffected when compared to the UAS control line (UAS-Lip3^{RNAi}/+). ***$p<0.001$ log-rank test for comparison of RNAi over-expression lines vs the respective driver control. n.s. $p>0.05$ log-rank test for comparison of RNAi over-expression lines vs UAS-lip3/+ line. da – whole body driver; FB and GS – Fat body 'specific' driver. Flies of each genotype were placed on starvation media after seven days on 1xSYA food (n=100/genotype). **(B)** Relative mRNA levels of *lip3* under control (black) or starved (48h – blue) conditions showed a reduction in expression of ~60%. n.s. $p>0.05$, *$p<0.05$, **$p<0.01$, ***$p<0.001$, Students t-test.

To determine whether lack of *lip3* induction is in fact limiting the starvation resistance of *foxo-null* flies, we decided to overexpress *lip3* in *dfoxoΔV3* flies and assess their starvation resistance. Therefore, we used the $S_1$106 *gene switch* driver that induces expression only in the presence of the drug RU486 (Giannakou et al., 2007; Roman et al., 2001). We observed an increase in dFOXO protein levels, consistent with a previous study where starvation was shown to induce *dfoxo* expression (Villa-Cuesta et al., 2010) (Figure 3.17A). Moreover, we saw some $Lip3^{HA}$ protein even in the absence of RU486 (Figure 3.17A), indicating leaky expression, resulting in a constitutive but mild overexpression. Nevertheless, $Lip3^{HA}$ overexpression was not sufficient to rescue the starvation sensitivity of *dfoxo-null* flies (Figure 3.17B), suggesting that additional dFOXO-dependent mechanisms are required for proper lipid mobilization.

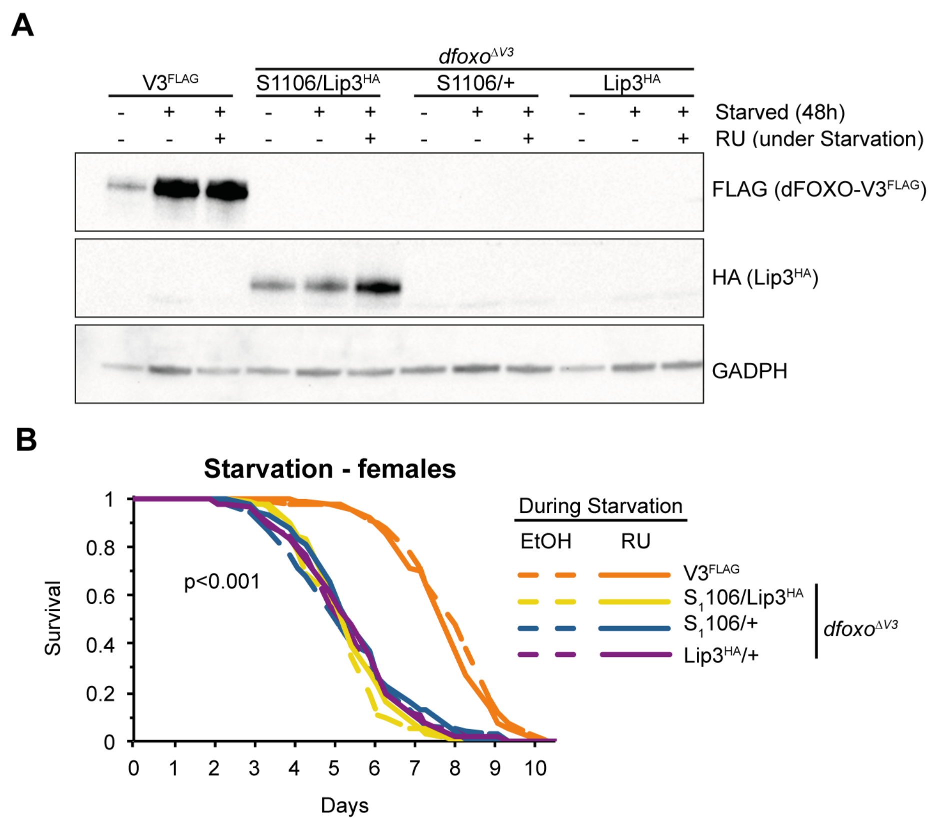

Figure 3.17. *lip3* overexpression in the fat body does not rescue the starvation sensitivity *dfoxo-null* flies. **(A)** Western blot on whole-body protein extracts from female flies showed $lip3^{HA}$ over-expression under the control of the $S_1$106GS-Gal4 inducible driver, even in the absence of RU. **(B)** $lip3^{HA}$ over-expression under the $S_1$106GS-Gal4 driver in the *dfoxo-null* background (ΔV3) did not increase nor restore wild type starvation resistance. ***$p<0.001$ log-rank test for comparison of *dfoxo-null* background lines (ΔV3) vs control ($V3^{FLAG}$). Flies of each genotype were placed on starvation medium after seven days on 1xSYA food (n=100/genotype).

TAG hydrolysis, to release FFAs as β-oxidation substrate and thereby increase energy production, is catalyzed by lipid-droplet associated enzymes, such as Bmm lipase and by a mechanism called lipophagy. Lipophagy involves the autophagic machinery, which engulfs lipid droplets and, after fusing with the lysosome, pH sensitive lipases, such as *lip4* and *lip3*, hydrolyze these lipids. In order to test whether dFOXO function is important for starvation-induced autophagy in *Drosophila* and whether this function requires dFOXO DNA binding ability, we did a western blot analysis with *dfoxo-null* (Δ*V3*), wild type replacement ($V3^{FLAG}$) and DBD mutant flies using an antibody against the autophagy-cargo protein Ref2p, known as p62 in mammals. Ref2p accumulates when autophagy is disturbed and can therefore be used as a marker for deficient autophagy (Nagy et al., 2015; Pankiv et al., 2007). Under long-term starvation conditions, Ref2p accumulated in *dfoxo-null* flies, but not in replacement ($V3^{FLAG}$) flies (Figure 3.18A-B), indicating that dFOXO is required for proper starvation-induced autophagy. Interestingly, Ref2p accumulation was much lower in the DBD mutants compared to *dfoxo-null* flies, suggesting that dFOXO, but not its DNA binding activity, is fundamental for starvation-induced autophagy.

Mammalian FOXO transcription factors are able to transcriptionally regulate multiple genes involved in autophagy, such as *atg5*, *LC3* (*atg8*) and *atg14*, in different tissues (Webb and Brunet, 2014). This function seems to be evolutionarily conserved since muscle-specific over-expression of dFOXO can up-regulate *atg8a* and, hence, induce autophagy (Bai et al., 2013). Moreover, *atg14* expression is critical for lipophagy induction in the mouse liver under starvation conditions (Xiong et al., 2012). Therefore, we tested the transcript levels of three important autophagy-related genes, *atg5*, *atg8a* and *atg14*. Starvation induced a mild, but consistent, expression of the three *atg* genes in replacement ($V3^{FLAG}$), *dfoxo-null* and DBD mutant flies (Figure 3.18C-E), indicating that dFOXO function is not required for their starvation-dependent induction. This observation suggests that lack of *atg* gene expression is not sufficient to explain the autophagy deficiency of *dfoxo-null* mutants and that there must be another currently unknown mechanism.

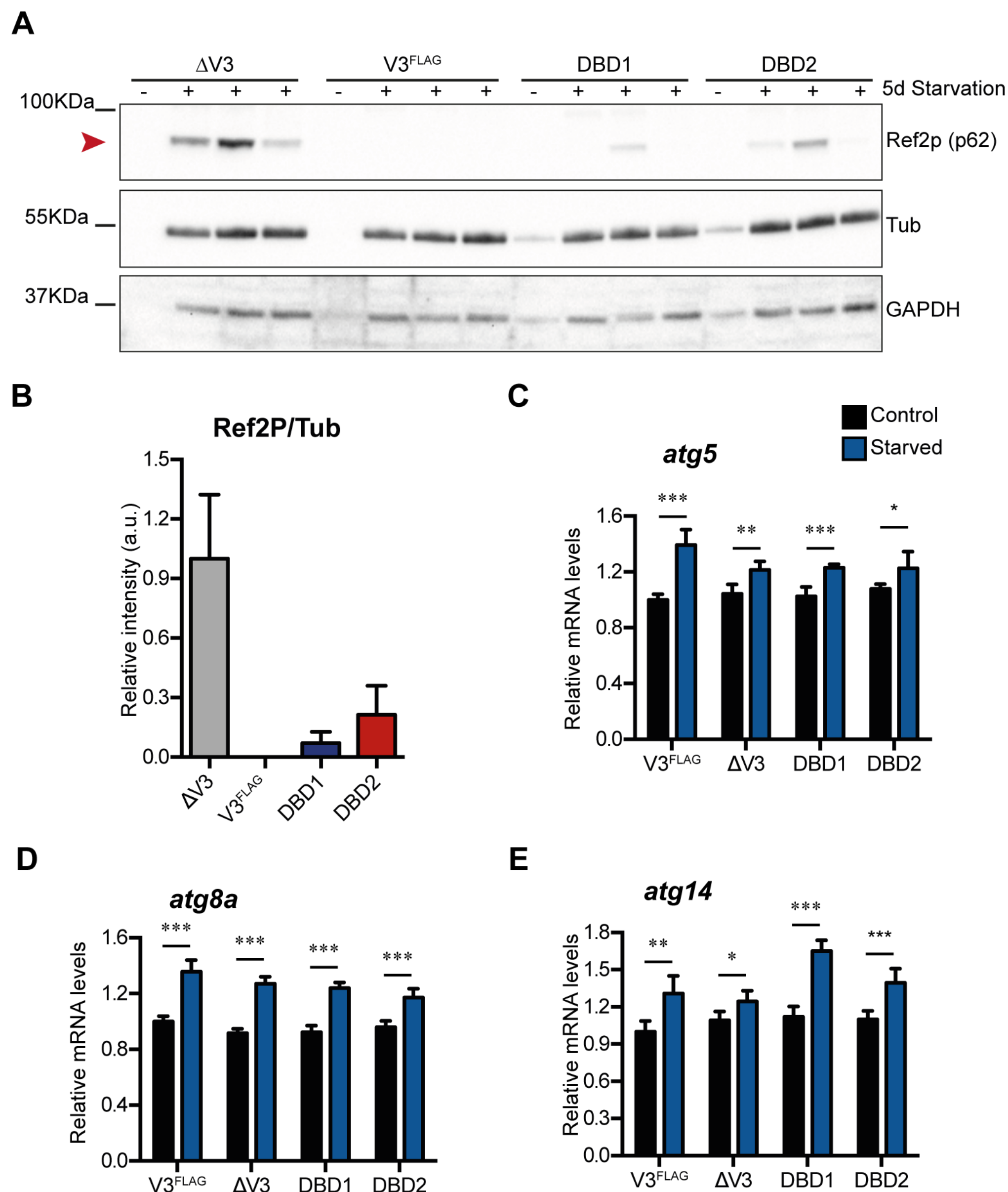

Figure 3.18. Autophagy is disrupted in fasted *dfoxo-null* flies and the dFOXO-DBD mutants rescue this phenotype. **(A)** Western blot on whole-body protein extracts from female flies showed the accumulation (improper autophagy) or absence (correct autophagy) of the autophagy associated protein Ref2p (p62) under starvation (five days). **(B)** Ref2p band quantification of the previous Western blot with respect to tubulin loading control (quantification was done in Image J). **(C-E)** Relative mRNA of autophagy related genes (*atg5*, *atg8a*, and *atg14*) evaluated by qPCR showed dFOXO independence for its induction under starvation. Control (black) or starved (48h) samples are compared. n.s. p>0.05, *p<0.05, **p<0.01, ***p<0.001, Students t-test.

In summary, it is possible that a combination between disturbed autophagy and improper expression of fasting-induced proteins/enzymes, such as *lip3*, are the reason behind the defective starvation response observed in *dfoxo-null* flies. With this in mind, we propose a model where starvation induces dFOXO nuclear retention and therefore induction of direct and indirect target genes, which in turn are involved in TAG usage as an energy source *via* cytoplasmic hydrolysis and/or lypophagy (Figure 3.19). In contrast, *dfoxo-null* flies would only be able to partially use TAGs through cytoplasmic hydrolysis, whereas the lipids engulfed by the autophagy machinery would be inaccessible to degradation by either lack of proper enzymes (*lip3*) or a currently unknown mechanism limiting autophagy (Figure 3.19). Future studies should 1) try to elucidate the relationship between dFOXO and starvation-induced autophagy and 2) identify the putative interaction partner facilitating transcriptional regulations of genes indirectly activated by dFOXO.

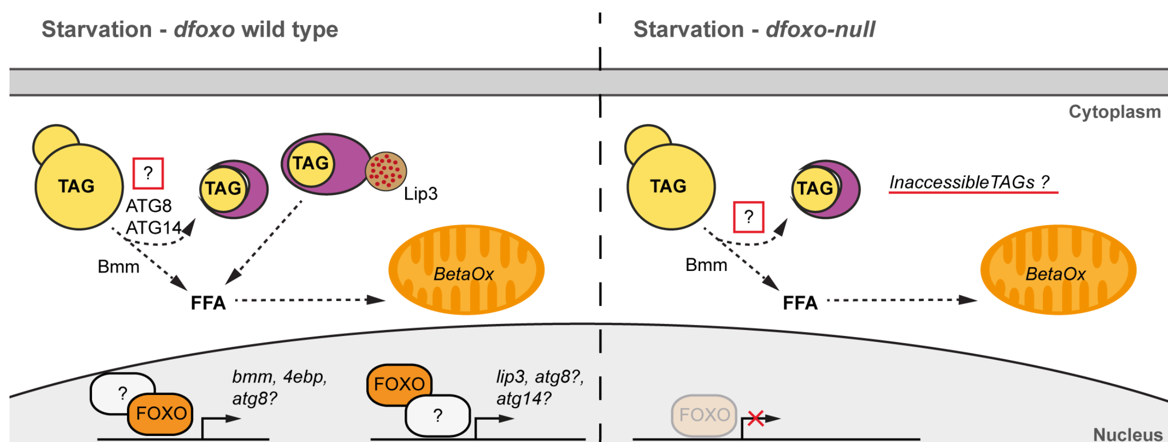

Figure 3.19. Schematic model of dFOXO action under starvation

In the presence of dFOXO (left), starvation induces dFOXO nuclear localization and the regulation of two different groups of target genes, direct targets, such as *bmm* (homolog of mammalian *atgl*) and *thor* (homolog of mammalian *4ebp*), and indirect targets, possibly regulated by dFOXO acting as a co-activator (such as *lip3*). At the same time, lipid droplets are used as an energy source by direct TAG hydrolysis by Bmm and other cytoplasmic lipases. In parallel, part of these TAGs can be processed by the autophagy machinery and hydrolysed in the autolysosome (resulting from autophagosome and lysosome fusion) by different lysosome associated lipases. In contrast, in the absence of dFOXO (right) there is no proper induction of fasting associated genes and, more importantly, the autophagy machinery does not seem to function properly. I propose that in the absence of dFOXO, the TAGs normally used by the autophagy machinery become sequestered, causing the flies to die, unable to fully use its lipid stores.

3.2.2.7 Generation of a putative dominant negative dFOXO

Hetero-allelic combinations of loss-of-function mutants *dfoxo*^{21} and *dfoxo*^{25}, have been traditionally used to study FOXO function in *Drosophila* (Giannakou et al., 2008; Jünger et al., 2003; Min et al., 2008). The *dfoxo*^{21} and *dfoxo*^{25} mutations were generated by EMS treatment that introduced premature stop codons within the DNA binding domain of dFOXO, resulting in truncated dFOXO proteins (Jünger et al., 2003). Recently, it was suggested that these truncated dFOXO proteins are still expressed and able to bind to DNA and may act as dominant negative rather than loss-of function *dfoxo* alleles (Alic et al., 2011; Slack et al., 2011).

In order to test if a shorter version of dFOXO could work as a dominant negative, we cloned a version called dFOXO-MAD (lacks Minimal Activation Domain), which contains the whole DBD and the NLS by introducing a stop codon at position 226 (Figure 3.20A). Consistent with this idea, cell culture over-expression of this protein termed MAD, was unable to induce transactivation of a luciferase reporter gene under a 4xFRE promoter (Figure 3.20B). Moreover, MAD remained nuclear even in the presence of insulin (Figure 3.20C), suggesting this protein cannot respond to nutritional cues and therefore remains in the nucleus.

To investigate the putative dominant negative role *in vivo*, we generated a *dfoxo-MAD* replacement line (Figure 3.21A) and characterized phenotypes associated with *dfoxo* loss-of function. Consistent with the *dfoxo-null* mutation, MAD mutants were short lived and were sensitive to oxidative stress (Figure 3.21B-E). Interestingly, comparable to the DBD mutants, the *dfoxo-MAD* flies were not sensitive to starvation (Figure 3.21F-G). Even though more experiments are required, these results imply that, if the DBD and MAD mutants regulate the starvation response by similar mechanisms, this would probably be through direct interaction with different proteins within the nucleus independent of both DNA binding and transactivation activity. It would therefore be interesting to further characterize the MAD mutant and its role in the starvation response.

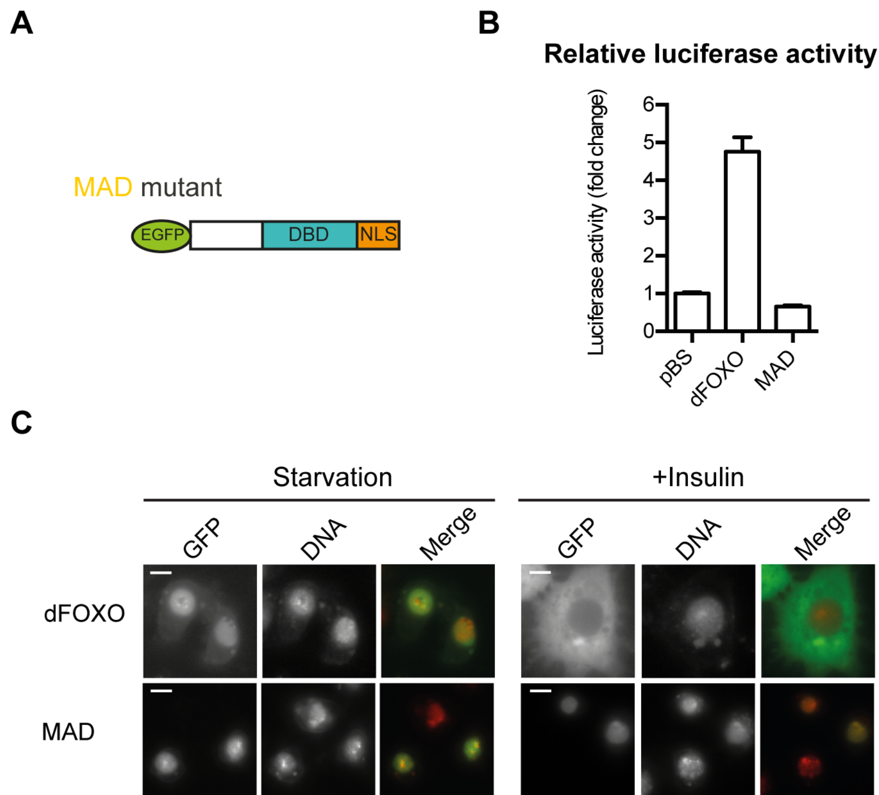

Figure 3.20. Removal of dFOXO – Minimal Activation Domain (MAD)
(A) Representation of dFOXO-MAD (removal of MAD) protein overexpressed in cell culture that lacks every amino acid following of the NLS (nuclear localization signal) and is N-terminally EGFP tagged (**B)** Luciferase reporter assay showed MAD removal abolished transactivation activity in *Drosophila* cell culture. S2-R+ cells were transfected with pBluescript (pBS), EGFP tagged dFOXO or EGFP tagged MAD along with reporter luciferase construcs 4xFRE-Luc. **(C)** dFOXO-MAD proteins localized in the cell nucleus independent of nutritional cues (starvation vs insulin) in *Drosophila* cell culture as shown by representative fluorescent microscopy images. S2-R+ cells transfected with EGFP tagged -dFOXO or -MAD and maintained in serum-free medium (2h) and later treated with insulin (10mM) for 10mins. Scale bar = 5μm.

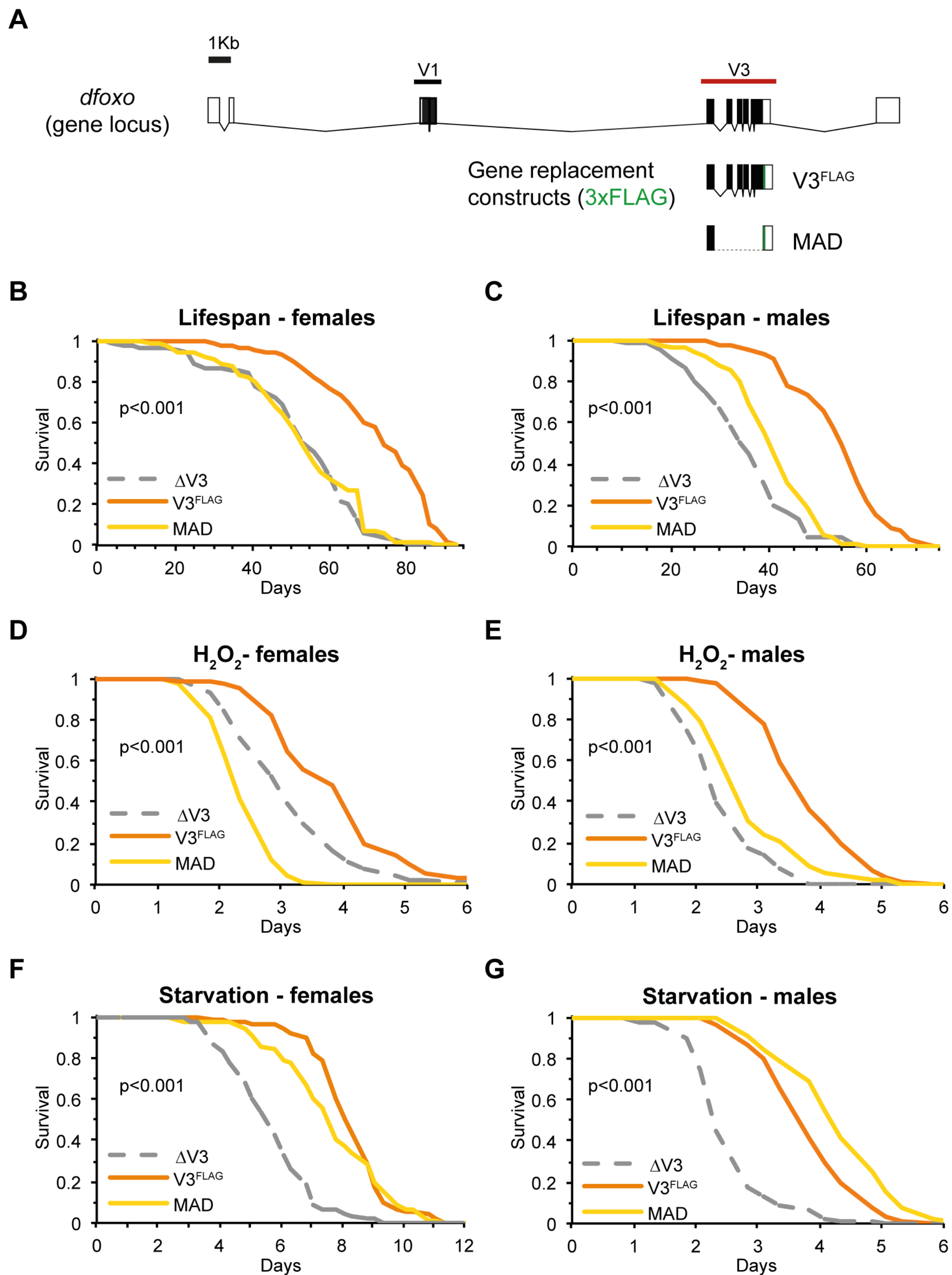

Figure 3.21. Generation and characterization of dFOXO-MAD mutant *in vivo*
(A) Depiction of the *dfoxo* locus and the V3^{FLAG} or MAD gene replacement constructs. Note that the MAD sequence lacks most of the coding exons of the V3 cluster but is still contains the C-terminal 3xFLAG tag and the unmodified 3'UTR. Boxes represent UTRs (white boxes) and the CDS (black boxes) within the exons. Regions arbitrarily denoted V1 (1Kb) and V3 (3Kb). **(B)** Female and **(C)** male lifespan assays showed MAD mutants are short lived when compared to V3^{FLAG} flies. ***$p<0.001$ log-rank test for comparison of *dfoxo-null* (ΔV3) or MAD vs control (V3^{FLAG}). Flies of each genotype were placed on 1xSYA food and followed throughout life

(n=100/genotype). **(D)** Female and **(E)** male ΔV3 and MAD flies were sensitive to oxidative stress when compared to $V3^{FLAG}$. ***p<0.001 log-rank test for comparison of *dfoxo-null* (ΔV3) or MAD vs control ($V3^{FLAG}$). Flies of each genotype were placed on H_2O_2 media after seven days on 1xSYA food (n=100/genotype). **(F)** Female and **(E)** male MAD flies, unlike ΔV3, were as resistant so starvation as control flies ($V3^{FLAG}$). ***p<0.001 log-rank test for comparison of *dfoxo-null* (ΔV3) vs control ($V3^{FLAG}$) or MAD. Flies of each genotype were placed on starvation media after seven days on 1xSYA food (n=100/genotype).

3.3 DISCUSSION

FOXO transcription factors are evolutionarily conserved downstream mediators of the IIS pathway (Piper et al., 2005). In addition to the IIS, several signaling pathways converge on FOXO proteins to regulate multiple cellular processes, such as development, metabolism, and ageing (Eijkelenboom and Burgering, 2013). Thus, a plethora of interaction partners and PTMs regulate FOXO proteins. However, the specifics of how a single transcription factor can be involved in such diverse range of activities are still unclear. In order to separate the different modes of FOXO action and their role in, for example, ageing modulation, it is fundamental to understand their regulation under different conditions.

Studies designed to elucidate the role of FOXO transcription factors were traditionally based on the generation of *foxo-null* or *foxo* over-expression mutants in multiple model organisms such as mice, worms and flies (Burgering, 2008). However, this kind of approach has multiple limitations. First, total removal of *foxo* genes in flies and worms perturbs different phenotypes, such as size, reproduction and longevity, but it does not provide an answer as to how FOXO regulates those phenotypes (Jünger et al., 2003; Kenyon et al., 1993; Puig et al., 2003; Slack et al., 2011; Tissenbaum and Ruvkun, 1998). In mammals it is even more complex, as *foxo1* null mutants are lethal due to improper angiogenesis, *foxo3* nulls had a mild age-dependent infertility and *foxo4* nulls have no obvious phenotype (Furuyama et al., 2004; Hosaka et al., 2004). Second, overexpression studies often overlook the effects of endogenous-wild type FOXO and cannot rule out secondary effects due to the over-expression (Giannakou et al., 2004; Samuel T. Henderson and Johnson, 2001; Hwangbo et al., 2004; Libina et al., 2003). Therefore, it is of great interest to better understand the role of FOXO proteins under less artificial conditions and at a higher resolution level, such as identification of functions associated with specific domains, interaction partners or PTMs. Thus, *Drosophila*, with its single FOXO homolog, its short lifespan, and the multiple tools available for genetic manipulation, constitutes a powerful model to study FOXO proteins.

3.3.1 Genomic engineering of the *foxo* locus

Gene-editing technologies have long been desired to study the functions associated with specific genes, and multiple paradigms, especially in *Drosophila*, have been developed to achieve modifications with single-nucleotide resolution (Venken et al., 2016). Among the different technologies, *Genomic engineering* appears as a highly efficient technique able to, first, reliably knock-out target genes and second, have high and fast levels of reintroduction of desired genetic sequences (Huang et al., 2009). Hence, we used genomic engineering on the *dfoxo* locus and generated three knockout parental lines that would allow us to modify the entire *dfoxo* gene. The generation of the 'knock-out parental line', where the endogenous sequence is replaced by an attP 'landing site', is normally seen as the limiting step in this technic (Huang et al., 2009). However, once generated, the reintroduction of any desired sequence in the attP site by the Φ31-integrase is highly specific and efficient, even when using large sequences (Huang et al., 2009; Venken et al., 2010). Hence, the novel generated *dfoxo*Δ-attP lines constitute an extraordinary tool that, not only permitted the generation of the mutants described in this thesis, but will also allow future researchers to modify the endogenous *dfoxo* gene in whichever preferred way.

Since the year 2012, a different tool for gene-editing has exploded in popularity, namely, the CRISPR-Cas9 system (clustered regularly interspaced short palindromic repeats-CRISPR-associated protein 9) (Jinek et al., 2012). This highly simplified system is based on a bacterial and archaeal adaptive immune system that protects them against foreign DNA (Bhaya et al., 2011). The engineered CRISPR-Cas9 system is composed of two components, a single guide RNA, which confers the specificity, and a nuclease (Cas9) able to induce double strand breaks. Thus, CRISPR-Cas9 constitutes a fast and versatile option to generate, among others, deletions, insertions or sequence replacements in diverse organisms, including *Drosophila* (Doudna and Charpentier, 2014). Worth noting, the CRISPR-Cas9 tools were only available after we had started this project (Bassett et al., 2013; Gratz et al., 2013; Jinek et al., 2012), and, even though this is a powerful technique, it has some limitations that our genomic engineering approach overcomes. For example, CRISPR-Cas9 is highly specific thanks to its 20 base-pair guide RNA, however, great efforts are still underway to try to minimize off-target effects (Koo et al., 2015). In contrast, the off-target effects of attB mediated integration are <6%, even for constructs over 120Kb (Venken et al., 2010). Moreover, CRISPR-Cas9 targeting is limited by the

presence of so-called protospacer adjacent motifs (PAMs), a short sequence that must be immediately downstream of the target DNA (Esvelt et al., 2013; Gasiunas et al., 2012). This limitation is not present with genomic engineering, as integration takes place at the previously integrated attP 'landing site'. In addition, nuclease-assisted gene replacement, such as CRISPR-Cas9, requires a donor sequence and is based on homologous recombination. This process is less efficient and has more stringent size limitations than those observed in integrase-mediated replacement (Venken et al., 2016). Last, attB/attP reinsertion permits unrestricted modifications of the target gene before reintroduction. For example, we generated knock-in lines that contained modifications at opposite ends of the integrated sequence *dfoxo-V3*, for example, mutations within the DBD and a carboxy-terminal tag.

In conclusion, we have generated a powerful tool that allows efficient *dfoxo* gene editing in *Drosophila*. This constitutes a novel way of studying mutations within the *dfoxo* gene and permitted us to separate different functions associated with this transcription factor.

3.3.2 FOXO functions dependent of DNA binding

Previous studies showed FOXO transcription factors may have functions independent of DNA binding (Matsumoto et al., 2006; Ramaswamy et al., 2002; Rudd et al., 2007). This observation is consistent with a study in the mouse liver showing that, unlike FOXO1 null mutants, FOXO1 DBD mutants have normal lipid biosynthesis after refeeding (Cook et al., 2015). This observation suggested that, at least in the liver, dFOXO modulates lipid synthesis independent of DNA binding. However, the exact mechanisms behind this putative role are not fully understood. Moreover, it is unclear whether the DNA-binding-independent functions are evolutionarily conserved or can modulate any of the other FOXO-dependent processes. Using the newly developed genomic engineering of the *dfoxo* locus, we generated two endogenous mutants that contain one (H150A) or two (N146A, H150A) point substitutions within the evolutionarily conserved DBD. Mutation of one (Ramaswamy et al., 2002) or both (Matsumoto et al., 2006) homologous residues in mammalian FOXO1 is sufficient to abolish DNA binding *in vitro* and in cell culture. Hence, we argued that mutation on one or both residues should be sufficient to abolish dFOXO DNA binding in *Drosophila*. In accordance, we see that both DBD mutants are unable to activate transactivation in cell culture, and DBD2 is unable to bind the *4ebp* promoter region *in vivo*. Yet, it is still possible that these DBD mutants are able to bind

DNA with a much lower affinity. However, we deem this possibility unlikely since the DBD mutants, much like *dfoxo-null* flies, have reduced fecundity, are sensitive to oxidative stress and are short lived. These observations have two connotations: first, DBD mutations do in fact interfere with dFOXO functions *in vivo* and second, the regulation of fecundity, redox stress and lifespan is fully dependent on dFOXO binding DNA. In agreement with our results, a recent study comparing FOXO target genes across tissues and organisms found a core set of conserved gene-targets implicated in the regulation of oxidative stress and ageing (Webb et al., 2016).

In conclusion, we have generated two independent fly lines (DBD1 and DBD2) that express a dFOXO protein unable to bind DNA *in vivo*. DBD mutant flies phenocopy *foxo-null* flies, as both have reduced fecundity, reduced oxidative stress resistance and reduced lifespan. In contracts, dFOXO-DBD mutants, but not *foxo-null* flies, yield flies of normal body size and are able to induce lipid mobilization under starvation.

3.3.3 Body size and developmental effects mediated by dFOXO

The regulation of body size in *Drosophila* has been extensively studied over the past two decades. Size determination depends on nutritional, i.e. amino acid (AA) abundance, and environmental cues, i.e. temperature, that in turn regulate signaling pathways and hormonal systems (Mirth and Shingleton, 2012). The signaling pathways, such as IIS and Target of Rapamycin (TOR), determine growth rate, whereas the hormonal systems seem to regulate growth duration (Mirth and Shingleton, 2012). Importantly, the crosstalk between the nutritional signaling pathways and the hormonal systems is in the end responsible for determining body size (Mirth and Shingleton, 2012).

Mutation of *dfoxo* gene produces smaller than wild type flies, similar to models of reduced insulin signaling (Clancy et al., 2001; Puig et al., 2003; Slack et al., 2011). These observations imply that dFOXO hyperactivation (reduced IIS) or hypoactivation (increased IIS or *dfoxo* removal) have a similar consequence for *Drosophila* body size. It is clear that IIS negatively regulates growth (Piper et al., 2005), but the reasons behind reduced body size in the *dfoxo-null* mutants are not entirely clear. However, dFOXO regulates expression of *Steppke*, a cytohesin required for correct IIS (Fuss et al., 2006), suggesting *dfoxo-null* mutants do not express normal levels of Steppke and therefore may also mimic this aspect of reduced IIS during development. Moreover, dFOXO is required

to achieve normal expression levels of *dilp 2, 3* and *5* (Slack et al., 2011). Mutation of these peptides, which are all secreted by the insulin producing cells (IPCs) in the *Drosophila* brain, yields smaller flies due to their regulation on systemic growth (Grönke et al., 2010).

In contrast to *dfoxo-null* flies, male and female DBD mutants have normal body weight after hatching, suggesting that FOXO-regulation of body size is independent of DNA binding. Similarly, eclosion rates are also restored to normal levels in dFOXO-DBD mutants. These observations suggest these developmental features are independent of DNA binding and could be consistent with a recent report by Koyama et al. In their study, they described a direct interaction between dFOXO and Ultraspiracle (Usp) that regulates the production of Ecdysone hormone to regulate body size (Koyama et al., 2014). The dFOXO-region interacting with Ups localizes downstream of the DBD and should not be affected by the DBD mutations. It is therefore possible that the regulation dFOXO exerts over Usp is independent of the ability of dFOXO to bind DNA, a possibility that requires experimental testing.

On the other hand, we also observed that developmental timing is only partially affected in dFOXO-DBD mutants. Moreover, *foxo-null* and DBD mutant flies do not develop in the absence of *dilp2-3,5*, a model of reduced IIS (Grönke et al., 2010), indicating that some developmental parameters do rely on dFOXO binding DNA target sequences. The mechanisms behind developmental regulation by dFOXO are still under investigation. However, previous studies suggest IIS is in fact a link between growth rate and duration (Mirth et al., 2014). Our results suggest this link could in fact be further separated based on dFOXO's ability to bind DNA.

3.2.4 Lipid metabolism regulation by FOXO

Mammalian FOXO1 orchestrates energy metabolism in response to insulin signaling in different tissues including the liver, muscle and adipose tissue (Kousteni, 2012). In *Drosophila*, dFOXO is also required to regulate energy balance in response to nutritional cues (Wang et al., 2011). Interestingly, lipid synthesis in the mouse liver seems to be regulated, at least partially, by FOXO1 independent of DNA binding (Cook et al., 2015). In contrast, our results indicate that lipid biogenesis under high nutrient availability is dFOXO-independent, but in response to starvation requires dFOXO presence but not its

ability to bind DNA. The former observation is consistent with previous studies in *Drosophila* that showed lipid biosynthesis to be independent of dFOXO (DiAngelo and Birnbaum, 2009). These results are also in line with adipose cell culture studies where FOXO1 was shown to regulate energy expenditure (Nakae et al., 2008). It would be therefore important to determine whether FOXO1 can regulate lipid synthesis and/or usage independent of DNA binding in the mammalian adipose tissue, since the discrepancy could arise from tissue-specific functions.

The mechanisms by which dFOXO regulates lipid mobilization under starvation are unclear. However, different target genes involved in lipid metabolism have been previously characterized. For example, a study reports *lip4*, a putative lysosomal associated lipase (Lipa), as a dFOXO target that is up-regulated under starvation in a dFOXO-dependent manner (Vihervaara and Puig, 2008). Our results however, are inconsistent with this observation. Normal expression of *lip4* under control conditions and correct induction upon starvation in the *foxo-null* flies indicates that dFOXO is not required for *lip4* transcriptional regulation. However, that does not rule out the possibility that dFOXO is able to bind and regulate this gene under different conditions. Worth noting, the regulation of *lip4* by dFOXO could be tissue specific and could therefore be masked in our qPCR results as we used whole body RNA. Similarly, *pepck*, the limiting enzyme during gluconeogenesis, was already shown to have reduced expression upon starvation of *foxo-null* flies (Wang et al., 2011). Interestingly, starvation induces greater than wild types transcription of this gene in the *foxo-nulls*. This could be seen as a compensatory mechanism due to the inefficient usage of fat stores. Of note, our outbred flies (w^{DahT}) have remarkably high levels of fat and are, in general terms, healthier than laboratory inbred lines used by others (such as w^{1118}) (Swindell and Bouzat, 2006; Toivonen et al., 2007). This difference in genetic background could explain some of the discrepancies observed in starvation resistance among different *Drosophila* studies.

On the other hand, starvation induces expression of *bmm* and *ATGL* in flies and mice respectively. These enzymes act as limiting factors for lipolysis, and are therefore fundamental mediators of the starvation response (Grönke et al., 2005; Zimmermann, 2004). Importantly, transcriptional regulation of *bmm* and *ATGL* was previously reported to be dependent on FOXO transcription factors (Chakrabarti and Kandror, 2009; Wang et al., 2011; Zhang et al., 2016). Our results confirm this observation, as *dfoxo-null* and DBD

mutants do not reach the normal levels of *bmm* induction under starvation. However, dFOXO-DBD mutants were able to fully mobilize their lipids under starvation, suggesting that *bmm* regulation, at least at the transcriptional level, is not required for correct lipid mobilization.

3.2.5 Lip3 could be a limiting factor for fat mobilization

In addition to *bmm* and *lip4*, dFOXO is thought to regulate *lip3*, as *dfoxo-null* flies are unable to induce its expression under starvation (Wang et al., 2011). Lip3 is another putative lysosomal associated lipase, homologous to mammalian Lipa. Mutation of the Lipa gene results in Wolman disease, which is characterized by the accumulation of cholesteryl esters and triglycerides in cells and tissues across the body (Anderson et al., 1994, 1993). This observation implies lysosomal lipases have a fundamental regulatory role in lipid usage in mammals.

In *Drosophila*, *lip3* is heavily induced by starvation and its expression is completely abrogated when flies are fed sugar (Zinke et al., 2002, 1999), suggesting that carbohydrate metabolism may be a key component on its regulation. In addition, and according to FlyAtlas, lipases *bmm*, *lip4* and *CG5966* are all expressed to some extent in the larval and adult tissues under fed conditions, whereas *lip3* levels are almost undetectable (Chintapalli et al., 2007). Moreover, *lip3* seems to be induced upon long-term starvation, as it is barely detectable after 6h starvation in adult flies (Chatterjee et al., 2014). These results suggest that *lip3* may in fact be fundamental for lipid usage under prolonged starvation. In accordance with this, our results suggest that *lip3* mRNA levels are very low under control conditions and its transcription is greatly induced under starvation. Interestingly, *lip3* transcription seems to be dependent on dFOXO, but not its ability to bind DNA, as dFOXO-DBD mutants are able to induce wild-type-like gene expression levels. However, Lipa regulation by FOXO1 is, at least partially, regulated by direct DNA interaction, as FOXO1 was shown to bind the Lipa promoter and regulate its expression *in vivo* (Lettieri Barbato et al., 2013), Thus, it remains to be determined whether Lipa regulation by FOXO1 could also occur in a DNA-binding-independent manner, as it seems to be the case for *lip3* in *Drosophila*.

In order to test whether *lip3* could have an upstream regulatory role on lipolysis, and therefore limit starvation sensitivity, we overexpressed $Lip3^{RNAi}$ in whole fly and in fat

tissue. $Lip3^{RNAi}$ over-expression reduced starvation resistance only marginally. However, the lack of starvation sensitivity of $Lip3^{RNAi}$ expressing flies could possibly be explained by the incomplete repression of the gene under starvation, since lip3 mRNA was reduced by ~60%. Hence, it would be important to evaluate the function of this lipase by generating a full knockout of the *lip3* gene to determine if it can act as a limiting factor for lipolysis, as Lipa seems to be in mammals (Anderson et al., 1994, 1993).

3.2.6 Lip3 regulation by dHNF4

dFOXO seems to work as a co-factor for *lip3* regulation, as either dFOXO wild type or a DBD mutant is required for *lip3* expression under starvation. However, the question of which transcription factor is dFOXO interacting with to regulate the starvation response, and at least *lip3* transcription, remains open. One strong candidate was nuclear transcription factor dHNF4 (Hepatic nuclear factor 4), since *dhnf4* mutant flies are unable to induce genes involved in lipid mobilization and ß-oxidation, and thus sensitive to starvation (Palanker et al., 2009). Among the deregulated genes is *lip3*, which is not induced upon starvation of *dhnf4-nulls*, similar to our observation on *dfoxo-null* flies. This result implies that *lip3* requires both dHNF4 and dFOXO for its expression under fasting. Motif detection analysis showed that the promoter region of *lip3* seems to contain a HNF4 motif. However, *Drosophila* dHNF4 over-expression seems to repress a luciferase reporter gene, cloned under the *lip3* promoter, in cell culture. The cell culture cell line used (S2R+ cells) expresses wild type dFOXO (Chintapalli et al., 2007); thus, our observation suggests *lip3* regulation is not merely dependent on the presence of dFOXO and dHNF4. One possible explanation for this the lack of induction is that S2-R+ cells are derived from embryonic tissue (Schneider, 1972) and may not recapitulate the conditions necessary for *lip3* induction. Moreover, the over-expressed dHNF4 may interfere with dFOXO-dependent *lip3* expression by sequestering this transcription factor, as mammalian cell culture studies suggest HNF4 can physically interact with FOXO1 (Hirota et al., 2003). Under these cell culture conditions, mammalian FOXO1 bound the DBD of HNF-4, repressing its ability to bind DNA (Hirota et al., 2003). In contrast, both FOXO1 and HNF4 could synergise to up-regulate the expression G6Pase, an enzyme involved in gluconeogenesis, under fasting conditions in mammalian cell culture and *in vivo* (Hirota et al., 2008). At the same time, FOXO1 could repress *glucose carboxykinase* (GcK), probably through an inhibitory interaction with HNF4 (Hirota et al., 2008). Hence, it would be important to determine how FOXO1 and HNF4 modulate their interaction and

whether this is conserved in flies. In addition, it is critical to create a positive control and verify that over-expressed dHNF4 protein is able to induce transcription in the *Drosophila* cell culture system.

Expression of six prominent dHNF4 target genes under starvation is not disturbed by mutations in the *dfoxo* gene, suggesting dHNF4-dependent starvation response does not require dFOXO. Thus, the question of which putative interaction partner could regulate starvation along with dFOXO is still unclear. A key mediator of nutrient sensing is the mechanistic Target of Rapamycin (mTOR), a kinase part of mTOR complex 1 (mTORC1) that is activated under nutrient abundance (Dibble and Manning, 2013). mTORC1 phosphorylates multiple targets and one of them, the recently described transcription factor *repressed by TOR* (REPTOR) (Tiebe et al., 2015), could be a putative dFOXO interaction partner. REPTOR is phosphorylated and therefore negatively regulated by mTORC1 under fed conditions and, analogous to IIS and dFOXO regulation, repression is abolished under starvation (Tiebe et al., 2015). Interestingly, REPTOR and its binding partner (REPTOR-BP) seem to bind the same enhancer regions as dFOXO proteins, and the two transcription factors seem to interact genetically (Tiebe et al., 2015). This observation opens the possibility of REPTOR/REPTOR-BP and dFOXO physically interacting, as they are both activated under starvation and share genomic binding loci. Therefore, future studies should address whether these proteins can indeed interact and, with that, modulate lipid usage under starvation. Moreover, our preliminary results with the dFOXO-MAD mutant, a mutant that lacks the N-terminal minimal activation domain, suggest that a shorter version of the protein is sufficient to rescue the starvation sensitivity observed in *dfoxo-null* flies. This observation indicates that a hypothetical interaction partner mediating the starvation response, perhaps REPTOR and/or REPTOR-BP, could interact with the N-terminal region of dFOXO, a possibility that will be addressed in the future.

3.2.7 dFOXO is required for starvation-induced autophagy.

In addition to cytoplasmic hydrolysis of TAG stores by enzymes such as ATGL and Bmm (Grönke et al., 2005; Zimmermann, 2004), starvation also induces autophagy (Neufeld, 2010). Autophagy is a process in which cytoplasmic components of the cell are engulfed by specialized membranes, to form the so called autophagosome, followed by fusion with the lysosome and therefore breakdown the cargo into its smaller parts (Neufeld, 2010).

The autophagy machinery can assist in TAG usage under starvation in a process called *lipophagy* (Singh et al., 2009). Our results indicate that autophagy is perturbed when *dfoxo-null* flies are starved, suggesting disturbed lipophagy might be at least partially responsible for inefficient TAG mobilization in *dfoxo-null* mutants. However, our results indicate that transcriptional induction under starvation of three fundamental autophagy related genes (*atg*) genes (*atg5*, *8a* and *14*) is not altered by removal of *dfoxo*. These genes were reported to be FOXO targets in different mouse tissues (Webb and Brunet, 2014), and *atg8a* seems to be a dFOXO target in the fly muscle (Bai et al., 2013). This observation suggests that, at least under full starvation and at this time point (48h starvation), dFOXO is not required to regulate those autophagy components at the transcriptional level. Yet, it is possible that dFOXO is required for starvation induced autophagy by regulating different genes. In accordance, FOXO1 and FOXO3 seem to be required for starvation induced autophagy in skeletal muscle (Hariharan et al., 2010; Mammucari et al., 2007).

Mutation of the *dfoxo* gene alters autophagy, however, it is hard to determine which of the different steps in autophagy is affected. There could be at least 5 different mechanisms affecting autophagy in an organism: reduced autophagy induction, enhanced autophagy repression, altered cargo recognition, inefficient autophagosome/lysosome fusion, or inefficient degradation of the cargo (Wong and Cuervo, 2010). Under nutritionally rich conditions, the presence of AAs activates mTORC1, which in turn would actively repress autophagy by phosphorylating ATG13 (Lum et al., 2005; Neufeld, 2010). Phosphorylation of ATG13 in turn prevents the formation of a complex with ATG1 and ATG17. Conversely, under reduced nutrients, ATG13 phosphorylation is lost, permitting complex formation, which increases ATG1 kinase activity and, therefore, autophagy induction (Kamada et al., 2000). Hence, enhanced autophagy repression or reduced autophagy induction, at least as regulated by mTORC1, are very unlikely to be the reason behind the observed autophagy deregulation, since full starvation should prevent activation of mTORC1.

In contrast to autophagy induction, our results indicate that dFOXO is required for *lip3* expression. If this putative lysosome-associated lipase is indeed fundamental for TAG hydrolysis, inefficient degradation of the cargo could be the culprit behind inefficient autophagy. However, our results demonstrate that *lip3* overexpression is not sufficient to

rescue the starvation sensitivity observed in *dfoxo-null* flies, suggesting that additional mechanisms might be in play. In order to test which autophagy step might be affected, future studies could determine whether TAGs accumulate in autolysosomes (structures formed from autophagosome and lysosome fusion events) or whether autolysosomes are formed at all. Exactly which mechanisms behind autophagy dysfunction are responsible for the observed phenotype will be addressed in the future.

FOXO ACETYLATION

4.1 INTRODUCTION

FOXO proteins modulate a plethora of cellular and organismal processes, including ageing and metabolism. It is therefore not surprising that these factors can be regulated by no less than six different PTMs (Daitoku et al., 2011; Zhao et al., 2011). In mammals, FOXO acetylation reduces DNA binding affinity and facilitates phosphorylation by AKT, inducing nuclear exclusion (Brent et al., 2008; Brunet et al., 2004; Matsuzaki et al., 2005; Qiang et al., 2010). Moreover, FOXO acetylation seems to modulate metabolism, as a knock-in mouse bearing a FOXO1 acetyl-null mutant relies on fat, and not sugar, as an energy source (Banks et al., 2011). In *Drosophila*, only dFOXO phosphorylation by different kinases, including IIS-activated AKT, has been widely studied (Mattila et al., 2008). More recently, acetylation was suggested to also influence dFOXO activity (Wang et al., 2011). However, the exact mechanism by which acetylation regulates dFOXO activity is not fully understood. To better understand the role of dFOXO acetylation I generated endogenous *dfoxo* mutants to either mimic or abolish acetylation in conserved lysine residues. These dFOXO mutants allowed the *in vivo* separation of functions associated with dFOXO acetylation.

4.2 RESULTS

4.2.1 Mutation of putatively acetylated residues

It was recently suggested that two Lys residues within the NLS could be acetylated to regulate dFOXO function in response to nutritional cues (Wang et al., 2011). This suggestion is based on evolutionary conservation of those two residues and on mutation studies in FOXO1 (Banks et al., 2011; Qiang et al., 2010; Wang et al., 2011). However, which specific dFOXO residues are acetylated is currently unknown. LAceP, a lysine acetylation prediction tool (Hou et al., 2014), highlights exclusively the five-lysine residues within the dFOXO-NLS as probable residues undergoing acetylation. Moreover, a protein alignment between human, worm and fly FOXO proteins showed that these residues are evolutionarily conserved (Figure 4.1A). This observation is specially intriguing, because lysine residues that are acetylated are more likely to be evolutionarily conserved (Weinert et al., 2011). Therefore, we generated two dFOXO mutants to either mimic (Lys substituted by Gln - 5KQ) or abolish (Lys substituted by Arg - 5KR) acetylation within those five Lys residues (Figure 4.1B).

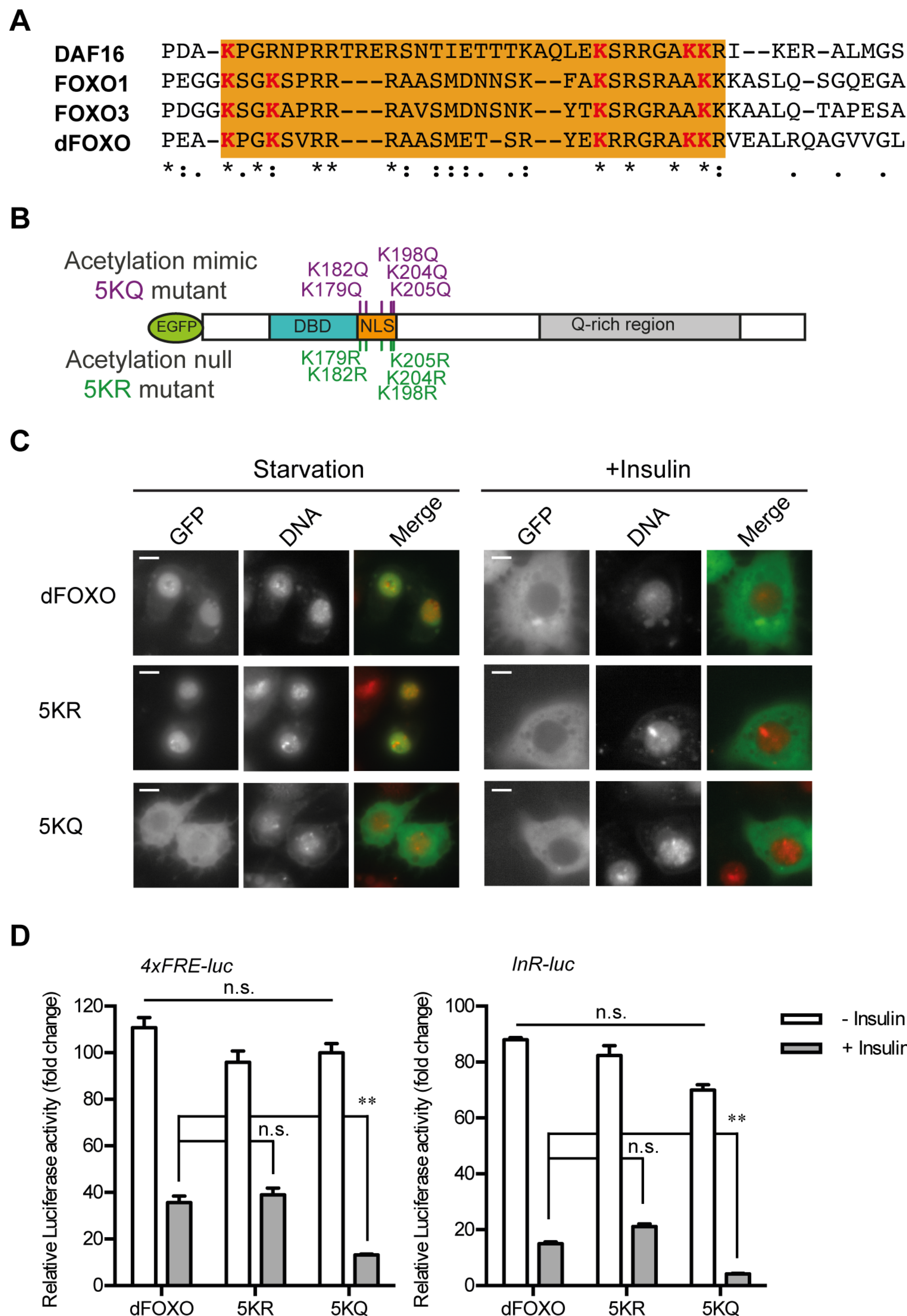

Figure 4.1. FOXO NLS is rich in lysine residues (K) and it is partially conserved throughout evolution. **(A)** Protein alignment of the FOXO NLS between mouse (FOXO1, FOXO3), worm (DAF16) and fly (dFOXO) showed a high degree of conservation. Orange background highlights the NLS. Identical (*), strongly similar (:) or weakly similar (.) residues between sequences. Red lysine residues are conserved in Drosophila. **(B)** Schematic representation overexpressed proteins in *Drosophila* cell culture with mutations to either mimic (5KQ) or abolish (5KR) lysine-acetylation. Both proteins have a N-terminal EGFP-tag. **(C)** Unlike dFOXO and acetyl-null (5KR) mutant, which localized mostly in the nucleus, acetyl-mimetic mutants (5KQ) were mostly cytoplasmic under starvation. Representative fluorescent microscopy images of *Drosophila* cells (S2-R+) transfected with EGFP tagged dFOXO, -5KR and -5KQ, maintained in serum-free

medium (2h) and later treated with insulin (10mM) for 10mins. Scale bar = 5µm. **(D)** Luciferase reporter assay showed dFOXO and both acetylation mutants have comparable levels of transactivation under normal transfection conditions for two independent reporter constructs (4xFRE-Luc or InR-Luc). In contrast, transfection in the presence of insulin yielded reduced transactivation activity of acetyl-mimic mutants (5KQ) when compared to dFOXO or acetyl-null mutant (5KR). S2-R+ cells were transfected, in the absence or presence of insulin (10mM), with pBluescript (pBS), EGFP tagged dFOXO, -5KR or -5KQ along with reporter luciferase constructs 4xFRE-Luc or InR-Luc. Luciferase activity shown as fold increase relative to pBS (1 fold – not shown). n.s. $p>0.05$, *$p<0.05$, **$p<0.01$, ***$p<0.001$, one-way ANOVA with post-hoc Dunnett's comparison with pBS. Luciferase assay performed by Ralf Meilenbrock.

Mammalian cell culture studies have shown that acetylated FOXO1 remains cytoplasmic under serum starvation (Banks et al., 2011; Qiang et al., 2010). Consistently, an eGFP-tagged dFOXO-5KQ (acetylation-mimic) protein was partially retained in the cytoplasm under starvation (Figure 4.1C). In contrast, the dFOXO-5KR (acetylation-null) protein localized to the nucleus under starvation and migrated into the cytoplasm upon insulin treatment comparable to the dFOXO wild type protein (Figure 4.1C). Both acetylation mutants were able to induce the expression of two different *dfoxo* luciferase reporter constructs (4xFRE and InR) to the same extent as the dFOXO wild type protein, implying that the acetylation-mimicking mutations do not fully prevent nuclear localization of dFOXO (Figure 4.1D). Interestingly, when reporter construct activity was measured under insulin treatment, the 5KQ mutant protein had a reduced luciferase activity (Figure 4.1D). This might suggest that the acetyl-mimic dFOXO protein has a higher probability to localize to the cytosol and is therefore more sensitive to the inhibitory effect of insulin treatment. In summary, these results suggest that the mutated lysine residues seem to have a direct effect on dFOXO regulation.

4.2.2 *In vivo* generation of dFOXO acetylation mutants

Taking advantage of the *dfoxo* genomic engineering tool described above, we generated endogenous dFOXO mutants containing mimicking or abolishing mutations of 5 lysine residues (Figure 4.2A, compare Figure 3.8). First, we evaluated the protein levels and observed that overall dFOXO protein levels were comparable between dFOXO wild type, 5KQ and 5KR mutants. In the western blot analysis dFOXO protein shows two prominent bands, whereby the upper band is thought to represent a phosphorylated version of dFOXO that includes the three AKT-dependent phosphorylation sites (Puig et al., 2003). Interestingly, in the acetyl-mimicking dFOXO 5KQ mutant, the upper band was weaker when compared to replacement wild type and acetyl-null (5KR), flies (Figure 4.2B). This

result might suggest a reduction in AKT-phosphorylation in the dFOXO 5KQ protein. With this in mind, we performed an immunoprecipitation on replacement ($V3^{FLAG}$) or acetyl-mutant flies and tested for AKT-dependent phosphorylation by using an antibody that recognizes AKT-phosphorylated consensus sites (Cell Signaling Technologies). Wild type dFOXO showed a homogeneous pattern with relatively equal abundance of, at least two, phosphorylated species (Figure 4.2C). It is worth highlighting that the phospho-Akt-substrate antibody detected both dFOXO bands, suggesting that AKT-dependent phosphorylation is probably not sufficient to explain the size shift between the two dFOXO bands on the western blot analysis. Intriguingly, the acetyl-mimic mutations (5KQ) shifted the 'band balance' towards the faster migrating, and presumably less phosphorylated form, whereas the acetyl-null mutation (5KR) seems to do the opposite (Figure 4.2C). This observation suggests that acetylation may indeed modify the AKT-dependent phosphorylation state of dFOXO *in vivo*.

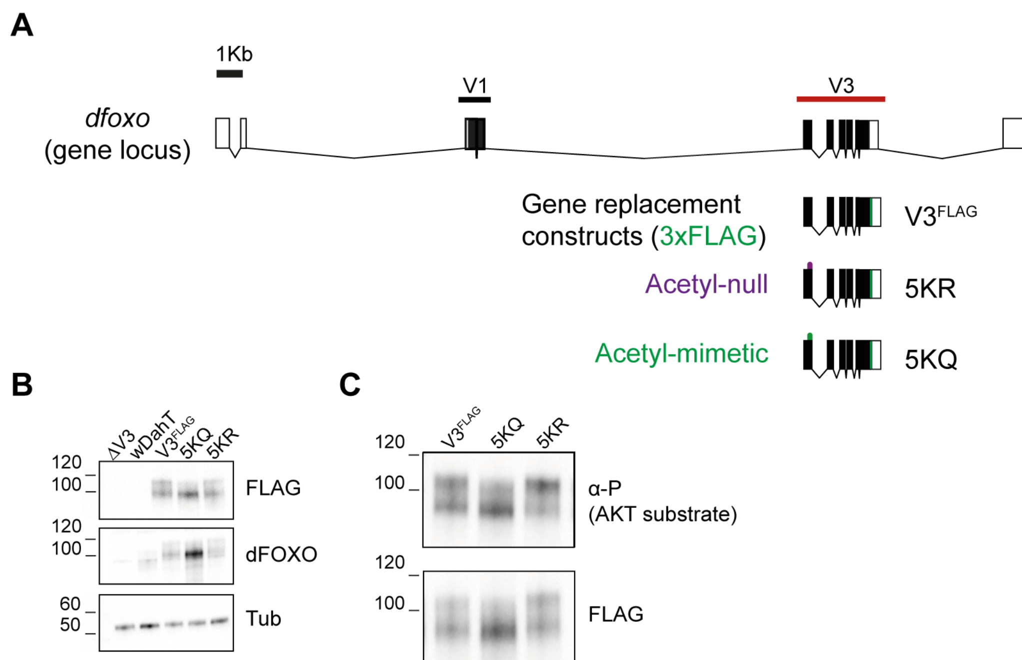

Figure 4.2. Generation and validation of dFOXO-acetyl-lysine mutants *in vivo*
(A) Depiction of the *dfoxo* locus and gene replacement constructs used to generate the dFOXO-acetylation mutant lines. Boxes represent UTRs (white boxes) and the CDS (black boxes) within the exons. Regions arbitrarily denoted V1 (1Kb) and V3 (3Kb). **(B)** Western blot on whole-body protein extracts from female flies confirmed proper expression in all gene replacement lines (dFOXO-$V3^{FLAG}$, V3-$5KR^{FLAG}$ and V3-$5KQ^{FLAG}$). **(C)** Western blot analysis of immunoprecipitated (IP) dFOXO ($V3^{FLAG}$) showed that under standard conditions both dFOXO bands were phosphorylated. In the 5KQ (acetyl-mimic) mutant dFOXO's lower band seemed to be more phosphorylated where as it was the opposite for 5KR (acetyl-null). Anti-P (phosphor akt-

substrate) recognises phosphorylated residues within an AKT phosphorylation motif. dFOXO had three of these motifs, which were phosphorylated in response to insulin signalling.

Preliminary phenotyping of 5KQ and 5KR mutant animals did not identify any obvious abnormalities of these mutants, with mutant flies having normal body weight, development and fecundity (as these data are still preliminary they were not included here). Moreover, both females and males have a normal lifespan (Figure 4.3A-B), and female mutants were as resistant to starvation and oxidative stress as wild type replacement flies (Figure 4.3C-D). These results suggest that, even though these acetyl-mutations may interfere *in vivo* with the phosphorylation state of dFOXO, this is not sufficient to affect these normal functions of this transcription factor.

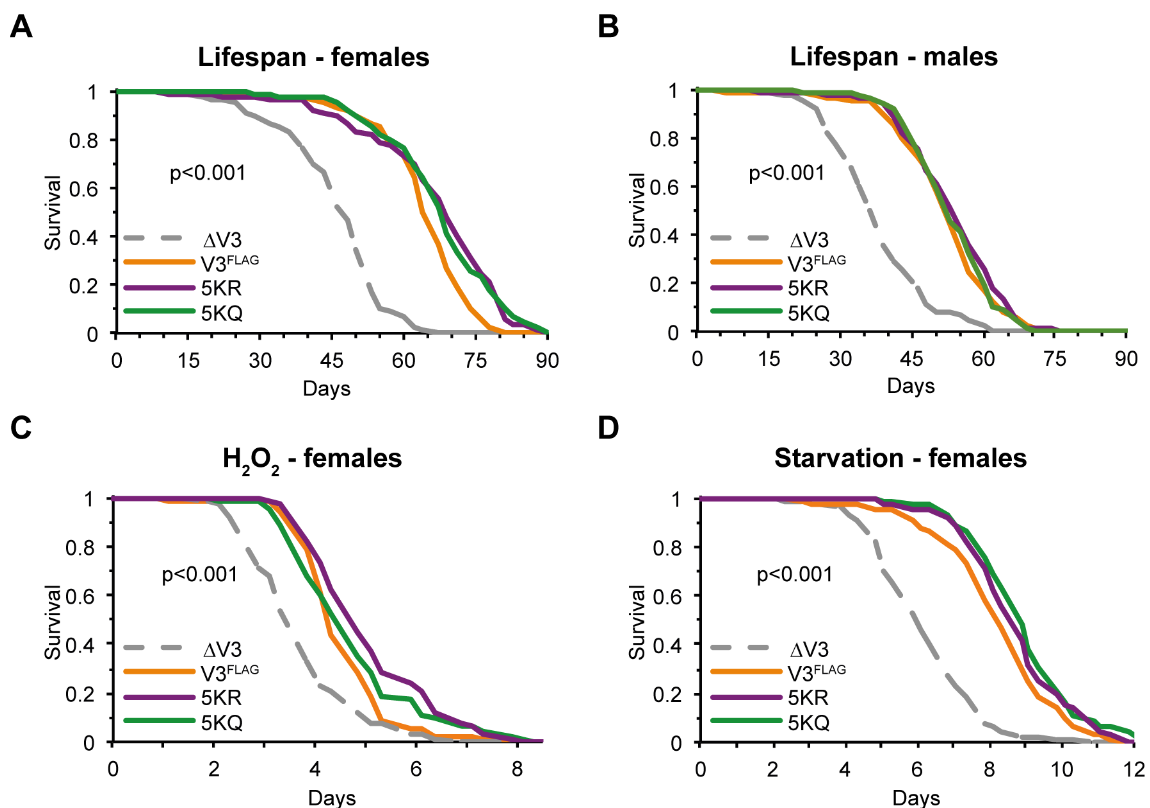

Figure 4.3. Mutation of acetyl-lysine residues does not interfere with normal dFOXO functions *in vivo*. **(A)** Female and **(B)** male lifespan assays showed acetyl-lysine mutants lived as long as gene replacement flies ($V3^{FLAG}$). ***$p<0.001$ log-rank test for comparison of *dfoxo-null* (ΔV3) vs $V3^{FLAG}$, 5KR or 5KQ. Flies of each genotype were placed on 1xSYA food and followed throughout life (n=100/genotype). **(C)** Female 5KR and 5KQ flies showed similar resistance to oxidative stress as control flies ($V3^{FLAG}$). ***$p<0.001$ log-rank test for comparison of *dfoxo-null* (ΔV3) vs $V3^{FLAG}$, 5KR or 5KQ. Flies of each genotype were placed on H_2O_2 media after 7 days on 1xSYA food (n=100/genotype). **(D)** Starvation assay for females showed 5KR and 5KQ mutants, unlike ΔV3 flies, were as resistant to starvation as $V3^{FLAG}$. ***$p<0.001$ log-rank test for

comparison of *dfoxo-null* (ΔV3) vs V3^{FLAG}, 5KR or 5KQ. Flies of each genotype were placed on starvation medium after seven days on 1xSYA food (n=100/genotype).

FOXO acetylation is highly regulated, both in mice and flies, by the nutritional state, where acetylation is reduced under starvation and increased after refeeding (Banks et al., 2011; Wang et al., 2011). Recent studies seem to reinforce the link between acetylation and the regulation of autophagy (Bánréti et al., 2013). Furthermore, acetylated FOXO1 is able to interact with ATG7 in the cytosol and induce autophagy in mammalian cell culture and *in vivo* (Zhao et al., 2010). With this in mind, we decided to test the response of the mutant flies to two diets to address if dFOXO acetylation might affect autophagy. Diet one, which served as a control, contained yeast, agar and no sugar, whereas diet two was composed of sugar and agar, but no yeast. The lack of yeast depletes amino acids from the diet and should induce autophagy (Mortimore and Schworer, 1977). In addition, along with replacement (V3^{FLAG}), *dfoxo-null* and acetylation mutant flies, we included the DBD mutants in order to test whether the response requires DNA-binding of dFOXO. As expected, flies required dFOXO to have a 'normal lifespan' under "no-sugar" conditions and this was fully dependent on dFOXO DNA binding ability but not on acetylation status (Figure 4.4A-B). *dfoxo-null* mutants (*ΔV3)* were also sensitive to the absence of yeast, demonstrating that dFOXO function is important for the response to amino acid starvation (Figure 4.4C-D). Interestingly, acetyl-mimicking (5KQ) flies were also sensitive to yeast deficiency, whereas dFOXO DBD mutants showed no (DBD1) or only a weak (DBD2) response. These findings implicate acetylation of dFOXO in the response to yeast starvation and suggest that that at least part of the response does not require dFOXO DNA binding ability (Figure 4.4C-D).

In the future, we will test these flies on a chemically defined diet (Piper et al., 2014) to verify if the short lifespan of dFOXO-5KQ mutants is caused by amino acid starvation. In addition, we plan to perform IPs on control versus amino acid-starved flies to see how the phosphorylation state of dFOXO changes under those conditions. Moreover, we will determine if autophagy status is altered on the different dietary regimes. The possibility of FOXO activity being affected by amino acids would give an additional layer of complexity to FOXO regulation and nutrient sensing.

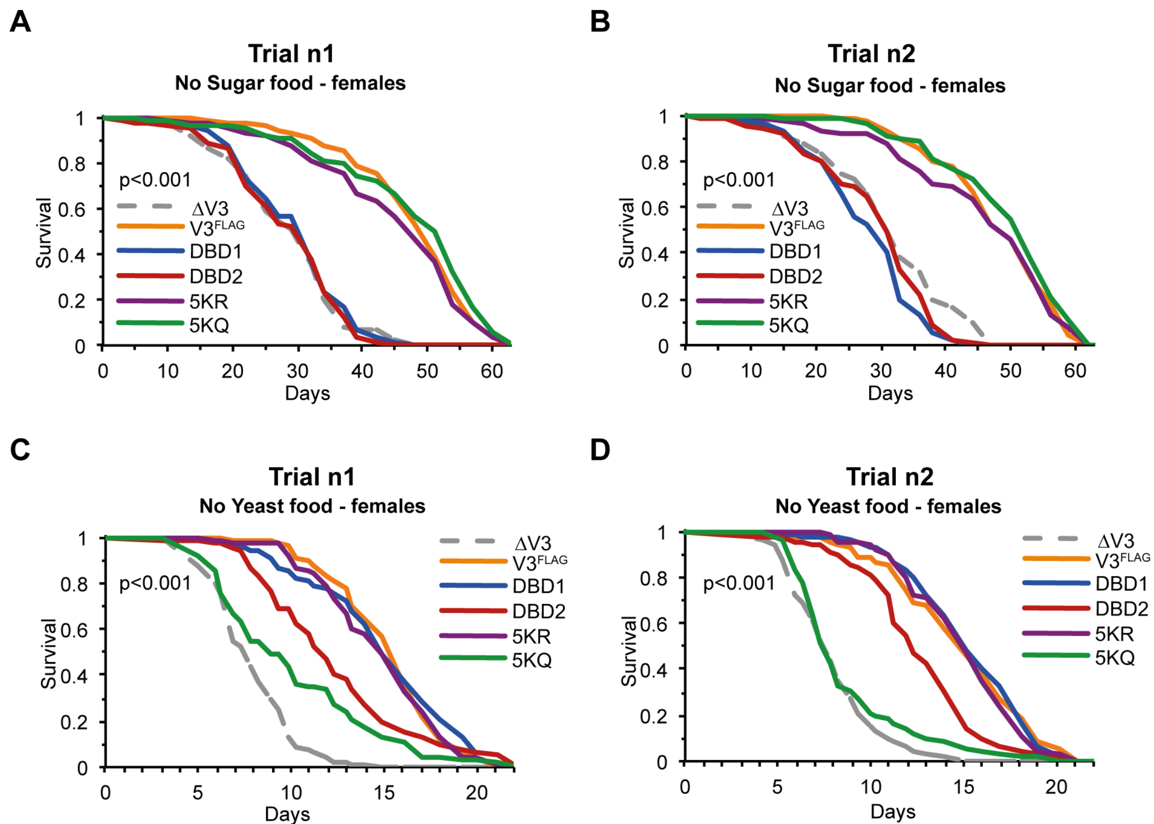

Figure 4.4. dFOXO acetylation could be required to modulate a response to amino acid starvation. (A-B) Trial #1 and #2 of female flies on no-sugar-food (1YA) showed *dfoxo-null* (ΔV3) and DBD mutant flies were sensitive whereas acetylation mutants (5KR and 5KQ) behaved as gene replacement flies ($V3^{FLAG}$). ***p<0.001 log-rank test for comparison of *dfoxo-null* (ΔV3), DBD1 or DBD2 vs $V3^{FLAG}$, 5KR or 5KQ. Flies of each genotype were placed on 1YA media after 7 days on 1xSYA food (n=100/genotype). **(C-D)** Trial #1 and #2 of female flies on no-yeast-food (1SA) showed *dfoxo-null* (ΔV3) and acetyl-mimetic (5KQ) flies were sensitive to the absence of yeast. In contrast, acetyl-null (5KR) and the DBD mutants were unaffected by this diet when compared to control flies ($V3^{FLAG}$). ***p<0.001 log-rank test for comparison of *dfoxo-null* (ΔV3) or 5KQ vs $V3^{FLAG}$, 5KR, DBD1 or DBD2. Flies of each genotype were placed on 1SA media after seven days on 1xSYA food (n=100/genotype).

4.2.3 Identification of dFOXO post-translational modifications

In order to better characterise dFOXO regulation, we plan to use dFOXO-V3^{FLAG} to identify novel post-translational modifications and protein interaction partners. This includes the putative but yet unidentified lysine acetylated residues. This system has the advantage that it would not require any artificial over-expression and would therefore minimise potential artificial effects. As a first step, I standardized an anti-FLAG IP protocol (see materials and methods) using whole fly extracts and evaluated efficiency of dFOXO purification by western blot (Figure 4.5A). In a preliminary assay, the eluted dFOXO protein has been evaluated twice by sequential digestion with trypsin and chymotrypsin followed by mass spectrometry. Mass spectrometry analysis was done in collaboration with the mass spectrometry core facility of the Max-Planck Institute for Biology of Ageing. The combined results cover 52.2% of the protein (Figure 4.5B). In order to maximize the number of post-translational modifications to be identified, and therefore have a better picture on how dFOXO is regulated, future efforts should further increase the protein coverage. Nevertheless, these trial experiments have already detected five phosphorylation residues (Figure 4.5B, red residues - S42, T44, S62, T63 and S66), four of which were previously unknown, one oxidized methionine (M21) and N-terminal acetylation. Phosphorylation of Threonine 44 (T44) is evolutionarily conserved and falls in an AKT phosphorylation motif composed by RxRxx(S/T), where x represents any amino acid. In contrast, the biological relevance of the remaining phosphorylated residues is currently unknown and awaits investigation.

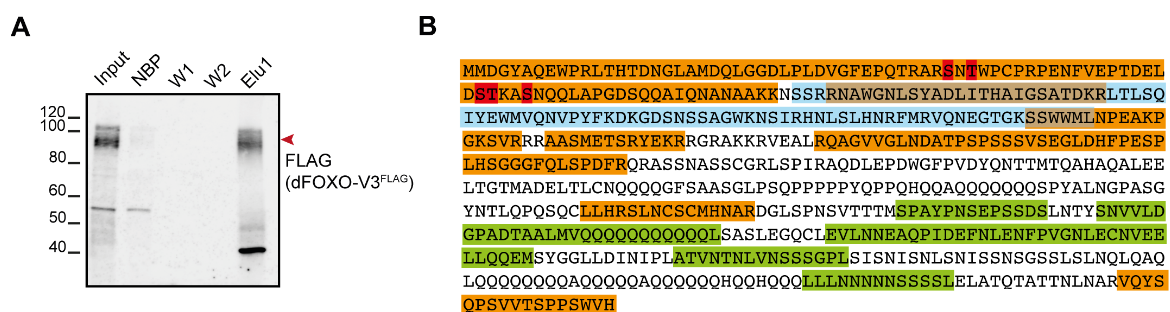

Figure 4.5. Validation of IP-MS protocol.
(A) Western blot using FLAG antibody (M2) showed proper enrichment of dFOXO protein in the elution following the described IP protocol for gene replacement flies (V3^{FLAG}). NBP (non-bound proteins), W (Wash), Elu1 (Elution 1). **(B)** Protein sequence of dFOXO highlights regions detected during MS analysis after trypsin digest (orange) or chymotrypsin digest (green). Phosphorylated residues detected are highlighted in red.

As described at the beginning of this thesis, identification of dFOXO interaction partners is critical in order to better understand its roles and ways of regulation. This endogenously tagged dFOXO-V3^{FLAG} tool can also be used to identify novel interaction partners under different stress conditions. This approach should broaden or understanding of the biology behind FOXO transcription factors. Moreover, the *dfoxo* gene-editing tool will allow the evaluation of any novel PTM or interaction partner by the generation of mutant alleles.

4.3 DISCUSSION

FOXO transcription factors are evolutionary conserved modulators of homeostasis. In order to participate in processes as diverse as body weigh determination, metabolism and ageing modulation, FOXO proteins are regulated by multiple PTMs, of which phosphorylation and acetylation are the best understood (Daitoku et al., 2011; Zhao et al., 2011). In mice, starvation prompts FOXO1 nuclear localization, whereas refeeding induces acetylation, subsequent AKT-dependent phosphorylation, and finally nuclear exclusion (Banks et al., 2011; Qiang et al., 2010). Therefore, acetylation of specific lysine resides within, or close to, the NLS, seem to play a critical role in FOXO regulation by reducing DNA binding affinity and facilitating phosphorylation (Brent et al., 2008; Brunet et al., 2004; Matsuzaki et al., 2005; Qiang et al., 2010). Moreover, a recent knock-in study demonstrated that a FOXO1 allele bearing acetyl-mimic mutations causes lethality during development, whereas acetyl-null mutant mice are viable and use fat, not sugar, as a preferred energy source (Banks et al., 2011). Similarly, refeeding seems to promote dFOXO acetylation in the fly (Wang et al., 2011). Moreover, cytoplasmic Daf-16, the worm FOXO homolog, seems to be constantly acetylated, and different kinds of stressors, such as heat shock or oxidative stress, induce deacetylation and therefore allow for nuclear localization (Chiang et al., 2012). Thus, regulation of FOXO factors by acetylation seems to be evolutionary conserved, however, the *in vivo* consequences of this modification are not fully understood.

To investigate whether acetylation could modulate any of the dFOXO-associated functions *in vivo*, we generated knock-in *dfoxo* alleles that either mimic (5KQ) or abolish (5KR) acetylation on conserved lysine residues within the NLS. We chose these residues based on 1) evolutionary conservation, since acetylated-lysine residues are more likely to be conserved (Weinert et al., 2011) and 2) these five residues being predicted to be acetylated by LAceP, a bioinformatics tool that predicts acetylation based on physicochemical characteristics of the amino acids surrounding lysine residues (Hou et al., 2014). Thus, even though it is still possible that dFOXO-acetylation takes place in different lysine residues, we deemed these five amino acids as the most likely candidates to undergo this modification.

dFOXO mutants that abolish acetylation (5KR) behave exactly as wild type flies for all tested phenotypes so far. These results, albeit preliminary, indicate that acetylation of lysine residues within the NLS does not modulate the way dFOXO regulates processes such as redox stress resistance, full starvation response and longevity. This observation could be in line with acetylation having a fundamental role in FOXO regulation upon refeeding in both mice and flies (Banks et al., 2011; Wang et al., 2011). However, we have not been able to reproduce detection of acetylated-dFOXO after refeeding with our $V3^{FLAG}$ (not shown), even after following the described protocol for flies (Wang et al., 2011). This could however be due to the different fly strains used, since the w^{DahT} flies used in this study are more resistant to starvation and it would therefore be important to standardize the starvation and refeeding times.

Similar to FOXO1 knockout mice (Furuyama et al., 2004; Hosaka et al., 2004), FOXO1 mice knock-in carrying an acetyl-mimic allele, where seven lysine residues were replaced by glutamine (7KQ), are developmentally lethal due to incomplete vascular development (Banks et al., 2011),. Worth noting, in addition to five lysine residues within the NLS, *foxo1-7KQ* mice have two extra residues mutated (Banks et al., 2011; Qiang et al., 2010), however, those residues are not evolutionarily conserved either in flies or worms. The developmental lethality observed in *foxo1-null* and *foxo1-7KQ* mice suggest FOXO1 is required to be in a non-acetylated state in order to allow correct angiogenesis (Banks et al., 2011). In contrast, *foxo3-null* and *foxo4-null* mice are viable (Castrillon et al., 2003; Hosaka et al., 2004), much like *daf16* mutant worms (Kenyon et al., 1993) and *dfoxo-null* flies (Puig et al., 2003; Slack et al., 2011) indicating that, under optimal conditions, only FOXO1 is required for mammalian development.

Unlike the *foxo1-7KQ* mice, acetyl-mimic mutations in dFOXO (5KQ) produce viable and seemingly healthy flies that are indistinguishable from wild type or acetyl-null mutant flies, with one exception. Yeast starvation, which is assumed to be an AA starvation diet, requires dFOXO to elicit a proper response, as shown by previous studies where *dfoxo-null* larvae and flies died prematurely on AA starvation diet (Hilliker et al., 2008; Slade et al., 2016). Consistent with this observation, *dfoxo∆V3* flies are sensitive to AA starvation. Interestingly, dFOXO mutants that mimic acetylation (5KQ) are also sensitive to this diet, unlike wild type ($V3^{FLAG}$) and acetyl-null (5KR) flies. This observation suggests dFOXO is required, in a non-acetylated state, to respond to AA starvation. Interestingly, the AA

starvation response has been associated with a decrease in histone deacetylation, at least in specific promoter regions of genes associated with this kind of stress (Shan et al., 2012). How AA starvation can modify the acetylation state of different proteins is still under investigation. Moreover, a not-fully defined mechanisms involved in AA starvation leads to the activation of c-Jun N-terminal kinase (JNK), which in turn phosphorylates and activates the histone acetyl transferase ATF2 (Averous et al., 2004; Bruhat et al., 2007; Chaveroux et al., 2009; Fu et al., 2011). This is interesting because JNK is also able to phosphorylate and activate FOXO proteins, specially under oxidative stress, in mammals, worms, flies and even *Hydra* (Eijkelenboom and Burgering, 2013). Hence, it is possible that AA starvation-induced JNK activation would also induce FOXO phosphorylation, which could be in turn affected by the acetylation status of the protein, or vice versa.

On the other hand, AA starvation represses mechanistic Target of Rapamycin (mTOR) complex 1 (mTORC1), preventing ATG13 phosphorylation and therefore inducing autophagy by allowing the formation of an ATG complex containing the kinase ATG1 (Kamada et al., 2000). Interestingly, mice over-expressing a FOXO1 protein containing mutations that make the protein constitutively nuclear and that inhibit its interaction with Sirt1 (NAD dependent histone deacetylase), inhibited glucose-deprivation-induced autophagy (Hariharan et al., 2010). This observation suggests that starvation induced autophagy, but this response seems to require FOXO1 in a non-acetylated state (Hariharan et al., 2010). This could indicate that our dFOXO acetyl-mimic mutants (5KQ) may be unable to induced autophagy under AA starvation, a possibility that will be addressed in the future.

The response to AA starvation seems to depend on dFOXO, but not on its DNA binding ability, since the dFOXO-DBD mutants are not (as) sensitive to this stress. This observation suggests that the role of dFOXO in AA starvation is not direct transactivation of target genes, and therefore it most likely behaves as a co-activator. However, it remains to be seen whether AA starvation actually induces dFOXO nuclear localization. In line with this idea, flies carrying a hypomorphic mutation for the *akt* gene are significantly longer lived than control flies under AA starvation (Slade et al., 2016). This result suggests that AA starvation induces AKT activation, and therefore possible dFOXO inactivation (cytosolic retention), which in turn would be detrimental for the AA starvation response. Furthermore, the AA starvation resistance seen in the *akt* mutant flies

is completely abolished in the absence of dFOXO (Slade et al., 2016), indicating that perhaps hypo-phosphorylated dFOXO, e.g. nuclear dFOXO, is fundamental to mediate this response. Nevertheless, this hypothesis should be tested in the future.

Our results indicate that acetylation of the mutated lysine restudies is detrimental for the nutritional response involved in AA, but not full, starvation. This observation has two connotations. First, since both acetyl-null and acetyl-mimic are resistant to full starvation, the absence of sugar overrules the presence or absence of acetylation on dFOXO. This observation could be consistent with our observation that lipid usage under full starvation is dependent on dFOXO but not its direct interaction with DNA, as acetylation was reported to interfere with DNA biding (Brunet et al., 2004; Daitoku et al., 2004; Matsuzaki et al., 2005). Second, it implies that AA starvation induces dFOXO deacetylation, which in turn is a prerequisite to exert the appropriate response to this kind of stress. Hence, it would be important to determine if HDAC4 mediates dFOXO deacetylation under AA starvation, just as it seems to do under full starvation (Wang et al., 2011). However, AA starvation seems to repress or down-regulate HDAC4 activity in mammalian cell culture (Palmisano et al., 2012) and it would therefore be important to test this *in vivo*. Nevertheless, other deacetylases could regulate this modification on dFOXO. For example, mammalian and worm FOXO proteins seem to be regulated by SIRT proteins, NAD-dependent histone deacetylase (Brunet et al., 2004; Chiang et al., 2012; Frescas et al., 2005; Wang et al., 2007). In accordance, a genetic link was recently reported between Sir2, the *Drosophila* homolog of Sirt1, and dFOXO (Palu and Thummel, 2016). However, whether this is a direct interaction is still unclear. Moreover, Sirt1-dependent deacetylation of FOXO1 was shown to be fundamental for glucose-starvation-induced autophagy (Hariharan et al., 2010), suggesting this as a possible mechanism regulating AA starvation survival in our dFOXO acetyl-mutant flies. In addition, FOXO acetylation seems to occur under refeeding conditions in both flies and mice (Banks et al., 2011; Wang et al., 2011). Hence, it would be interesting to assess whether the dFOXO acetylation mutants are able to elicit a proper refeeding response after full or AA starvation.

Acetylation seems to regulate AKT-dependent phosphorylation of FOXO proteins (Banks et al., 2011; Matsuzaki et al., 2005; Qiang et al., 2010). Consistent with these observations, our results indicate that acetylation of the NLS lysine residues seems to

modulate dFOXO migration on SDS-PAGE studies. Acetyl-null mutants seem to migrate normally while acetyl-mimic dFOXO seems to accumulate in the lower band. Traditionally, the SDS-PAGE migration shift from the lower to higher band has been attributed to phosphorylation of the highly conserved AKT-substrate residues. However, we can detect phosphorylation on AKT-motifs in both bands. Previous studies have shown that FOXO3 migration-shift depends exclusively on AKT-dependent phosphorylation of residue S315 (Brunet et al., 1999). Interestingly, the observed shift in dFOXO seems to be fully dependent on phosphorylation, as phosphatase treatment collapses both bands into the lower molecular weight, *in vitro* (Puig et al., 2003) and *in vivo* (Alic et al., 2011). Whether phosphorylation of the last AKT-phosphorylated residue facilitates any other phosphorylation, or is solely responsible for the migration-shift, is currently unknown. Our results suggest that acetylation of the lysine residues within the NLS may specifically regulate phosphorylation of the FOXO3 S315 homologous residue (dFOXO S259) and thus modulate whatever the cause for the migration shift is.

It is worth noting that acetyl-mimic modifications, e.g. replacement of lysine by glutamine residues, are a great approach to better understand this modification. Lysine residues are positively charged at physiological pH and this charge is abolished upon acetylation. Hence, glutamine mimics the charge disappearance associated with lysine-acetylation. However, this mimic does not recapitulate acetyl-lysine mediated interaction with proteins containing a so-called *Bromodomain* (Filippakopoulos and Knapp, 2014). Therefore, any conclusions drawn from such mutations should consider this limitation. Nevertheless, the observation that acetyl-mimic dFOXO flies, but not acetyl-null, are sensitive to AA starvation indicates that the regulation of this response is most likely independent of any putative acetyl-lysine mediated interaction.

In conclusion, our results suggest that dFOXO-acetylation may play a critical role in the AA starvation response. Future studies should address whether this response is in fact triggered by AA deprivation, for example by using a chemically defined diet (Piper et al., 2014), and determining the mechanisms behind FOXO acetylation in response to AA availability signalling. In addition, we have developed an IP-MS coupled system that will allow us to detect dFOXO-acetylated residues. Moreover, this tool will also allow identification other PTMs, helping us better understand of dFOXO regulation *in vivo*.

CONCLUSION

5.1 CONCLUSIONS AND FUTURE PERSPECTIVES

In this study, we described the generation and validation of a genetic tool that permits reliable modification of the endogenous *dfoxo* locus. Thus far, we have used this tool to generate novel mutant alleles that abolish DNA binding, allowing us to dissect different dFOXO-regulated functions in the fly. Being a transcription factor, it is no surprise dFOXO's ability to bind DNA is required for most of its known functions, such as fecundity, oxidative stress resistance and lifespan. Remarkably, dFOXO is able to regulate body weight and lipid usage under starvation independent of DNA-binding. The mechanisms by which the DBD mutants modulate starvation resistance are still unclear. However, our experiments suggest a possible synergistic effect between two systems. The first system refers to direct and indirect regulation of enzymes involved in lipolysis, such as *bmm* and *lip3*, and the second one refers to modulation of starvation-induced autophagy. Both systems are probably working in concert to allow proper mobilization of lipids under starvation. Hence, the next step would be to identify which proteins are involved in the indirect gene regulation and to identify how exactly is autophagy being limited in the absence of *dfoxo*. Moreover, it would be interesting to determine which transcription factor dFOXO is interacting with to modulate the starvation response.

The results associated to our second set of mutant *dfoxo* alleles, which mimic or abolish acetylation of evolutionarily conserved lysine residues within the dFOXO NLS, have three major connotations: first, acetylation does not seem to play a role in many of the functions associated with dFOXO, such as development, full starvation and ageing. Second, acetylation seems to play a fundamental role in dFOXO phosphorylation *in vivo*, but the biological relevance of this observation is still unclear. Third, dFOXO is required in a non-acetylated state to respond to AA starvation. Even though preliminary, these results are the firsts steps to better understanding how dFOXO is associated to amino acid sensing, a link not previously described.

Finally, the tools generated here will allow future research to further separate dFOXO-associated functions, by better understanding the regulation of this transcription factor. For example, researchers will be able to detect dFOXO interaction partners and PTMs in a system with minimal artificial effects. Hence, endogenous dFOXO levels could be monitored biochemically (FLAG tag) or *in vivo* (mCherry tag) to assess the role this transcription factor plays in the multiple functions with which it is associated.

Once we have a better understanding of how FOXO factors are regulated, and in turn are able to modulate a great number of cellular processes, we will be able to separate the beneficial and detrimental effects of FOXO manipulation in ageing studies. With that, we might be a step closer to preventing age-associated diseases, even if dFOXO does not turn out to be our internal *fountain of youth*.

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SUPPLEMENT

Supplementary Table S1. Primers used during this PhD thesis.
Fw – forward. Rv – reverse.

Stock number (SOL)	Oligo name	Sequence
202	pGX-attP 5´SpeI	CTCGACACCGGTATAACTTCGTATAATG
205	pGX-attP 3´KpnI	CACTACGCCCCCAACTGAGAGAAC
238	Foxo primer	GAGAACAACAAGAAGATAAGTCCGCC
376	Foxo sequencing 1	GGCCCAGGCTGACCCACAC
377	Foxo sequencing 2	CCGGCAAGTCATCCTGGTG
378	Foxo sequencing 3	CCCGCCCCCGCCCTATC
379	Foxo sequencing 4	GCGGCCTGCTGGACATCA
380	Foxo sequencing 5	CGGCTACCACAAATCTGAATGCTC
381	Foxo sequencing 6	ATTGCGACGCGATGAGTTCTTCT
382	Foxo ORF 5´primer	CACCATGATGGACGGCTACGCGC
383	Foxo ORF 3´primer	CTAGTGCACCCAGGATGGTGGC
384	Foxo 25 3´ primer	CACATTCTGGACCATTCACTCGTAAATC
385	Foxo DBD 1x 5 primer	GGAAGAACTCCATACGTGCCAATCTGTCGCTGC
386	Foxo DBD 1x 3´ primer	GCAGCGACAGATTGGCACGTATGGAGTTCTTCC
387	Foxo DBD 2x 5´ primer	TGGAAGGCCTCCATACGTGCCAATCTGTCGCTGCAC
388	Foxo DBD 2x 3´ primer	GTGCAGCGACAGATTGGCACGTATGGAGGCCTTCCA
393	Foxo 3KR 5´ primer	CCGGAGGCCAGGCCCGGCAGGTCTGTGCGCCG
394	Foxo 3KR 3´ primer	CGGCGCACAGACCTGCCGGGCCTGGCCTCCGG
397	Foxo 3KR 5´ primer II	CCGGTACGAGAGGCGGCGCGGCAGGGCC
398	Foxo 3KR 3´ primer II	GGCCCTGCCGCGCCGCCTCTCGTACCGG
463	pGE attB amp 5´	GGGAATAAGGGCGACACGGA
514	Foxo 2KQ 5´	CCGGAGGCCCAGCCCGGCCAGTCTGTGCGCCG
515	Foxo 2KQ 3´	CGGCGCACAGACTGGCCGGGCTGGGCCTCCGG
516	Foxo 3KQ 5´	CCCGGTACGAGCAGCGGCGCGGCAGGGCC
517	Foxo 3KQ 3´	GGCCCTGCCGCGCCGCTGCTCGTACCGGG
561	Foxo-5KQ 5’	CGCGGCAGGGCCCAGCAGCGGGTGGAGGCA
562	Foxo 5KQ 3’	TGCCTCCACCCGCTGCTGGGCCCTGCCGCG
563	Foxo 5KR 5’	CGCGGCAGGGCCAGGAGGCGGGTGGAGGCA
564	Foxo 5KR 3’	TGCCTCCACCCGCCTCCTGGCCCTGCCGCG
572	dFOXO G. engineering 1st Arm fw	GCTGCTAGGCCGCCTTTGAGCAGCTGTTCCGGATTGACTTGGCCTCGC CAGCGGCCGCCACAACATACGAGCCGGAAGCATA
573	dFOXO G. engineering 1st Arm rv	TGTATGAGTACTCTGAACAAGATATATAGTCATGTACAACTTTAAAT GATGCGGCCGCATGTGCGCGGAACCCCTATTTG
574	dFOXO G. engineering 2nd Arm fw	ATTTGTGGCTTTGATTATGAAGATTAAACTTAAGCTTAGTAAGTATAA AAACTAGTCACAACATACGAGCCGGAAGCATA
575	dFOXO G. engineering 2nd Arm rv	ATAGCAAAGACAGAGGCGACACGGAAGCAGCCGCGCCCTCGATTCT GTTCCTCGAGATGTGCGCGGAACCCCTATTTG
576	dFOXO G. engineering 3rd Arm fw	GAACCAAAGACTAAAGACTAAACAAATCGGCCAGATACAATTTATG ATGAGCGGCCGCCACAACATAGAGCCGGAAGCATA
577	dFOXO G. engineering 3rd Arm rv	ATTGCTTACAAATCGTTACATCAGCGCGATGAGTTCCCAAGGATTTG CAAGGTACCATGTGCGCGGAACCCCTATTTG

578	dFOXO G. engineering 4th Arm fw	AACTGCTAATACTCCGCAGGATAACTTAAGTTTAACTTAAGTTAGCT GTAGGCGCGCCCACAACATACGAGGGAAGCATA
579	dFOXO G. engineering 4th Arm rv	GTGGCACATCTAATCACAGGCCTTGGCATACAGTCAGTCAGTCAGTC AATCTCGAGATGTGCGCGGAACCCCTATTTG
580	dFOXO Arm1 sequencing	CCACGTTTATCGGCGTATATCT
581	dFOXO Arm1 sequencing	GTAAGCTGTGACGCAGTAGAT
582	dFOXO Arm1 sequencing	AAGGCCTGCAGATATAATCATACA
583	dFOXO Arm1 sequencing	CGCGCGACCCTATCTAAATTAT
584	dFOXO Arm1 sequencing	TGAGTACCTGAAGTGCATTTCAAT
585	dFOXO Arm1 sequencing	AATGTGCGAGGCATTTAAACAAGA
586	dFOXO Arm1 sequencing	GAAGAATGAGGAGCAAAACGAAAT
587	dFOXO Arm2 sequencing	TTTACATCGCAGCATACGTAAATA
588	dFOXO Arm2 sequencing	ATTTTTGCTGATACTTCAGCACTC
589	dFOXO Arm2 sequencing	GTTTGTCTATGGATAATCGAACTA
590	dFOXO Arm2 sequencing	ATTGAACCGGTGCCAAGTATT
591	dFOXO Arm2 sequencing	GCCTTATCATAATGGGCCTTAT
592	dFOXO Arm2 sequencing	GGGAATGAGAACAAATACGAGA
593	dFOXO Arm2 sequencing	TACCACTGACTGATGATTGCATAA
594	dFOXO Arm3 sequencing	TTATGTTGTCACTCGCAGCCAT
595	dFOXO Arm3 sequencing	GGGTTTAAACCGTTGAATATGTC
596	dFOXO Arm3 sequencing	TTGTGATTCACGCTCGATGCA
597	dFOXO Arm3 sequencing	AATGCTCTCGCGTTCTTTAGG
598	dFOXO Arm3 sequencing	AACAGAGAAGCAGCTTCCACT
599	dFOXO Arm3 sequencing	TTTAGCCTGACTAACAGTGGG
600	dFOXO Arm3 sequencing	CGTTCCGAGAAATCCAGAAATC
601	dFOXO Arm4 sequencing	TTAAGTCCTTTTCAATTTGGGTGC
602	dFOXO Arm4 sequencing	TTAAAACTGAGATTCGACACGGTT
603	dFOXO Arm4 sequencing	CAATCGCATGACGTGCTAATAA
604	dFOXO Arm4 sequencing	CCCCCAAACTTCTATTCTTTGA
605	dFOXO Arm4 sequencing	AATTAGCGACAGGATTTGTCC
606	dFOXO Arm4 sequencing	ATAATGACGTGACAGGAGCTA
607	dFOXO Arm4 sequencing	ATGGATGGAAGTGAAGTCAAGT
628	dFOXO Arm1 sequencing extra	CGCTCGGTACAAATCTTCAGGTGG
629	dFOXO Arm2 sequencing extra	CAGCATTGAAAAGGGGCAACCGGT
630	dFOXO Arm3	CCCCGATTCATTCGGCTAATTGCT

	sequencing extra	
631	dFOXO Arm3 sequencing extra	AAGTACCTTTTCGGTAAAGACCTT
632	dFOXO Arm3 sequencing extra	TACTTGGCTGGATTTGCTTTACCA
633	dFOXO Arm3 sequencing extra	CGCCGGACACACACAGCGAAAATG
634	dFOXO Arm4 sequencing extra	GCACCCCCGTTAAATATGCACTGG
635	dFOXO Arm4 sequencing extra	TAACTTCTGCCTTTTTCCCGGCAC
661	Before Arm1 dFOXO	CGTCGCCTGTCTGATTCCCCCTGA
662	After Arm2 dFOXO	TACACGTTCGAGCCCCCTTTGCAC
663	Before Arm3 dFOXO	CGTCTGCTGCCATCTTGCTAAAGC
664	After Arm4 dFOXO	CGCTTTATGGCCGCCACTCCTTGG
665	dFOXO small deletion fw	TGCAACTGCACAACGAACTTTTCT
666	dFOXO small deletion rv	ATAGATTTCCCCATGCATTGCG
667	dFOXO large deletion fw	GCTATCTCCAGAGCTGAAGTTT
668	dFOXO large deletion rv	CATACGTATGCACATGATTGACTG
669	dFOXO medium deletion fw	TGCACAACCGCTTTATGAGGGT
670	dFOXO medium deletion rv	CAGTTGATAGTTACCTGTGGAG
681	dFOXO replacement V2 fw	TTAAATACAAATCTAATCAAGCTACTAAAGATTAATTTAAAATTACTT TGGCTAGCCACAACATACGAGCCGGAAGCATA
682	dFOXO replacement V2 and V3 rv	TATTTGGGATAAAGGGTACAAAAATATCTCAGTTTAATATCACTTTA ATTGGCGCGCCATGTGCGCGGAACCCCTATTTG
683	dFOXO replacement V3 fw	ATATATTTTGCAAAAACCGAAATATATTGAATGTAAAAGAATTGCAA AATGCTAGCCACAACATACGAGCCGGAAGCATA
690	dFOXO Exon sequencing	CTTTAGTAGCTTGATTAGATTTGT
691	dFOXO Exon sequencing	AATAGACTCACAGCAGGCTATA
692	dFOXO Exon sequencing	TCTTTTACATTCAATATATTTCGG
693	dFOXO Exon sequencing	AGATACTCCTTGCTATTATATCTT
694	dFOXO Exon sequencing	AGTGGCGGTGGCTTCCAATTAT
695	dFOXO Exon sequencing	TTCAGTGCCGCCTCGGGACT
696	dFOXO Exon sequencing	CCAGCTTGGAAGGTAATTATGA
697	dFOXO Exon sequencing	CAGTTGCTGCTAAATAATAACAAC
698	dFOXO Exon sequencing	TCCATCATGGGGTAGAAAAGTT
699	dFOXO Exon sequencing	GAGAACGAGAGAGAAACATATATG
700	dFOXO Exon sequencing	TAACGATCATCCTTGCGATTTGGA
713	Tags V1short PCR fw	GTATGTTGTGGCTAGCCAAAGTAATTTTAAATTAATC
714	Tags V1short PCR rv	TTGGATCTGGCCCGGGTCTGTGGCTCGA
717	FOXO Tags seq1	ATTGATTGCGAAGCTTTCACATTC
718	FOXO Tags seq2	CTGTGGCTCGAAGCCCACGT
719	FOXO Tags seq3	TCGAACTCGTGGCCGTTCAC

720	FOXO Tags seq4	TCCCCTCAGTTCATGTACGG
721	FOXO Tags seq5	CAACATCAAGTTGGACATCACCTC
722	Verify FOXO V1 or V2 reinsertion fw	ACAAGATATATAGTCATGTACAAC
723	Verify FOXO V1 or V2 reinsertion rv	CAAATCTAATCAAGCTACTAAAGA
724	Verify FOXO V1 reinsertion rv	AGAAAACGTAGTGCAATTTGTGGC
725	Verify FOXO V3 reinsertion fw	ATTGCTTACAAATCGTTACATCAG
726	Verify FOXO V3 reinsertion rv	GCAAAAACCGAAATATATTGAATG
727	Verify FOXO V2 or V3 reinsertion rv	TTGAAACCGAACGGTGCGGT
728	shortV3 Tags PCR fw	TCACAGCCCAGCGTGGTGACCTCGCCACCATCCT
729	shortV3 Tags PCR rv	AGTTATGGTACCGGCGCGCCAATTAAAGTGATATTA
791	dFOXOΔV1 end of Arm1 fw	CGACATATATGTATGAGTACTCTG
792	dFOXOΔV1 loxP rv	GCATACATTATACGAAGTTATGG
793	dFOXOΔV1 attP fw	GCGGGCTAGCACATATGC
794	dFOXOΔV1 Beginning of Arm2	CGTAGTGCAATTTGTGGCTTTG
809	dFOXO-V3-DBD1 fw	CGTTCTCGTTACAGAACTCCATACGTGCCAATCTGTCGCTGC
810	dFOXO-V3-DBD1 rv	GCAGCGACAGATTGGCACGTATGGAGTTCTGTAACGAGAACG
811	dFOXO-V3-DBD2 fw	CGTTCTCGTTACAGGCCTCCATACGTGCCAATCTGTCGCTGC
812	dFOXO-V3-DBD2 rv	GCAGCGACAGATTGGCACGTATGGAGGCCTGTAACGAGAACG
813	dFOXO-V3 fw	CATGCAATGCGGCCGCTAGCATTTTGCAATTCTTTTACA
814	dFOXO-V3-MAD rv	CAGGATGGTGGCGAGGTCACCCTACTTGTCATCGTCATCCTTG
817	dFOXO-V3 rv	CGAAGTTATGGTACCGGCGCGCCAATTAAAGTGATATTAAACTGAGA
823	pUbiP-EGFP-dFOXO seq	CCGACCACTACCAGC
824	dFOXO-V3 rv for tags	AGGATGGTGGCGAGGTCACCACGCTGGGCTGTGAGTA
883	dFOXO-V3 fw	GCAATGCGGCCGCTAGCATTTTGC
884	dFOXO-V3 rv	GTACCGGCGCGCCAATTAAAGTGA
885	dFOXO-V3 shorter for seq rv	GTTGATAGTTACCTGTGGAGCGGA
886	dFOXO-V3 shorter for seq fw	TGGAGAACTTTCCCGTGGGCAATC
887	dFOXOΔV3 end of Arm3 fw	GACGAGGTCTGGTGAAGACA
988	Thor1 ChIP-qPCR fw	GAATGCGATTGGCGTTTAGT
989	Thor1 ChIP-qPCR rv	CTCGCCTTGAGCTCTTGTTT
1092	lip3-promoter-fw-long	TCTATCGATAGGTACCCGCCTATATCAGTAGCTAATATG
1093	lip3-promoter-fw-short	TCTATCGATAGGTACTTGCTGGCGTGATTCAGGCA
1094	lip3-promoter-rv	CCGGAATGCCAAGCTCGCAGTACTATGATCCGTG
1127	UAS-Lip3 genotype fw	ATGACAAGAGGAGCGTTAAAAGTG
1128	UAS-Lip3 genotype rv	TAGGTGTTGCCTCGGGCATT
1139	Lip3 promoter seq extra#1	GTCCAATAGTCCACCGTAAC
1140	Lip3 promoter seq	CATAACATTGGCATTCTTGGCC

	extra#2	
1160	3xHA-HNF4 fw	TATGACGTCCCGGACTATGCAGGATCCTATCCATATGACGTTCCAGA TTACGCTGGTAGCGGCAGCGGTAGCATGATGAAGCATCCGCAGGA
1161	HNF4 rv	GATCCTCTAGACTAGCTAGCCTAGTAACCAGTCTCTGGCT
1162	3xHA-HNF4 fw2	CGCAGAATAATCCAACTAGTATGTACCCATACGATGTTCCTGACTAT GCGGGCTATCCCTATGACGTCCCGGACTATG
1167	3xHA-HNF4 seq1	GTTGGCGTCGATAAATAAGTT
1168	3xHA-HNF4 seq2	TGCATTATGTGGCTCTCCGA
1169	3xHA-HNF4 seq3	AAGTGGGACTAACAGCAGTC
1170	3xHA-HNF4 seq4	ATCACAAGGCACTGTCCAGA
1171	3xHA-HNF4 seq5	ATGATTCCTTCCGGGCCTA

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Victor Bustos