ANALYSIS OF THE FORKHEAD BOX SUBGROUP 'O' (FOXO) FAMILY OF TRANSCRIPTION FACTORS AND THE TOXIC EFFECTS OF Gal4 IN DROSOPHILA MELANOGASTER

JAMIE M. KRAMER

Analysis of the forkhead box subgroup 'O' (FOXO) family of transcription factors and the toxic effects of Gal4 in *Drosophila melanogaster*.

by

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Abstract

The insulin signalling pathway provides a conserved mechanism used by metazoan animals to regulate growth, metabolism, and behaviour in response to environmental cues. The FOXO transcription factors regulate cellular function in the presence of low levels of insulin signalling. This thesis describes the identification of the *Drosophila melanogaster* homologue of FOXO (dFOXO), that has high sequence conservation in the forkhead box DNA binding domain and Akt phosphorylation sites, when compared to analogous genes in mice, humans, and *Caenorhabiditis elegans*. Overexpression of dFOXO in the developing eye leads to a characteristic phenotype resulting from growth reduction. This phenotype can be rescued by co-expression of upstream PI3K signalling components, unless FOXO is mutated to be non-responsive to Akt phosphorylation. Ubiquitous expression of dFOXO at different stages of larval development closely replicates the effects of starvation, and endogenous dFOXO activity is modulated by the presence of nutrients. These results suggest that dFOXO is important for the response of Drosophila larvae to starvation. Analysis of dFOXO loss of function suggests that dFOXO is required for the adaptation of young larvae to starvation, but is not required during starvation in the later stages of larval development. A role for dFOXO in larval wandering is suggested by the observations that loss of dFOXO leads to reduced height of pupation, overexpression of dFOXO induces wandering, and endogenous dFOXO activity is increased during the wandering stage of the larval life cycle. In summary, dFOXO may regulate growth, behaviour, and starvation resistance in response to reduced PI3K signalling in Drosophila.

Studies in Chapter 1.2 utilize the UAS/Gal4 ectopic expression system. We have shown that Gal4 causes a rough eye phenotype in adult flies when expressed at high levels during eye development. The rough eye phenotype is characterized by a loss of ommatidial organization and is accompanied by a high level of apoptosis in the eye imaginal discs where Gal4 is expressed. These phenotypes are essentially eliminated through co-expression of the caspase inhibitor, p35. This suggests that Gal4 has the capability to act as a toxic protein that activates the cellular apoptotic machinery. These results should be taken into consideration in the design and analysis of any experiment involving the use of the UAS/Gal4 system.

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List of Abbreviations

4E-BP - eIF-4E binding protein (4E-BP)
act - actin
AdipoR1/2 – adiponectin receptors 1 and 2
AEL – after egg laying
AFX - acute lymphocytic leukemia-1 fused gene from chromosome X
AH – after hatching
ALS – acid labile subunit
AR - androgen receptor
arm - armadillo
BAD - Bcl-2 antagonist causing cell death
BIM - Bcl-2 interacting mediator of cell death
CDK – cyclin dependent kinase
CBP - CREB binding protein
CCLR - cell culture lysis reagent
CEBPβ - CAAT-enhancer binding protein β
CK1 - casein kinase 1
CNS – central nervous system
Crm1 – chromosomal region maintenance protein 1
daf – dauer formation
dAkt – Drosophila Akt
dEGFR - Drosophila epidermal growth factor receptor
Diap1/2 – Drosophila inhibitor of apoptosis 1 and 2
dILP – Drosophila insulin-like peptide
dInR - Drosophila insulin receptor
dMyc – Drosophila Myc
DN – dominant negative
dPTEN – Drosophila PTEN
dRas1/2 – Drosophila Ras 1 and 2
DYRK1A/B – dual-specificity tyrosine-phosphorylated and regulated kinase 1A and B
dFOXO – Drosophila FOXO
DNA – deoxyribonucleic acid
eIF-4E - eukaryotic initiation factor 4E
ER - estrogen receptor
FasL - Fas ligand
FKHR - forkhead in rabdomyosarcoma
FKHRL1 - FKHR-like 1
FOXO – forkhead box, subgroup 'O'
FRE – FOXO recognition element
G6Pase – glucose 6-phosphatase
GADD45 – G_2 arrest and DNA damage repair gene 45
GMR – glass multiple reporter
GR - glucocorticoid receptor

GS - glycogen synthase
GSK3β - glycogen synthase kinase 3β
Hh – hedgehog
HNF4 - hepatic nuclear factor 4
hs – heat shock
HST – heat shock treatment
IκB - inhibitor of NF-κB
IGF – insulin-like growth factor
IGFR – IGF receptor
IIS – insulin/IGF signalling
IKK - IκB kinase
InR – insulin receptor
IRS – insulin receptor substrate
JAK – janus kinase
JNK – jun N-terminal kinase
LPL - lipoprotein lipase
Luc - Luciferase
MAPK – mitogen activated protein kinase
mFoxo – Murine FOXO
MLL - mixed lineage leukemia
Mnb - minibrain
MnSOD - manganese superoxide dismutase
NES - nuclear export sequence
NF-κB - nuclear factor-κB
NGF - nerve growth factor
NLS - nuclear localization sequence
PAX3/7 – paired box 3 and 7
PBS – phosphate buffered saline
PDK1 - phosphoinositide-dependent kinase-1
PDK4 - pyruvate dehydrogenase kinase 4
PEPCK - phosphoenolpyruvate carboxykinase
PGC-1α - PPARγ coactivator 1α
PH – pleckstrin homology
PI3K – phosphotidylinositide 3-kinase
PIP_2 - phosphotidylinositide 4,5-bisphosphate
PIP_3 - phosphotidylinositide 3,4,5,-triphosphate
PPARγ - peroxosome proliferator activated receptor γ
PR - progesterone receptor
PTB – phosphotyrosine binding domain
PTEN - phosphatase and tensin homologue
RAR - retinoic acid receptor
Rheb – Ras homologue enriched in brain
RLU – relative light units
ROS – reactive oxygen species

RTK – receptor tyrosine kinase
S6K – ribosomal subunit 6-kinase
SCP2 - sterol carrier protein
SEM – scanning electron microscope
SGK - Serum and glucocorticoid-regulated kinase
SH-2 – Src homology 2
SIRT1 - silent information regulator of transcription 1
STAT – signal transducer and activator of transcription
Tb - tubby
TGF-β – transforming growth factor β
TOP - 5' terminal oligopyrimidine tract
TOR - targaet of rapamycin
TR - thyroid hormone receptor
TSC1/2 - tuberous sclerosis complex 1 and 2
UAS – upstream activation sequence
Wnt – wingless and int-1

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Co-authorship statement

The following statement clarifies the roles played by multiple authors in the manuscript chapters of this thesis, 1.2, 1.3, 2.2, and 2.3. In accordance with the requirements of the School of Graduate studies, my role in the completion of these manuscripts chapters is defined with respect to the following categories: *i) design and identification of the research proposal; ii) practical aspects of the research; iii) data analysis*; and *iv) manuscript preparation.*

i) design and identification of the research proposal; and ii) practical aspects of the research: Chapter 1.2: BES initiated the research project by identifying FOXO biometrically, obtaining dFOXO cDNA (LD05569), creating UAS-dFOXO transgenic fly, sequencing LD05569 (honours student JTD), and cloning mFoxo cDNAs and the FRE-luc cDNA into the transformation vector (masters student JTL). JMK created transgenic flies containing mouse FOXO cDNA and the FRE-Luc transgene and performed all overexpression studies including the genetic manipulations required for these studies and the subsequent phenotypic analysis. Chapter 1.3: all experiments were conceived and performed by JMK. Chapter 2.2: All experiments were initiated performed and conceived of by JMK. Chapter 2.3: As follow up to Chapter 2.2, AFMH created fly strains and performed EM analysis, while JMK performed staining and fluorescent microscopy of larval tissue. These practical contributions were judged as being equal by all parties involved, however these experiments were initially conceived of by JMK.

iii) data analysis: JMK collected and analysed all data with the exception of; 1) sequencing of LD05569 in chapter 1.2, 2) SEM analysis in chapter 2.3.

iv) manuscript preparation: All manuscripts were prepared by JMK with critical review from BES, and in some cases AFMH.

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Chapter 1

Identification and regulation of the FOXO family of transcription factors in Drosophila: implications in the developmental response to starvation

Chapter 1.1: Introduction and Overview

1.1.1 - Signal Transduction in Metazoans

Life on earth is remarkably diverse, yet amazingly simple. An estimated 1.7 million species have been described and it is possible that there are anywhere between two and ten million species living on the planet. The majority of these species are rarely seen by humans because of their small size or remote location, but we have only to look around ourselves to witness the great array of plants, animals, and insects inhabiting the earth. Despite the great diversity of organisms there is a high level of conservation in the fundamental cellular processes that define life. DNA replication, transcription, translation, membrane transport, protein structure, nucleic acid structure, and many metabolic pathways are cellular features that are shared among all life forms and are thought to have evolved about 3.2 billion years ago in the eubacteria [1]. Even the common traits that define eukaryotic cells probably evolved about 2 billion years ago in prokaryotes and the early single celled eukaryotes. These traits include the formation of cilia and flagella, the existence of membrane bound organelles, the ability to perform endo- and exocytosis, the processes of mitosis and meiosis, the dynamic formation of an actin/tubulin cytoskeleton, the CDK/cyclin mechanism for the control of cell cycle, and the formation of histone/DNA complexes [1]. Indeed, it is amazing to see such diversity in organisms that have evolved using what seems like a limited set of core cellular processes.

During metazoan development, cellular processes such as growth, metabolism, proliferation, survival, and differentiation, are controlled by a diverse array of signalling molecules [2-8]. The first evidence that such molecules existed came from experiments

using the amphibian embryo in the early 1900s. In 1924, Spemann and Mangold generated a two-headed salamander by transplanting a small piece of tissue from one embryo to another [9]. As the cells surrounding the transplanted tissue would normally not have developed into a second head, the transplanted cells must have communicated with adjacent cells to directly influence their development. During eighty years, the following progress has been made in identifying the mechanisms by which cell signalling occurs.

Genetic and biochemical studies have revealed a general mechanism for cell signalling that is conserved among metazoan animals. Usually a signalling molecule, the ligand, activates a transmembrane receptor by binding to the extracellular domain and inducing phosphorylation of an intracellular domain [2-8]. This leads to the activation of kinases that phosphorylate each other sequentially resulting in the activation or repression of effector molecules [2-8]. Surprisingly there are only seven main signalling pathways known to control the development of metazoan animals [7]. These include the TGF-β pathway [5], the Wnt pathway [2], the Hedgehog (Hh) pathway [4], the receptor tyrosine kinase (RTK) pathway [8], the Notch/Delta pathway [6], the JAK/STAT pathway [3], and the nuclear receptor pathway [10]. All of these pathways conform to the generalized scheme of signal transduction with the exception of the nuclear receptor pathway, in which the ligands cross the cell membrane and bind to nuclear receptors inside the cell [10]. All of the different signalling pathways show amazing flexibility and can be used in various combinations during development to perform different functions in different

situations. As a result, a limited set of signalling mechanisms can direct all aspects of development, leading to the formation of many different cells, tissues, and body types.

The importance of the core signalling pathways in the regulation of cellular processes is highlighted by the prevalence of human diseases that are caused by disruption of signalling genes [11-20]. Many human cancers can be linked to defects in components of one or more signalling cascades [11, 12, 14, 15, 18, 19]. In these cases, disruption of normal signalling can lead to deregulation of growth and the formation of tumours. Neurodegenerative diseases can also result from disruption of signalling genes that normally promote neuronal survival or direct neuronal development [13, 16, 19, 20]. Additionally, insulin resistance in diabetic individuals may result from disruption of signaling genes regulating nutrient homeostasis [19, 20]. Thus, medical science can benefit greatly from 'basic' research that contributes to the understanding of the components and mechanisms controlling cell signalling networks. The conservation of signalling pathways in vertebrates and invertebrates has allowed the use of model organisms such as *Drosophila melanogaster* and *Caenorhaebditis elegans* to elucidate the components of the different pathways and the developmental contexts in which they occur [1, 7, 21]. To extend this work, mammalian cell culture studies have elucidated many of the biochemical mechanisms through which these signalling pathways operate [2-8]. These combined efforts are beginning to reveal a complex picture, in which signalling events elicit a response that is dependent on cell type, developmental stage, signal strength, and interactions with other signalling cascades. The advent of molecular techniques has rapidly increased the progress made in understanding these processes, but

we are still a long way from having a true understanding of how cells interpret the constant barrage of signals that they receive.

Model organisms provide an excellent system to determine the physiological importance of signal transduction genes. Chapters 1.2 and 1.3 of this thesis describe the analysis of the Drosophila homologue of the forkhead box subgroup 'O' (FOXO) family of transcription factors. FOXO transcription factors are inhibited by PI3K/Akt signalling that is conserved in nematodes, mammals, and flies [22, 23]. Previous to this work, the mechanisms of PI3K/Akt signalling had been described in mammals, *C. elegans*, and in part, in Drosophila [24-26]. This thesis describes the identification of the solitary FOXO family member in Drosophila and an analysis of its regulation by upstream signalling components and nutrients [27, 28]. This work is the first published study to examine a FOXO transcription factor in Drosophila, and provides evidence of an evolutionarily conserved role for these transcription factors in the adaptive response of metazoan animals to fluctuations in nutrient availability [27, 28]. Subsequent sections of the introduction will provide background information on IIS signalling and a review of the literature describing the function and regulation of the FOXO family of transcription factors.

1.1.2 – The IIS pathway: ligands, receptors, and adaptors

The IIS pathway has emerged as an important regulator of growth in response to developmental signals and nutrients (Figure 1) [21, 29, 30]. The main activators of IIS

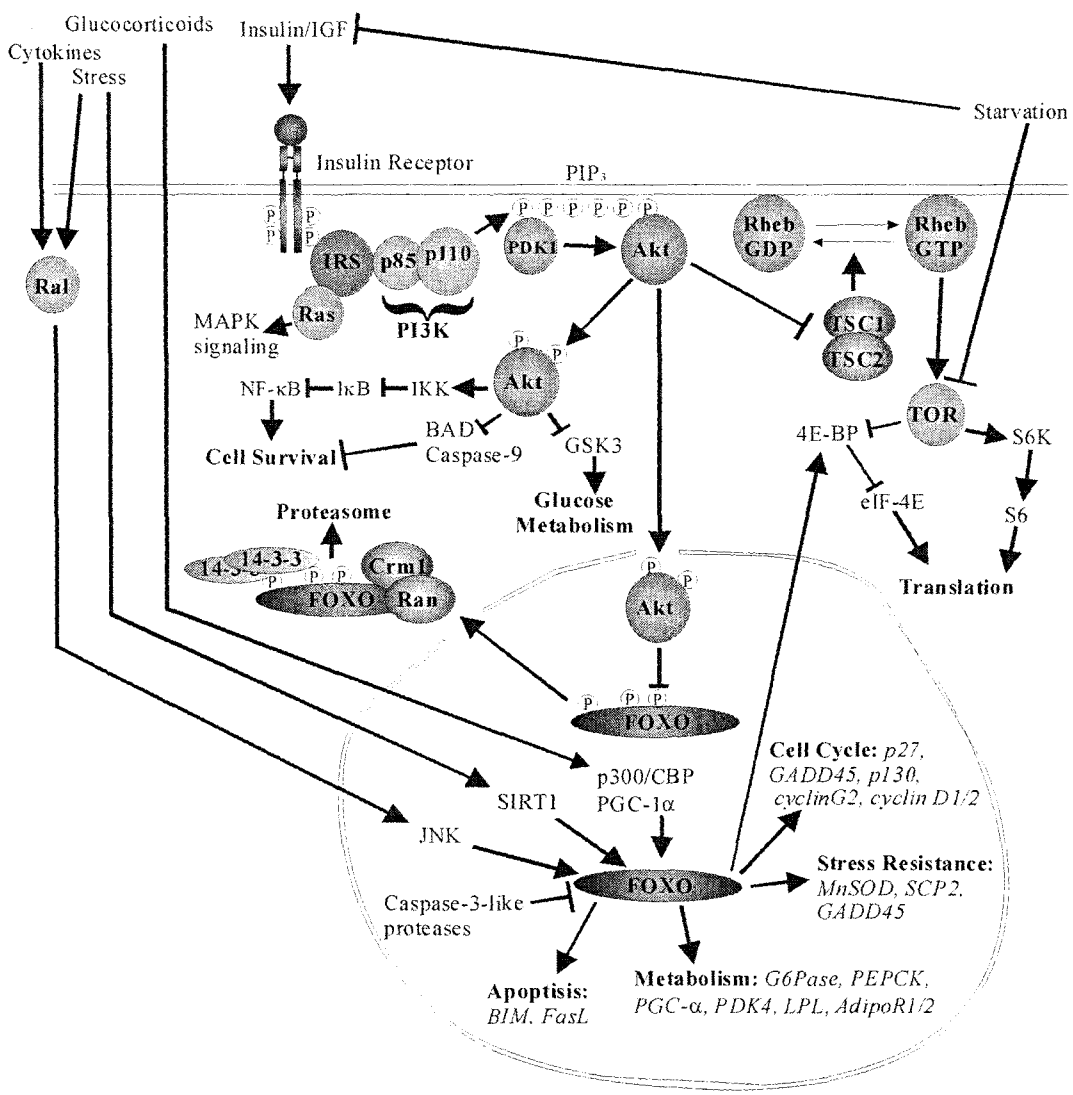

Figure 1 - Insulin/Insulin-like growth factor signaling and the regulation of the FOXO transcription factors. See text for details.

signalling in mammals are insulin, IGF1, and IGF2. Insulin is produced in the pancreatic β-cells in response to circulating nutrients [31] and IGF-1/2 are produced primarily in the liver in response to growth factors and nutritional cues [30, 32]. The insulin receptor (InR) and the IGF receptor (IGFR) belong to the receptor tyrosine kinase (RTK) family of transmembrane receptors [33-35]. These receptors are composed of two extracellular α-subunits and two intracellular β-subunits that are linked together by disulfide bridges. The α-subunit is responsible for ligand binding while the β-subunit signals to intracellular signalling molecules through it tyrosine kinase domain [35]. InR and IGFR belong to the class II family of RTKs that contain cysteine-rich motifs in the α-subunit that are important for ligand binding [36, 37]. Binding of a ligand to the extracellular domain of the RTK initiates the tyrosine kinase activity of the intracellular domain, and autophosphorylation of the β-subunit at several tyrosine residues [36, 37]. Tyrosine phosphorylation of the β-subunit creates binding sites for proteins that have phosphotyrosine binding (PTB) domains (Figure 1). The first of these to be discovered, the insulin receptor substrate 1 (IRS1), was identified by immunoprecipitation using phosphotyrosine antibodies against cell extracts from insulin-stimulated cells [38]. These antibodies revealed a 185 kDa protein belonging to a family of IRS proteins that contain several characteristic domains, including a PTB domain, a PH (pleckstrin homology) domain, and SH-2 binding domains [21, 36, 37]. The SH-2 binding domain provides a link between InR/IGFR and two downstream pathways, the Ras/MAPK pathway, and the phosphoinositide 3-kinase (PI3K) pathway. The Ras/MAPK pathway is a highly conserved signalling mechanism that is used in eukaryotes from yeast to mammals [39].

Both Ras and PI3K are activated by binding to IRS though their SH2 binding domains (Figure 1). The remainder of this discussion will focus on the components of the IIS system that function downstream of PI3K.

1.1.3 - Intracellular signal transduction in response to IIS activation: PI3K and Akt

PI3K phosphorylates phosphoinositide residues at the 3' hydroxyl group of the inositol ring, converting phosphotidylinositide 4,5-bisphosphate (PIP_2) to phosphotidylinositide 3,4,5,-triphosphate (PIP_3). There are three classes of PI3K proteins, of which class I is the major isoform that is activated by insulin [40]. Class I PI3K exists as a heterodimer with an 85 kDa (p85) adaptor subunit and a 110 kDa (p110) catalytic subunit. In mammals, there are four isoforms of p110, each encoded by a different gene, and seven versions of p85 that are generated by alternative splicing of three different genes [40, 41]. The adaptor subunit binds to phosphotyrosine residues in IRS proteins through its SH2 domain, leading to the recruitment of the 110 kDa catalytic subunit (Figure 1). Once bound to IRS, PI3K becomes activated and is able to catalyse the formation of PIP_3 on the inner surface of the plasma membrane (Figure 1) [40, 41]. The lipid phosphatase, PTEN (phosphatase and tensin homologue), can negatively regulate PI3K signalling through the dephosphorylation of PIP_3. PTEN is classified as a tumour suppressor because of its negative effect on cell growth and because mutations in the human *PTEN* gene have been linked to a variety of tumours [42]. Thus, PI3K and PTEN act in opposition to each other to control cell function.

The generation of PIP_3 is critical for the activation of signalling components downstream of PI3K [40]. PIP_3 residues recruit signalling molecules to the plasma membrane by binding to proteins with PH domains [40]. Two of the major PH-domain-containing proteins that are influenced by the presence of PIP_3 are Akt (also known as protein kinase B) and phosphoinositide-dependent kinase-1 (PDK1) (Figure 1) [24, 40, 41]. Akt is a serine/threonine kinase that is recruited to the membrane and phosphorylated in response to the generation of PIP_3 [24, 41, 43]. Although the mechanism of Akt activation is not well understood, at least one of the phosphorylation events is mediated by PDK1 [24, 41]. There are three homologues of Akt in mammals that can elicit their effect by altering enzyme activity directly, or through modulation of downstream signalling pathways and transcription factors. Akt is phosphorylated after insulin stimulation and relocates from the membrane, to the cytoplasm and the nucleus, where it phosphorylates multiple proteins involved in the regulation of cell survival, metabolism, cell cycle, transcription, and translation (Figure 1) [24, 41, 43, 44]. The minimum motif required for Akt to recognize its substrate is Arg-Xaa-Arg-Yaa-Zaa-Ser/Thr-Hyd, where Xaa is any amino acid, Yaa and Zaa are usually small residues other than glycine, and Hyd is a bulky hydrophobic residue such as phenylalanine or leucine [45]. The first Akt substrate to be identified was glycogen synthase kinase 3β (GSK3β), a kinase that phosphorylates and inhibits glycogen synthase (GS). Phosphorylation by Akt inhibits GSK3β, leading to GS activation and possibly to the increased storage of sugars as glycogen in response to insulin [41, 43, 46]. Other Akt targets include, the cell death genes, BAD (Bcl-2 antagonist causing cell death) and caspase 9, the cell cycle

inhibitor, p21, TOR and the FOXO family of transcription factors [24, 41, 43, 44]. Phosphorylation of these targets by Akt may have effects on cell survival and/or cell proliferation.

The proliferation and growth of cells requires a high capacity for protein synthesis. Akt can influence the cellular capacity to make proteins by modulating the TOR (target of rapamycin) pathway (Figure 1). TOR is an evolutionarily conserved nutrient sensing kinase that phosphorylates ribosomal S6 kinase (S6K) and eukaryotic initiation factor 4E (eIF-4E) binding protein (4E-BP) [47, 48]. TOR-mediated phosphorylation activates S6K activity [47, 48]. S6 may be involved in promoting translation of mRNAs containing a 5' terminal oligopyrimidine tract (TOP), which often encode for components of the translational apparatus [49]. TOR mediated phosphorylation of 4E-BP leads to its dissociation from eIF-4E [48]. 4E-BP binding inhibits eIF-4E, which is part of a ribosome binding complex that promotes translation through interaction with the 7-methyl-guanosine cap of mRNA [50]. Thus, TOR activity leads to increased ribosome biosynthesis and the selective increase in translation of proteins making up the translational apparatus [47, 48, 51].

Akt modulates TOR activity through TSC 1 and 2 (tuberous sclerosis complex 1 and 2) (Figure 1) [21, 47, 52, 53]. These two proteins exist in a complex that can bind to the small GTPase Rheb, to turn on its GTPase activity. Rheb is a member of the Ras family of GTP-binding proteins and activation of its GTPase activity leads to the conversion of Rheb-GTP to Rheb-GDP [52]. Rheb-GDP is inactive, whereas Rheb-GTP is active and can promote TOR kinase activity. Akt prevents activation of Rheb GTPase

activity through phosphorylation of TSC2, leading to the dissociation of the TSC1/2 complex [21, 47, 52, 53]. Thus, Akt regulates the translational capacity of the cell through modulation of TSC/Rheb/TOR signalling.

Often, cell signalling events cause major changes in cellular activities that are mediated by alterations in gene transcription. Akt can alter the transcriptional profile of the cell through two main mechanisms, activation of nuclear factor-κB (NF-κB) and inhibition of FOXO (Figure 1). NF-κB promotes transcription of genes involved in cell survival. Although the physiological significance is still unsure, Akt may relieve the repression of NF-κB by its cytosolic inhibitor, IκB (inhibitor of NF-κB) [24, 41]. IκB binds to NF-κB in the cytoplasm, preventing it from entering the nucleus and activating gene transcription. IκB is targeted for degradation after phosphorylation by IKK (IκB kinase). Akt has been shown to activate IKK, suggesting that Akt, via IKK, can promote cell survival by activation of NF-κB-mediated transcription. Alternatively, Akt may effect cellular processes through inhibition of gene transcription. Akt-mediated inhibition of the FOXO transcription factors is well documented [22] and is believed to be one of the critical consequences of Akt activation. The following sections will provide a detailed review of the biochemical regulation of the FOXO transcription factors.

1.1.4 - Discovery of the FOXO transcription factors in human cancer

The FOXO proteins belong to a family of transcription factors that contain a highly conserved 100 amino acid forkhead box DNA binding domain made up of three

α-helices separated by two large loops. Members of the human FOXO family were identified as genes disrupted through chromosomal translocations seen in patients with alveolar rhabdomyosarcoma [54, 55] and acute lymphocytic leukemia [56-59]. The forkhead in rhabdomyosarcoma (FKHR) gene was found as a fusion of two genes, FKHR with either PAX3 [55] or PAX7 [54], in patients with rhabdomyosarcoma. The resultant fusion proteins contain the entire PAX3/7 DNA binding, domain fused to the transactivation domain of FKHR (Figure 2). These fusion proteins displays PAX-like DNA binding, with aberrant regulation of PAX target genes, and the ability to induce the transformation of cultured cells [60]. However, a PAX3/FKHR fusion protein did not cause tumour formation in mice, leading to the hypothesis that disruption of the FKHR gene is an important factor contributing to cellular transformation [61]. Two other human FOXO homologues, FKHR-like 1 (FKHRL1) and acute lymphocytic leukemia-1-fused-gene-from-chromosome X (AFX), were identified in chromosomal translocations resulting in fusion to the mixed lineage leukemia (MLL) gene [58, 59]. These chimeras contain the forkhead transactivation domain, fused to the DNA binding domain of MLL. The FOXO transactivation domain was necessary for cellular transformation [62, 63], thus, it appears that disruption of normal FOXO activity is an important step in the progression of certain types of cancer.

The identification of the FOXO transcription factors in human cancer led to increased interest in these proteins. Additional homologues have since been identified in mice, chicks, zebrafish [64], nematodes [65], fruit flies [27, 66, 67], and rats [68]. A unified nomenclature was devised for the forkhead/winged helix genes in which FOX

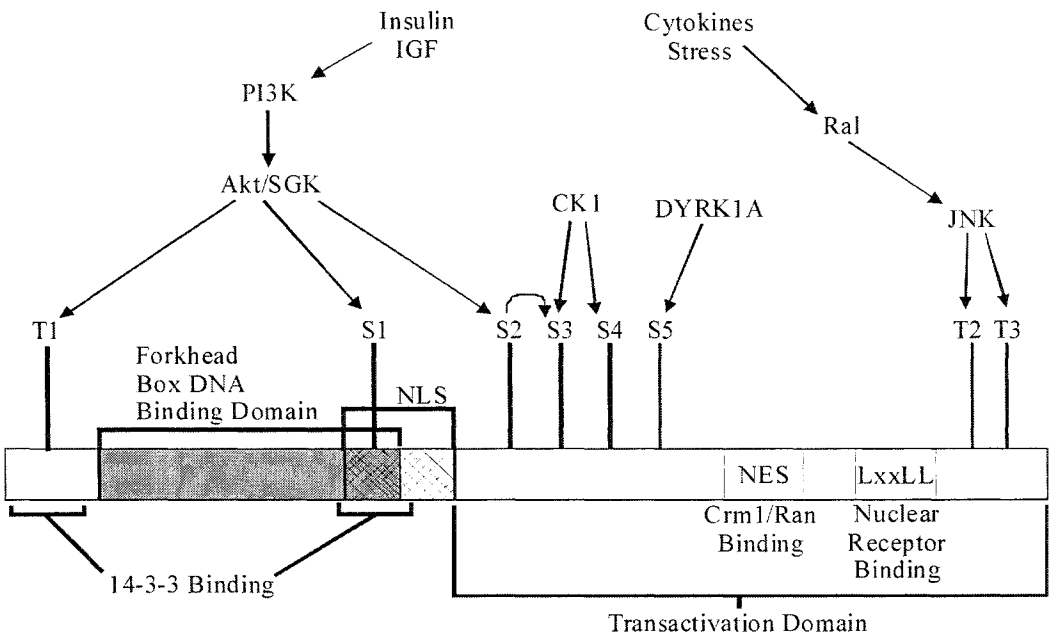

Figure 2 - Schematic organization of domains and phosphorylation sites in the FOXO family of transcription factors. See text for details.

(forkhead box) is the abbreviated name for the entire family, with sub-groups that are defined by the letters A to O [69]. Thus, FKHR, FKHRL1, and AFX, were renamed to FOXO1, FOXO3a, and FOXO4, respectively [69]. Although the precise role of these transcription factors in cancer is still largely undefined, research within the last six years has resulted in greater understanding of the function of the FOXO family members and the factors that regulate their activity.

1.1.5 - Regulation of FOXO through nuclear shuttling, proteasomal degradation, and proteolytic cleavage

The first evidence showing that FOXO factors are regulated by insulin signalling came from genetic studies examining dauer larvae formation in *C. elegans*. Dauer larvae

arise as a developmental adaptation to nutritional stress or crowding [70]. Mutations in *daf-2*, an insulin receptor homologue, and *age-1*, a homologue of the PI3K catalytic subunit, lead to arrested development in the dauer larvae stage. Mutations in the *C. elegans* FOXO homologue, *daf-16*, negate the effects of *daf-2* and *age-1* mutations [65], suggesting that the main purpose of daf-2/age-1 signalling is to antagonize daf-16. These results led biochemists to test this possibility in mammalian cells. Indeed, it was shown that activation of PI3K/Akt signalling leads to phosphorylation of FOXO and causes it to be sequestered in the cytoplasm, while inhibition of PI3K/Akt signalling leads to dephosphorylation and increased nuclear localization of FOXO (Figure 1) [71-78]. Three Akt phosphorylation sites were identified that are conserved in all of the FOXO family members. These sites include a threonine residue near the N-terminus (T1), and two serine residues, one in the DNA binding (S1) domain, and one just outside the C-terminal side of the DNA binding domain (S2) (Figure 2) [71-78]. Preventing the phosphorylation of these sites by mutating them to alanine residues renders FOXO proteins resistant to cytoplasmic translocation in response to Akt activation [71, 75, 77, 78]. Serum and glucocorticoid-regulated kinase (SGK) is a closely related kinase that can also phosphorylate the T1, S1, and S2 sites in response to IIS (Figure 2). However, while Akt shows a preference for phosphorylation of the S1 site, SGK preferentially phosphorylates the S2 site [79]. The physiological significance of these preferences is unclear, yet it is possible that these two kinases act in redundant or parallel pathways.

Regulation of nuclear shuttling is one of the main controls over FOXO activity [22, 80]. Shuttling of FOXO is regulated by a nuclear localization sequence (NLS) in the

DNA binding domain and a nuclear export sequence (NES) in the transactivation domain (Figure 2). Nuclear export of FOXO is dependent on several components of the nuclear export machinery including Crm1 and the small GTPase, Ran [71, 81, 82]. These factors are thought to mediate nuclear export of FOXO by binding to the NES (Figure 1 and 2). Studies that examined point mutations in the phosphorylation sites of FOXO have revealed an intricate mechanism that regulates the paradoxical activities of the NLS and NES motifs [22, 68]. The NLS of FOXO may be disrupted upon phosphorylation of the S1 site, as this residue is located within the NLS, and addition of a negative charge at this site by substituting a glutamate residue leads to inhibition of nuclear import [81]. Mutation of the S1 site to alanine prevents phosphorylation of T1 and S2, thus the S1 site acts as a gate-keeper for the phosphorylation of the other residues [75, 83]. Phosphorylation of T1 and S1 may also promote export from the nucleus and the cytoplasmic accumulation of FOXO by providing a consensus sequence for binding to 14-3-3 proteins [72, 84] (Figure 2). Binding of 14-3-3 at these residues increases the rate of Crm1/Ran mediated nuclear export and inhibits the potential for nuclear import by antagonizing the NLS [84]. Phosphorylation of the S2 site is also important in promoting the nuclear export of FOXO proteins, by providing a consensus sequence for the phosphorylation of an additional site, S3, by casein kinase 1 (CK1) [82]. In turn, phosphorylation of the S3 site provides a primer for CK1-mediated phosphorylation of another serine residue, S4. S2, S3, and S4 are immediately adjacent to each other and to an additional phosphorylation site, S5 (Figure 2). S5 is constitutively phosphorylated by the dual specificity kinase, DYRK1A, reducing FOXO-mediated gene transactivation

[85]. Disruption of the stretch of adjacent phosphorylation sites from S2-S5 does not affect binding to Crm1 or Ran, but does inhibit nuclear export [82]. This stretch of phosphorylated serine residues may provide a 'patch' of negatively charged amino acid side chains that is necessary for nuclear export [82]. In summary, S1 phosphorylation allows subsequent phosphorylation events that act to fine tune the nuclear shuttling of FOXO.

Additional phosphorylation of FOXO occurs via the small GTPase, Ral, and its downstream effector, JNK (jun N-terminal kinase) (Figures 1 and 2). Ral/JNK signalling is activated by multiple factors, including oxidative stress and cytokines. FOXO4 has been shown to undergo Ral-dependent phosphorylation at two threonine residues near the C-terminus of the protein, T2 and T3 (Figure 2) [86, 87]. Phosphorylation of T2 and T3 is mediated by JNK, which is activated through Ral in response to oxidative stress and cytokines [88]. Phosphorylation at these sites is required to achieve the full effect of FOXO4 on gene transactivation [86, 88]. Ral/JNK signalling may provide an IIS-independent mechanism of FOXO activation that occurs in response to oxidative stress. However, these Ral-dependent phosphorylation sites are not conserved in all FOXO family members, suggesting a role specific to the function of FOXO4.

In addition to nuclear shuttling, PI3K/Akt signalling can modulate FOXO activity by regulating the stability of the protein in the cell. This occurs through two independent mechanisms; (1) targeting FOXO to the ubiquitin proteasome system [89, 90], and (2) proteolytic cleavage (Figure 1) [91, 92]. Phosphorylation of FOXO increases the ubiquitination of FOXO and reduces FOXO levels in a proteasome-dependent manner

[89, 90]. Proteasomal degradation of FOXO is dependent on Akt phosphorylation and cytoplasmic localization [89]. This provides an additional mechanism for downregulation of FOXO after nuclear export. In contrast, the proteolytic cleavage of FOXO may provide a mechanism of inhibition that does not depend upon nuclear export [91, 92]. FOXO proteins that are constitutively nuclear due to a nuclear export-disabling mutation in the NES can still be inhibited [93]. Both FOXO1 and FOXO3a have been shown to undergo proteolytic cleavage mediated by caspase-3-like proteases (Figure 1) [91, 92]. This cleavage results in the release of a large N-terminal fragment (532 amino acids for FOXO1) that is transcriptionally inactive [91, 92] and may act to inhibit gene transactivation by the full length FOXO [92]. Indeed, the regulation of FOXO activity is very complex and involves many cellular processes, including nuclear shuttling, phosphorylation, ubiquitination, protein-protein interactions, and proteolytic cleavage.

1.1.6 - Targets of FOXO and regulation of transcription through interactions with co-activators

The regulation of FOXO gene transactivation is as complex as the regulation of FOXO localization in the cell. FOXO proteins can activate the transcription of many genes involved in the regulation of apoptosis [72, 94, 95], cell cycle arrest [22], metabolism [96], and stress resistance [97-100] (Figure 1). Activation of gene transcription by FOXO occurs when FOXO binds to DNA at a consensus sequence called the FOXO recognition element (FRE). Recent research suggests that the effect of FOXO on target gene expression is also influenced by interactions with transcriptional co-

activators, nuclear receptors, acetylases, and deacetylases [22, 68, 80, 101]. Thus, a myriad of factors are able to influence the activity and specificity of FOXO-mediated gene transactivation.

The FOXO proteins can influence the fate of many different cell types. FOXO induces apoptosis in cultured blood cells and neurons. In lymphocytes, FOXO has been shown to cause apoptosis in the absence of cytokines by activating transcription of BIM (Bcl-2 interacting mediator of cell death), and the Fas ligand (FasL) [72, 94]. In cultured sympathetic neurons, FOXO causes cell death in the absence of NGF (nerve growth factor), through transcription of BIM [95]. Many other cell types, such as fibroblasts, undergo cell cycle arrest in response to FOXO expression. FOXO promotes G1 arrest in these cells by activating expression of $p27^{KIP1}$ [86, 102-107] and, possibly, by promoting the stability of $p27^{KIP1}$ through a post-translational mechanism [107]. FOXO homologues have also been implicated in the expression of p130, a promoter of cell senescence [98], and cyclin G2, which accumulates during G_0 and inhibits cell cycle entry [108]. In addition, FOXO3a can promote G2 cell cycle arrest through transcription of GADD45 in response to oxidative stress [99, 100]. FOXO also may inhibit the cell cycle by down-regulation of the expression of cyclins D1 and D2, both being required for cell cycle progression [109, 110]. The ability of FOXO to down-regulate transcription appears to be independent of FRE binding [109], suggesting that FOXO acts as a transcriptional corepressor through protein-protein interactions with other transcription factors.

In mammals, insulin levels decline in response to decreased blood glucose, and the body shifts from synthesis to mobilization of energy storage molecules, such as glycogen and triglycerides. In addition, gluconeogenesis is increased in the liver in order to provide the brain with an essential source of carbohydrates. When circulating insulin levels decline, the FOXO homologues may affect metabolism in various tissues and organs [96]. FOXO proteins can activate the expression of several gluconeogenic enzymes including, G6Pase (glucose-6-phosphatase) [111, 112], G6Pase transporter [113], PEPCK (phosphoenolpyruvate carboxykinase) [111, 114, 115], and PGC-1α [116] (Figure 1). FOXO-mediated expression of these genes may promote gluconeogenesis in the liver during periods when insulin production is decreased [96]. In muscle cells, FOXO can promote the expression of PDK4 (pyruvate dehydrogenase kinase 4) [117] in order to maintain glucose levels during starvation. FOXO can also promote the utilization of fat stores in muscle cells of starving animals through expression of genes involved in fatty acid metabolism, such as LPL (lipoprotein lipase) [118], and AdipoR1/2 [119]. Finally, FOXO may prevent the formation of fat cells through expression of p21, an inhibitor of adipocyte differentiation [120]. These findings suggest that FOXO acts in a tissue specific manner to regulate metabolism during periods of decreased blood insulin in mammals.

Genetic studies in *C. elegans* [121] and Drosophila [66] have implicated the FOXO transcription factors in the cellular response to oxidative stress. In mammalian cells, FOXO proteins appear to be activated in response to oxidative stress and can influence the transcription of several stress resistance genes (Figure 1) [97-100]. The

growth arrest and DNA damage-response gene (GADD45) is induced by FOXO in response to oxidative stress and mediates FOXO-induced G2 arrest and DNA repair [99, 100]. G2 arrest in response to stress may allow more time for DNA repair after damage has incurred prior to mitosis. FOXO may also provide protection from reactive oxygen species (ROS) by promoting expression of manganese superoxide dismutase (MnSOD) [98] to detoxify oxygen free radicals, and sterol carrier protein (SCP2) to protect fatty acids from peroxidation [97]. As a result, FOXO may promote the long term survival of cells by increasing their resistance to oxidative stress and DNA damage.

FOXO-mediated gene transcription is regulated through protein-protein interactions with multiple cofactors [122-124]. The presence or absence of these cofactors in certain cell types may influence the tissue specific pattern of FOXO-mediated gene expression. FOXO interacts with p300/CBP (CREB binding protein) to mediate the effect of glucocorticoids on the expression of the IGFBP1 gene [124]. FOXO also mediates the effects of glucocorticoids on G6Pase and PEPCK transcription, through interaction with PGC-1α (peroxisome proliferator-activated receptor co-activator α) in the liver [123]. Thus, by binding to coactivators, FOXO can integrate signals from multiple circulating factors, including glucocorticoids and insulin (Figure 1). FOXO can also interact with the cofactors, DYRK1A/B, to promote G6Pase expression in a manner that is independent of DYRK1A/B kinase activity [122]. In addition, FOXO may regulate endometrial differentiation through transcriptional coactivation of CEBPβ (CAAT-enhancer binding protein β) [125]. These studies show the diversity of the

FOXO transcription factors in their ability to control cell function through protein-protein interactions.

Another mechanism responsible for the regulation of FOXO-mediated gene transcription occurs through interactions with nuclear receptors. Nuclear receptors are signalling molecules that bind to their ligands inside the cell, leading to receptor dimerization and subsequent regulation of gene transcription [10]. FOXO homologues may bind to nuclear receptors through a conserved LxxLL motif located near the C-terminus of the protein (Figure 2). FOXO family members have been shown to interact with the oestrogen receptor (ER) [126, 127], the progesterone receptor (PR) [127], the thyroid hormone receptor (TR) [127], the glucocorticoid receptor (GR) [127], the retinoic acid receptor (RAR) [127], the androgen receptor (AR) [128], the peroxisome proliferator activated receptor γ (PPARγ) [129], and hepatic nuclear factor 4 (HNF4) [130]. Interactions with these receptors and the resulting effects on transcription are variable, depending on the specific receptor involved. For example, binding to ER is ligand-dependent, whereas binding to RAR is ligand-independent [127]. Interactions between FOXO and ER have been shown to augment ER-mediated transcription and inhibit FOXO-mediated transcription [126]. However, FOXO has also been shown to repress ER-mediated transcription [127], reflecting differences in cell type or target promoter sequences. FOXO acts to repress HNF4-mediated transcription in the absence of insulin, yet HNF4 seems to have no effect on FOXO mediated transcription [130]. In contrast, PPARγ and FOXO show mutual repression of transcriptional activity [129]. Clearly, the interaction of FOXO family members with nuclear receptors has specific effects on

transcription, which may result in the fine tuning of gene expression profiles in physiological systems.

Additional regulation of FOXO gene transactivation occurs through interactions with acetylases and deacetylases. The cofactor, p300/CBP, interacts directly with FOXO and promotes its acetylation at several sites [124, 131, 132], while SIRT1 (silent information regulator of transcription 1) interacts with FOXO and causes its deacetylation [23, 133-135] (Figure 1). Acetylation by p300/CBP inhibits FOXO-mediated transactivation of $p27^{KIP1}$ expression [131]. To oppose this, SIRT1 deacetylation causes an increase in expression of $p27^{KIP1}$ and the stress resistance genes GADD45 and MnSOD [23, 133, 134]. SIRT1 deacetylation can also decrease expression of the pro-apoptotic protein BIM [133, 135] and can disrupt the ability of dFOXO to induce apoptosis [135]. Oxidative stress is required for the interaction of FOXO with SIRT1, suggesting that SIRT1 deacetylase activity acts to promote cell cycle arrest and inhibit apoptosis in cells that are undergoing oxidative stress [22, 23, 80, 101].

1.1.7 - Physiological function of the FOXO transcription factors: insights from model organisms

Biochemical analysis of the IIS pathway and the FOXO transcription factors has revealed many potential functions for this signalling cascade [22, 80]. Analysis of FOXO target genes and coactivators has suggested a role for these transcription factors in the regulation of apoptosis, cell cycle, metabolism, and stress resistance [22, 80]. However, it is difficult to determine the physiological or developmental significance of these

findings within the context of cultured cells. Studies in model organisms, such as *C. elegans*, mice, and Drosophila are providing insights into the physiological functions of the FOXO family members. Consistently, analysis of the FOXO homologues in whole organisms reveals that these transcription factors are critical regulators of stress resistance, longevity, and glucose metabolism in the absence of IIS activity.

Caenorhaebditis elegans

The FOXO transcription factors were first placed downstream of insulin signalling by analysis of mutants regulating dauer formation in *C. elegans* [65, 136, 137]. The dauer larvae forms as a developmental response to starvation or crowding that is characterized by arrest of growth at a sexually immature stage along with altered metabolism to increase the storage of fat [70]. Mutations in the *C. elegans* homologues of the insulin receptor (*daf-2*) [65, 136-138], PI3K (*age-1*, *daf-23*) [65, 136, 139], and Akt (*akt1* and *akt2*) [140] cause constitutive dauer formation and increased lifespan through a mechanism dependent on the activity of the FOXO homologue, *daf-16* [65, 136, 137, 140]. Mutations in insulin signalling components in *C. elegans* can also cause daf-16-dependent resistance to oxidative damage, UV stress, and hypertonic stress [25, 121, 141]. In addition, forced activation of *daf-16* in the nematode is sufficient to induce dauer formation [142], increased stress resistance [143], and increased longevity [143]. Exposure of *C. elegans* to starvation, heat, and oxidative stress causes a relocation of daf-16 to the nucleus, and daf-2 and akt 1 and 2 are able to inhibit daf-16 nuclear localization in fed animals. Microarray analysis has demonstrated that daf-16 may promote increased

longevity and stress resistance by upregulating the expression of several groups of genes encoding stress resistance proteins, heat shock proteins, antimicrobial proteins, and metabolic enzymes [141, 144]. These studies suggest a global role for FOXO homologues in the response of *C. elegans* to environmental factors and in the protection of cells against stress.

Mice (Mus musculus)

Mutational analysis of *FOXO* homologues in mice has revealed the importance of these factors in diabetes and in mammalian development [120, 145-148]. Loss of function of *FOXO1* in mice causes embryonic lethality due to multiple factors including impaired vascular development [148]. In contrast, mice lacking *FOXO3a* and *FOXO4* survive to adulthood with minimal defects [147, 148]. No noticeable abnormalities have been described for loss of *FOXO4* [148]. However, loss of *FOXO3a* leads to age-dependent infertility and abnormal follicular cell development [147, 148]. FOXO3a may act to suppress follicle cell differentiation in the early stages of follicle growth [147]. Thus, in the absence of *FOXO3a*, follicular activation occurs too early, leading to oocyte death and premature infertility [147].

Analysis of *FOXO1* mutant heterozygous mice revealed that *FOXO1* haploinsufficiency can rescue the diabetic phenotype observed in mice that are; 1) heterozygous for InR [146]; 2) homozygous for loss of IRS [145]; and 3) fed a high fat diet (diet-induced diabetes) [120]. In addition, overexpression of activated FOXO in the liver or the pancreas is sufficient to induce a diabetic-like phenotype [146]. Thus, the

symptoms of diabetes result, in part, from the overactivation of FOXO in selected tissues. The transcription of *FOXO1* mRNA is increased in starved mice [117, 118, 149], and this increase is reversed when animals are re-fed [117, 149]. These experiments in mice show the importance of the FOXO homologues in mammalian development, and in the physiological response of adult mammals to reduced food intake.

Drosophila melanogaster

Like *C. elegans*, Drosophila has a single FOXO homologue (dFOXO) that regulates growth [27, 66, 67], stress resistance [66, 150], longevity [150-152], and the developmental response to starvation [27, 28]. Loss of the Drosophila insulin receptor substrate, *chico*, leads to the generation of adults that are reduced in size [153]. Analysis of flies with loss of function mutations in *dFOXO* show that it is required for the reduction of growth observed in *chico* mutant flies [66]. In addition, overexpression of dFOXO can mediate growth reduction in the developing eye of Drosophila [66, 67, 154]. These studies indicate that dFOXO is a negative regulator of growth, and in conjunction with evidence from mammalian cell culture, suggest that FOXO homologues act to inhibit cell cycle progression.

Expression of dFOXO in the adult Drosophila fat body yields increased oxidative stress resistance [150] and lifespan [150, 151]. dFOXO has been shown to upregulate the expression of 4E-BP [66, 67], and loss of function in either dFOXO or 4E-BP results in sensitivity to oxidative stress [66, 155]. Expression of 4E-BP in a dFOXO mutant background is sufficient to restore oxidative stress resistance [155]. In Drosophila, JNK

signalling also extends lifespan and increases stress resistance [152]. The increased lifespan observed upon increased JNK signalling is negated by heterozygosity for dFOXO [152]. These studies suggest that dFOXO mediates the effects of JNK signalling as well as loss of insulin signalling, and that 4E-BP is a critical downstream regulator of FOXO-induced resistance to oxidative stress.

Analysis of the FOXO transcription factors in Drosophila has shed light upon their wider physiological importance. Chapters 1.2 [27] and 1.3 [28] tell a story that begins with the identification of dFOXO and ends by determining the role for dFOXO in the developmental adaptation of Drosophila larvae in response to nutritional stress. Chapter 1.2 describes dFOXO overexpression experiments that demonstrate interactions between dFOXO and upstream components of the PI3K/Akt signalling pathway. Additional overexpression studies show that dFOXO can mimic the effects of starvation on Drosophila larvae. Chapter 1.3 provides an analysis of the effects of starvation on endogenous dFOXO activity, and the effects of dFOXO loss of function during starvation. Evidence presented in chapter 1.3 supports a model where dFOXO is regulated by the presence of nutrients and is partly required for the developmental adaptation of Drosophila larvae to starvation. Evidence from chapters 1.2 and 1.3 suggest an additional role for FOXO in the regulation of larval wandering behaviour. These studies provide a foundation, correlating physiological responses to dFOXO activity, with respect to control of growth, behaviour, and starvation response.

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Chapter 1.2

Expression of Drosophila FOXO regulates growth and can phenocopy starvation

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1.2.1 - Abstract

Background: Components of the insulin signalling pathway are important regulators of growth. The FOXO (forkhead box, sub-group "O") transcription factors regulate cellular processes under conditions of low levels of insulin signalling. Studies in mammalian cell culture show that activation of FOXO transcription factors causes cell death or cell cycle arrest. The *Caenorhaebditis elegans* homologue of FOXO, Daf-16, is required for the formation of dauer larvae in response to nutritional stress. In addition, FOXO factors have been implicated in stress resistance and longevity.

Results: We have identified the *Drosophila melanogaster* homologue of FOXO (dFOXO), which is conserved in amino acid sequence when compared to the mammalian FOXO homologues and Daf-16. Expression of dFOXO during early larval development causes inhibition of growth and alterations in feeding behavior. Inhibition of larval growth is reversible upon discontinuation of dFOXO expression. Expression of dFOXO during the third instar or at low levels during development leads to the generation of adults that are reduced in size. Analysis of the wings and eyes of these small flies indicates that the reduction in size is due to decreases in cell size and cell number. Overexpression of dFOXO in the developing eye leads to a characteristic phenotype with reductions in cell size and cell number. This phenotype can be rescued by co-expression of upstream insulin signalling components, dPI3K and dAkt, however, this rescue is not seen when FOXO is mutated to a constitutively active form.

Conclusions: dFOXO is conserved in both sequence and regulatory mechanisms when compared with other FOXO homologues. The establishment of Drosophila as a model

for the study of FOXO transcription factors should prove beneficial to determining the biological role of these signalling molecules. The alterations in larval development seen upon overexpression of dFOXO closely mimic the phenotypic effects of starvation, suggesting a role for dFOXO in the response to nutritional adversity. This work has implications in the understanding of cancer and insulin related disorders, such as diabetes and obesity.

1.2.2 - Background

The biological control of the size of an organism is one of the most elusive concepts in biology. What mechanisms determine the size differences between species? What genetic and environmental factors contribute to variations of size within a species? How does an individual regulate the size of its organs to maintain proportion with the rest of the body? Although much remains unanswered, it is clear that the size of an individual is directly related to the number of cells it has, and the size of these cells [1-3]. Thus, the final size of an organism is determined by the number of cell divisions that occur during development, and the amount of growth these cells undergo. When considering the size difference between two organisms, such as a mouse and a human, it is obvious that the main cause of the size difference is the total number of cells [2]. Intuitively, this may lead to the conclusion that the size of an organism is related to the rate of cell proliferation during development. However, experimental evidence shows that there are more subtle controls involved [4, 5]. For example, increasing or decreasing cell proliferation in the Drosophila imaginal discs does not alter the final size, but instead

produces discs with either an increased number of small cells or a decreased number of large cells [4, 5]. These studies indicate that there must be a genetically predetermined total cell mass and a mechanism for sensing this critical size.

Studies in Drosophila demonstrate that the evolutionarily conserved insulin signalling pathway is involved in the control of body size, through alterations of cell size and cell number [1]. Seven Drosophila insulin-like peptides (dILPs) have been identified that are able to promote organism growth when expressed ubiquitously during development [6, 7]. The dILPs activate cell signalling through the Drosophila insulin receptor (dInr), a receptor tyrosine kinase that can promote growth when overexpressed in the developing eye [6, 8]. Loss of function mutations in *dInr* are lethal during embryogenesis [8]. However, reduction of dInr levels through combination of weak heteroallelic mutations [9], or through partial loss of function mutations [6], reduces growth and leads to the development of small adults that have reduced cell size and number. In mammals, the insulin receptor promotes signalling through adaptor proteins, the insulin receptor substrates (IRS) 1-4, which are required to activate phosphoinositide-3-kinase (PI3K) [10, 11]. PI3K is a lipid kinase that phosphorylates inositide lipids on the inner surface of the cell membrane, leading to the activation of the serine/threonine kinase, Akt. Once activated, Akt phosphorylates many substrates that are involved in the regulation of metabolism, cell death/survival, and cell proliferation. Negative regulation of insulin signalling occurs through the tumor suppressor, phosphatase and tensin homolog (PTEN). PTEN removes phosphates from inositide lipids, thus acting in opposition to PI3K. This signalling mechanism appears to be conserved in Drosophila,

and the Drosophila homologues of IRS 1-4 (chico), PI3K (dPI3K), Akt (dAkt) and PTEN (dPTEN) have all been individually implicated in the regulation of cell size, and cell number [1]. Flies that are homozygous for a null mutation in *chico* are smaller than normal due to a reduction in cell size and cell number [12]. Null mutations in *dAkt* are lethal [13], however, rescue of *dAkt* mutants through ectopic expression of *dAkt* during embryogenesis results in a small fly phenotype [14] similar to that seen with *chico* mutants and through reduction of dInr activity. Clearly, components of the insulin signalling pathway act to control body and organ size through regulation of cell size and cell number during development.

In addition to developmentally predetermined size control, many cells and organisms can alter their size according to environmental stimuli, such as nutrient limitation. When Drosophila larvae are raised under nutrient limited conditions the adults are smaller than well-fed flies [15, 16]. This phenomena appears to be phenocopied in the generation of small adults through inhibition of Drosophila insulin signalling [6, 9, 12, 14]. Interestingly, expression of *dILPs 3*, *5*, and *7* has been linked to the availability of nutrients [7]. These dILPs are produced in neurosecretory cells in the larval brain where they are released into the circulatory system [7]. These studies indicate that nutritional signals may regulate body size by modulating the circulating levels of dILPs 3, 5, and 7.

Newly hatched Drosophila larvae require a nutritional signal to initiate the cell cycle in mitotic tissues [17]. Well-fed larvae increase their body mass very rapidly due to replication of cells in mitotic tissues. In contrast, larvae hatched into conditions of amino

acid starvation live in a state of developmental arrest for several days until nutrients become available to initiate the cell cycle [16, 17]. Dominant negative inhibition of dPI3K in developing Drosophila larvae has been shown to phenocopy the effects of amino acid starvation [18]. Expression of dPI3K in subsets of cells in the imaginal discs of starved larvae allows these cells to divide in the absence of nutritional signals [18]. Expression of dPI3K in the fat bodies of starved larvae significantly reduces their survival, thus conferring starvation sensitivity in these larvae [18]. This suggests that Drosophila insulin signalling may play a protective role in the response to starvation.

An insulin-like signalling pathway involved in the response to nutrient limitation also exists in the nematode, *Caenorhaebditis elegans*. When *C. elegans* are raised under conditions of nutrient limitation, they enter an alternate developmental stage called the dauer larvae. The dauer stage is characterized by arrest of growth at a sexually immature stage along with altered metabolism to increase the storage of fat [19]. Mutations in components of the insulin signalling pathway in *C. elegans* lead to dauer larvae formation and increased life span [20-24]. A null mutation in the *C. elegans* gene, Daf-16, negates dauer formation and the life expanding effect of these mutations [21, 25, 26]. Thus, in *C. elegans*, Daf-16 is necessary for dauer formation and may be the primary mediator of cell function during low levels of insulin signalling.

Daf-16 is the *C. elegans* homologue of a highly conserved group of Akt phosphorylatable forkhead transcription factors, the FOXO (forkhead box, subgroup "O") transcription factors. These transcription factors were first discovered as proto-oncogenes, which were disrupted as a result of chromosomal translocations leading to

acute lymphocytic leukemia and rhabdomyosarcoma [27, 28]. Three versions of FOXO have been identified in humans (FOXO1, FOXO3a, and FOXO4; formerly known as FKHR, FKHR-L1, and AFX) and mice (Foxo1, Foxo3, and Foxo4), and additional homologues have been identified in zebrafish and chickens [29]. The FOXO transcription factors share a highly conserved forkhead box DNA binding domain in the N-terminal half of the protein, and three highly conserved Akt phosphorylation sites. Mammalian cell culture studies have shown that in the absence of Akt signalling, FOXO is able to activate gene transcription and cause cell death, cell cycle arrest, or cell senescence [30, 31]. In the presence of activated Akt, FOXO becomes phosphorylated and is sequestered in the cytoplasm through facilitation of 14-3-3 binding [32-35], and/or disruption of a nuclear localization signal [34, 36]. The down-regulation of FOXO in this manner is, possibly, one of the most important consequences of Akt-mediated signalling.

Based on evidence from studies in *C. elegans* and mammalian cell culture, it appears that FOXO transcription factors are a critical mediator of low levels of insulin signalling. To investigate this further, we have identified and characterized the *Drosophila melanogaster* version of *FOXO*. We show that Drosophila FOXO (dFOXO) retains the conserved domains seen in other organisms and is involved in the regulation of growth. Of special interest is that dFOXO appears to have an effect upon feeding behavior, and may be a key player in the response of Drosophila larvae to nutritional stress.

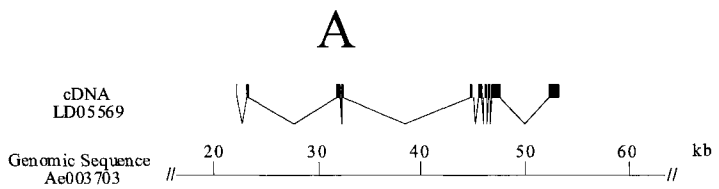
1.2.3 - Results

dFOXO retains the functional domains found in Daf-16 and the mammalian FOXO homologues.

The *dFOXO* gene consists of 10 exons and is spread out over approximately 31 kb in polytene chromosome section 88A within the genomic scaffolding region, AE003703, of the Berkeley Drosophila Genome Project (BDGP) (Figure 1A). *dFOXO* encodes a theoretical protein of 463 amino acids (Figure 1B). Analysis of the complete Drosophila genome for additional *dFOXO* homologues revealed none.

Alignment of dFOXO with the human homologues of FOXO and Daf-16a1 using ClustalW [37] (Figure 1B) revealed that although the overall identity of amino acids is not high, the identity in the forkhead box DNA binding domain is between 74 and 86 percent. The Akt phosphorylation sites are also well conserved in their relative position in the protein, and in sequence. The T1 site is located at T24 in dFOXO, the S1 site at S160, and the S2 site at S239. These sites align with the human FOXO homologues in the ClustalW alignment, however the Daf-16 S1, and S2 sites are slightly out of alignment (Figure 1B). All three of the potential Akt phosphorylation sites in dFOXO fit the Akt consensus target sequence (RxRxxS/T).

Other notable features found in FOXO include a DYRK1A phosphorylation site, a 14-3-3 binding site, a nuclear localization signal (NLS), a nuclear export signal (NES), and Ral-dependent phosphorylation sites. A DYRK1A phosphorylation site was confirmed experimentally in FOXO1 at S329 [38]. This serine residue is conserved in



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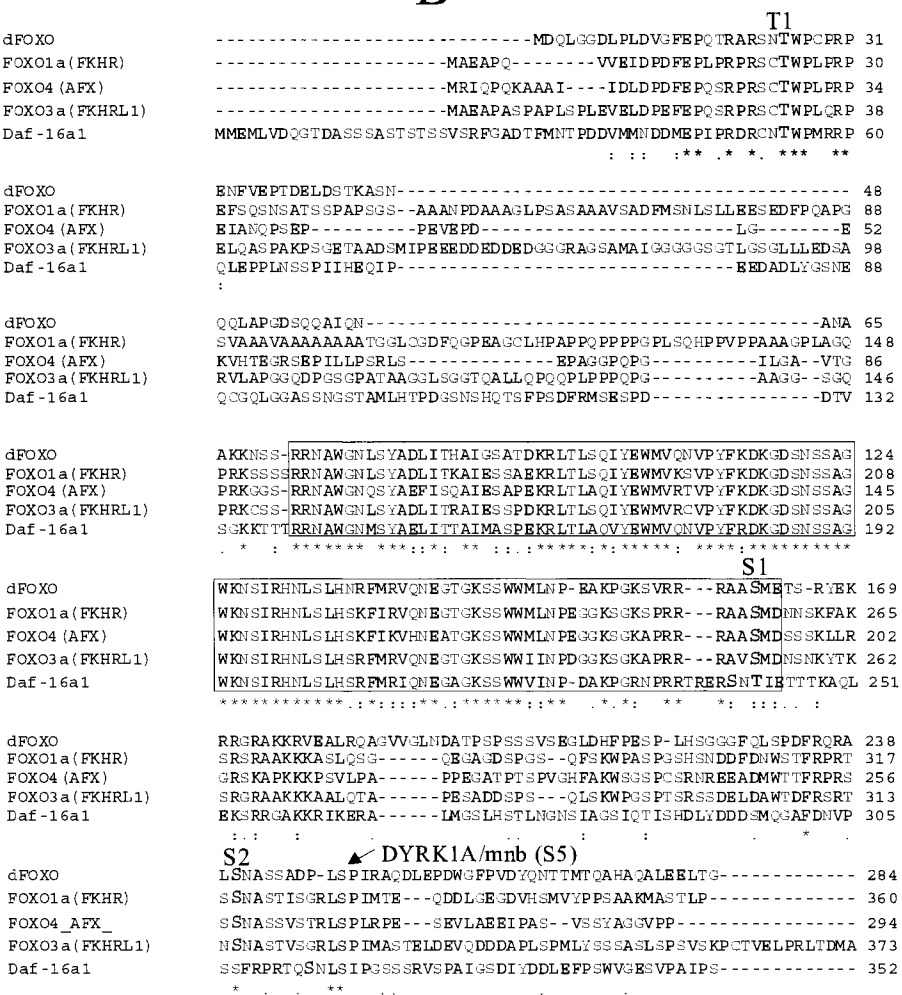

Figure 1: *dFOXO* encodes a protein that retains important functional domains found in other FOXO homologues. (A) Schematic representation of the dFOXO cDNA clone LD05569 and its location in the genomic scaffolding, region AE003703, of the BDGP sequence. (B) ClustalW alignment of the proposed dFOXO amino acid sequence with that of mammalian homologues (FOXO1a, FOXO3a, and FOXO4) and Daf-16a1. We have shown the alignment up to amino acid 284, for dFOXO, and to the corresponding amino acid in the alignment for the other homologues. Highlighted are: the T1, S1, and S2 Akt target sequences (yellow shading); the potential DYRK1a/mnb phosphorylation site (arrow, and grey shading); and the forkhead box DNA binding domain (black box). "*" indicates nucleotides that are identical in all sequences in the alignment, ":" indicates conserved substitutions, according to the chemical nature of the amino acids, and "." indicates semi-conserved substitutions. Colors indicate the chemical nature of the amino acid; Red = small hydrophobic (including aromatic), Blue = Acidic, Magneta = Basic, and Green = basic amino acids with hydroxyl groups and/or amine groups.

human FOXO3a (S324), FOXO4 (S267), Daf-16a1 (S317), and dFOXO (S248) (Figure 1B). In addition, the sequence surrounding this site in dFOXO ($LS^{248}PI$) is identical to that in FOXO1. The high conservation of this sequence suggests that dFOXO could be phosphorylated at this site by the Drosophila homologue of DYRK1A, minibrain (mnb).

Binding to 14-3-3 proteins is thought to be important for the sequestration of FOXO factors in the cytoplasm [30, 31]. 14-3-3 proteins normally bind to a consensus site containing a phosphoserine residue, either $RSxS^{P}xP$, or $RxxxS^{P}xP$ [39]. In the case of dFOXO, the sequence surrounding the T1 Akt phosphorylation site corresponds to the motif with a substitution of threonine for serine. It has been shown experimentally that 14-3-3 does bind to the T1 site in FOXO1 [40], FOXO3a [33], and Daf-16 [32], hence, it is also likely this region functions as a 14-3-3 binding site in Drosophila.

The current model for FOXO deactivation suggests the existence of an NES, causing constitutive localization of FOXO in the cytoplasm in the absence of a functional NLS [31]. A non-conventional NLS was identified in human FOXO4 from amino acids 180-221 [36]. The corresponding sequence in dFOXO (amino acids 147-194) is 38% identical and 66% similar in amino acid content (Figure 1B). This similarity suggests that this region may act as an NLS in dFOXO as well. A leucine-rich NES has been identified in FOXO1 (368 MENLLDNLNL 377) and the conservation of this sequence is high in FOXO3a, FOXO4, and Daf-16 [30]. The corresponding region in dFOXO retains two of the important leucine residues (281 LTGTMADELTL 291), however, the remaining sequence is more divergent.

FOXO4 has previously been shown to be phosphorylated in a Ral-dependent manner at threonines 447 and 451 [41]. However, these sites do not appear to be conserved in the other human FOXO homologues, Daf-16, or dFOXO, indicating that Ral-dependent phosphorylation of FOXO may be specific to FOXO4.

dFOXO expression during development phenocopies starvation and alters feeding behaviour.

Drosophila larvae feed continuously up to the age of 5 days after egg laying (AEL). During this time the appetite and growth rate of the larvae is enormous. If young larvae are deprived of food, they do not grow and tend to disperse randomly [16, 17, 42]. When the food supply is replenished, the larvae immediately move towards it and continue eating until they approach pupation. If the food supply is again depleted, the larvae will disperse again [42]. We utilized the UAS/Gal4 ectopic expression system [43] to overexpress dFOXO in the developing larvae under the control of the *ActGal4* driver [44]. This resulted in complete developmental arrest of the larvae, which remained as first instar for up to 7 days (Figure 2A), similar to the life expectancy of starved larvae [16-18]. This trend was also seen using a constitutively active version of *Murine* Foxo1 (mFoxo1) containing an alanine substitution at the T1 (T24A), and S1 (S253A) Akt phosphorylation sites (mFoxo1-AA) [45] (Figure 2A). In addition, larvae expressing dFOXO and mFoxo1-AA were often found to be wandering far from their food supply. We monitored feeding behavior by assessing the number of larvae away from their food at 48 and 72 hours AEL. Larvae expressing dFOXO and mFoxo1-AA

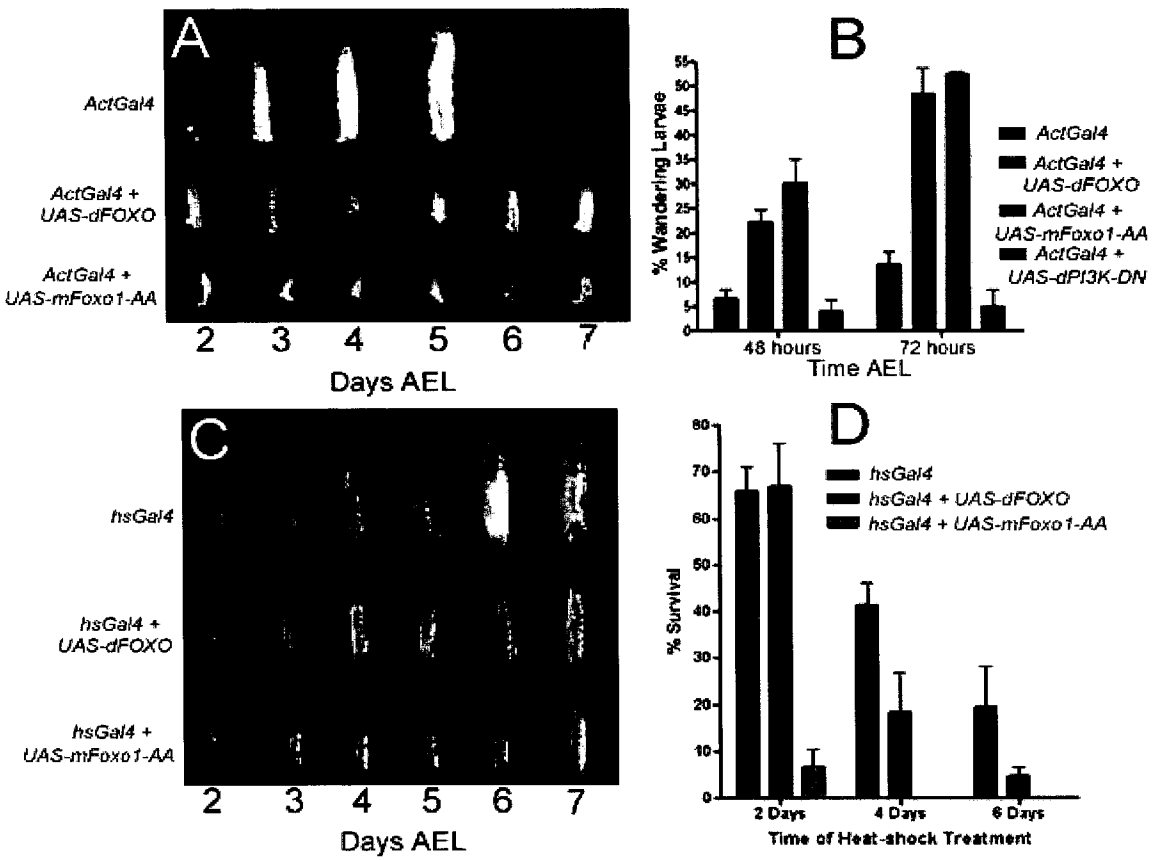

Figure 2: Expression of dFOXO in first instar larvae phenocopies starvation and influences feeding behavior. Expression of dFOXO and mFoxo1-AA early in larval development using the (A) *ActGal4* and (C) *hsGal4* driver lines leads to developmental arrest similar to that seen in starved larvae. Developmentally arrested larvae are capable of surviving for up to seven days after egg laying (AEL). (B) Expression of dFOXO (red bars) and mFoxo1-AA (green bars) leads to alterations in feeding behavior when compared to controls (grey bars). The percentage of wandering larvae is significantly greater in larvae expressing dFOXO and mFOXO1-AA at 48 hours and 72 hours AEL (p=0.05). Expression of dPI3K-DN (blue bars) did not increase larval wandering. (D) Developmental arrest is reversible upon removal of dFOXO expression (red bars), but not upon removal of mFOXO1-AA expression (green bars). Grey bars represent the controls. Each bar reflects the average of three separate trials, with 50 larvae per trial. Genotypes are; (A-top, B-grey bars) *w; ActGal4/+*, (A-middle, B-red bars) *w; ActGal4/+; UAS-dFOXO/+*, (A-bottom, B-green bars) *w, UAS-mFoxo1-AA/w; ActGal4/+*, (C-top, D-grey bars) *w; hsGal4/+*, (C-middle, D-red bars), *w; hsGal4/UAS-dFOXO*, (C-bottom, D-green bars) *w, UAS-mFoxo1-AA/w; hsGal4/+*, (B- blue bars) *w; ActGal4/dPI3K-DN.*

showed a 3-4 fold increase in wandering over larvae expressing Gal4 alone (Figure 2B). Thus, dFOXO expression drastically alters feeding behavior and is able to induce a starvation type response in larvae which have an adequate food supply.

In Drosophila, PI3K consists of an adaptor subunit, dp60, and a catalytic subunit, dp110. Unexpectedly, expression of an inhibitory or "dominant negative" version of dp110 (*UAS-dPI3K-DN*) [46] under the control of *ActGal4* did not lead to increased larval wandering (Figure 2B). Expression of this construct also did not appear to inhibit larval growth, whereas other negative regulators of insulin signalling do [18]. The inability of this construct to inhibit growth and induce wandering behaviour suggests that it does not have a complete dominant negative effect.

Larvae that are developmentally arrested by starvation are able to resume growth upon acquisition of food [17]. We examined if larvae expressing dFOXO could resume growth upon termination of dFOXO expression. To do this we utilized the *hsGal4* driver [47]. dFOXO was expressed in the larvae by heat shock treatment (HST) at 37 degrees Celsius for 10 minutes every 24 hours. This treatment was sufficient to inhibit growth while allowing controls to survive to adulthood with a 48 hour delay in the time to pupation (Figure 2C). When dFOXO expression was discontinued after 2, 4, and 6 days of HST, developmentally arrested larvae were able to recover with decreased levels of survival as time progressed (Figure 2D). Significant lethality was also observed in controls, suggesting that low survival rates were partially due to expression of Gal4, shown to induce apoptosis [48], and/or by the HST itself (Figure 2D). Nevertheless,

developmental arrest caused by dFOXO is clearly reversible as these individuals could be returned to their normal path of development.

dFOXO performs an analogous function to C. elegans, *Daf-16.*

The formation of dauer larvae in *C. elegans* is a developmental response to nutrient limitation [19]. The dauer larvae provides a temporary defense mechanism allowing the nematode to persevere until nutrients are available, at which point development can continue. Interestingly, constitutive activation of Daf-16 by mutation of its Akt phosphorylation sites to alanine residues causes obligatory dauer larvae formation [49]. We found a similar result in the Drosophila larvae using the constitutively active mFoxo1-AA [45]. This construct had an effect similar to that of dFOXO when expressed under the control of *ActGal4* (Figure 2A), and *hsGal4* (Figure 2C). Upon removal from HST, larvae expressing mFoxo1-AA did not resume growth but remained in a state of developmental arrest until death (Figure 2D). Although a few larvae did survive to adulthood after 2 days of HST, none of the larvae were able to continue development after 4, or 6 days of HST (Figure 2D). Out of 450 larvae examined at all time points, only 10 expressing mFoxo1-AA survived, when compared to 110 and 180 for larvae expressing dFOXO, and Gal4 alone, respectively. Presumably this occurs because Akt is unable to deactivate mFoxo1-AA (Figure 4P), allowing it to continue functioning long after expression is induced. Taken together, this data suggests that dFOXO is evolutionarily conserved in function, possibly playing a role in the response to nutritional adversity, as seen in the formation of dauer larvae in *C. elegans.*

dFOXO inhibits growth through alterations in cell size and cell number.

Expression of dFOXO in the third instar larvae caused significant lethality, however, rare flies that did survive were much smaller than control flies (Figure 3A), showing a phenotype similar to that caused by mutations in *chico* [12], *dAkt* [14] and *dInr* [6, 9]. Expression of *dFOXO* under the control of the ubiquitous low level Gal4 drivers, *armadillo-Gal4*, and *hsGal4* (raised at 25 °C with no heat shock) had very little effect on growth (data not shown). In contrast, increasing expression of *dFOXO* using the *hsGal4* driver in flies raised at 29 °C lead to the development of small adults, which were approximately half the weight of control flies (Figures 3B and 3D). Analysis of the wings of these flies showed that the wing area was reduced by nearly one third and that this reduction was due to a decrease in both cell size and cell number (Figures 3C and 3D). SEM analysis of the eyes revealed reductions in both ommatidia number and ommatidia area, which reflect cell number and cell size, respectively (Figures 3E and 3F). These results implicate dFOXO in the control of body size through alterations in cell size and cell number.

Regulation of FOXO by PI3K/Akt pathway is conserved between mammals and flies.

When dFOXO is expressed in the developing eye under the control of the *GMR-Gal4* driver [50], the eye is smaller, lacking many ommatidia and nearly all of the mechanosensory bristles (Figure 4E). The remaining ommatidia are arranged in the typical hexahedral array and cross sectional analysis revealed that all of the normal

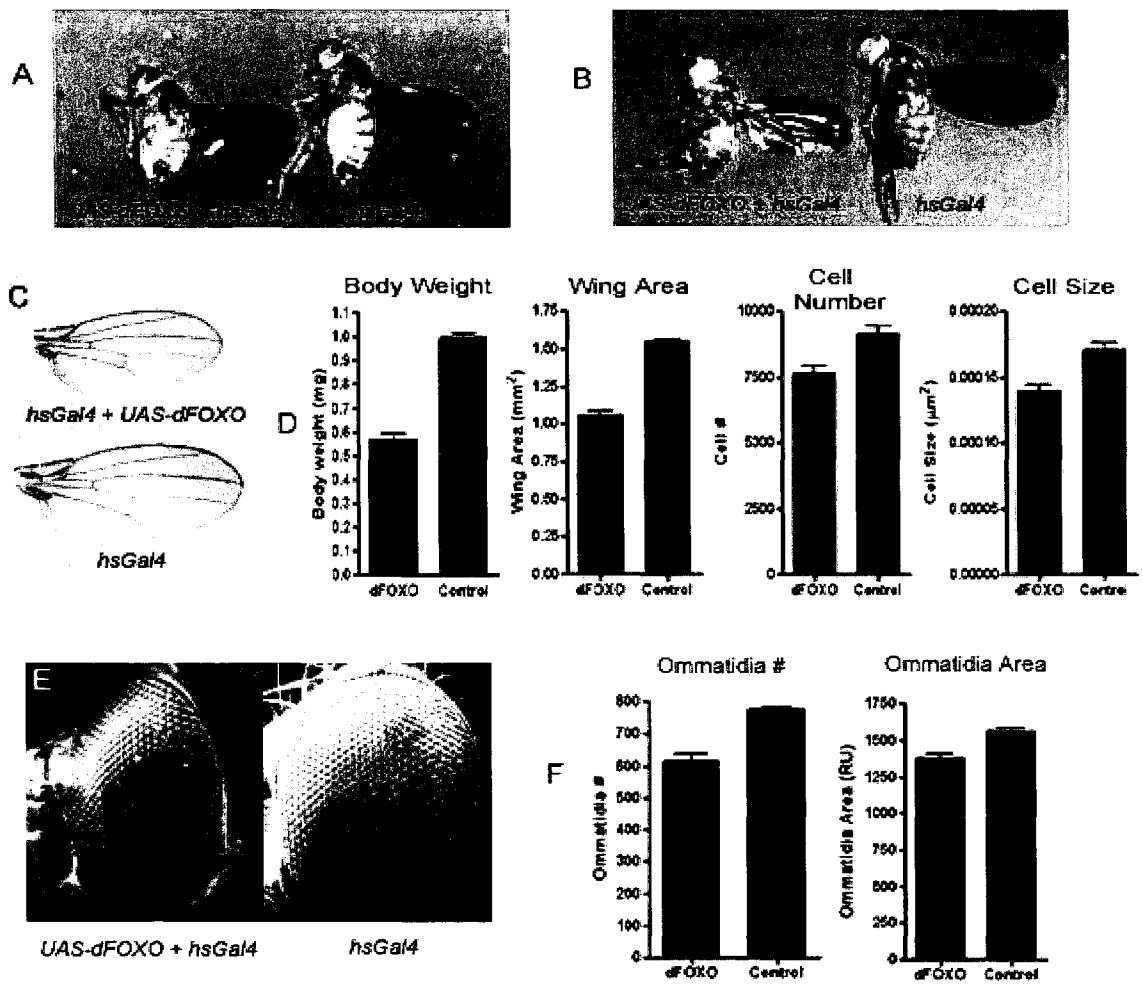

Figure 3: dFOXO reduces growth through alterations in cell size and cell number. (A) Expression of *UAS-dFOXO* in the third larval instar produces small flies (left) when compared to controls (right). *w; hsGal4/CyO* flies were crossed to *w; UAS-dFOXO/UAS-dFOXO* flies and the progeny were heat shocked at 36°C for 4 hours during the early third instar. (B) Flies of the genotype *w; hsGal4/+; UAS-dFOXO/+* (left) were smaller than *w; hsGal4/+* (right) flies when raised at 29°C. (C) The wings of *w; hsGal4/+; UAS-dFOXO/+* flies raised at 29°C were smaller than control wings (scale bar=1 mm). (D) Flies expressing dFOXO (red bars) also showed a significant reduction in body weight, wing area, cell number, and cell size when compared to control flies (grey bars) (p=0.005). (E) Flies expressing dFOXO had smaller eyes than control flies (scale bar = 150 m), and (F) their eyes were reduced in both the number of ommatidia and the area of the ommatidia (red bars) when compared to controls (grey bars) Genotypes are; (A-left, B-left, C-top, D-red bars, E-left, F-red bars) *w; hsGal4/+; UAS-dFOXO/+*, (A-right, B-left, C-bottom, D-grey bars, E-right, F-grey bars) *w; hs-Gal4/+*.

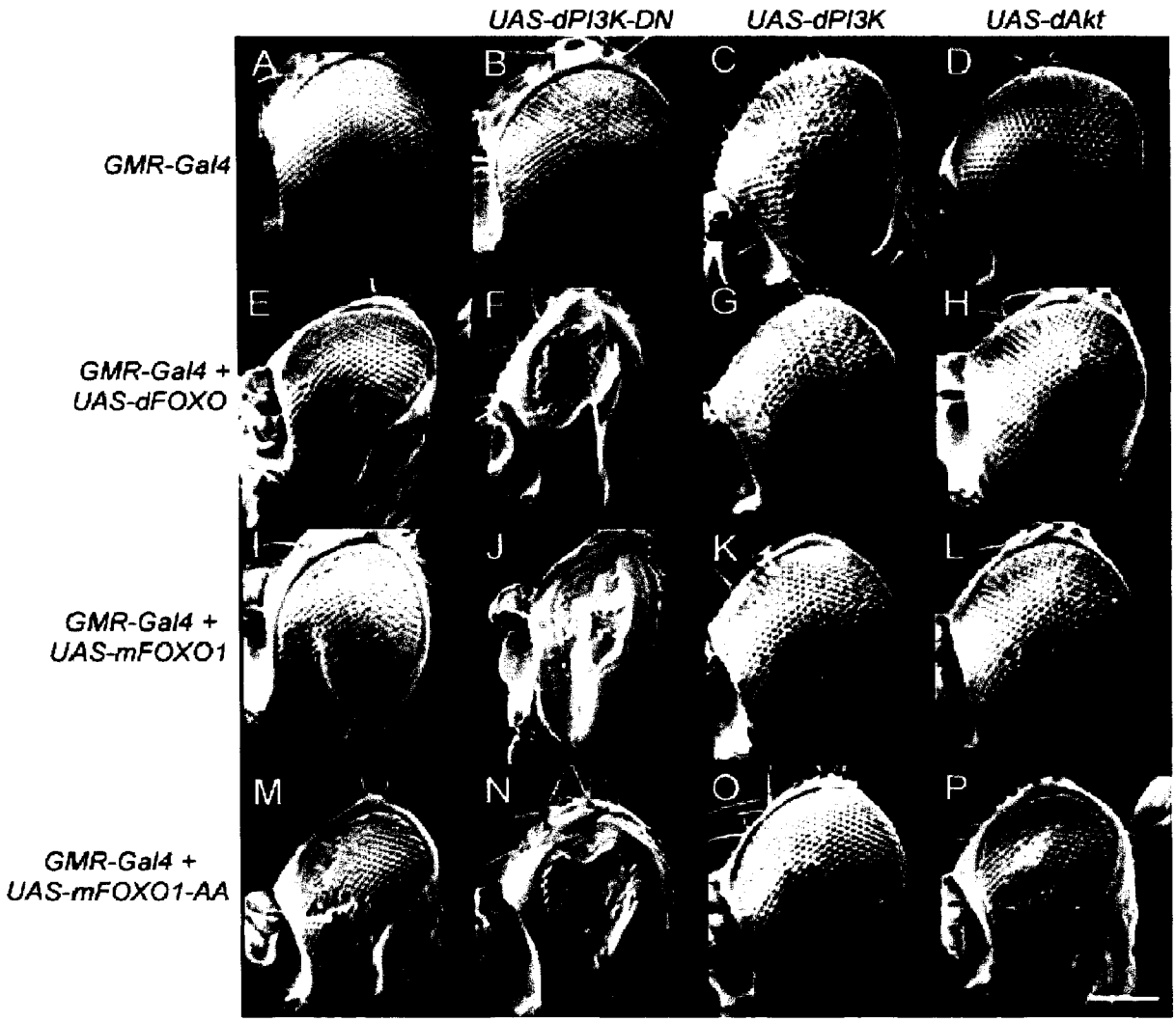

Figure 4: Regulation of dFOXO through insulin signaling is conserved between mammals and flies. The *GMR-Gal4* driver was used to drive the expression of (B) dPI3K-DN, (C) wild type dPI3K, (D) dAkt, (E) dFOXO, (I) mFoxo1, and (M) mFoxo1-AA, both alone and in various combinations (F-H, J-L, N-P) as indicated through the rows and columns in the figure (scale bar = 150 m). Genotypes are: (A) *w; GMR-Gal4/+*, (B) *w; UAS-dPI3K-DN/GMR-Gal4*, (C) *w; UAS-dPI3K/GMR-Gal4*, (D) *w; UAS-dAkt/GMR-Gal4*, (E) *w; GMR-Gal4/+; UAS-dFOXO/+*, (F) *w; UAS-dPI3K-DN/GMR-Gal4; UAS-dFOXO/+*, (G) *w; UAS-dPI3K/GMR-Gal4; UAS-dFOXO/+*, (H) *w; UAS-dAkt/GMR-Gal4; UAS-dFOXO/+* (I) *w; GMR-Gal4, UAS-mFoxo1/+*, (J) *w; GMR-Gal4, UAS-mFoxo1/UAS-dPI3K-DN*, (K) *w; GMR-Gal4, UAS-mFoxo1/UAS-dPI3K*, (L) *w; GMR-Gal4, UAS-mFoxo1/UAS-dAkt*, (M) *w, UAS-mFoxo1-AA/w; GMR-Gal4/+*, (N) *w, UAS-mFoxo1-AA/w; GMR-Gal4/UAS-dPI3K-DN*, (O) *w, UAS-mFoxo1-AA/w; GMR-Gal4/UAS-dPI3K*, (P) *w, UAS-mFoxo1-AA/w; GMR-Gal4/UAS-dAkt.*

photoreceptor cells are present (Figure 4E, data not shown). Thus, it appears that dFOXO expression causes a reduction in the number of cells but does not interfere with cellular differentiation and the organization of the ommatidia themselves. We have used this eye phenotype to test for interactions between dFOXO and other components of the insulin signalling pathway.

Expression of *dPI3K-DN* under the control of *GMR-Gal4* leads to the formation of relatively normal eyes with fewer and smaller cells [46] (Figure 4B). When dFOXO is co-expressed in the developing eye with dPI3K-DN the eye is nearly obliterated (Figure 4F). In contrast, co-expression of dAkt, and wild type dPI3K with dFOXO causes nearly complete rescue of the phenotype, restoring the ommatidia and nearly all of the mechanosensory bristles (Figures 4G and 4H). Thus, diminishing PI3K/Akt signalling (through overexpression of dPI3K-DN) allows for greater activity of dFOXO, and enhancing PI3K/Akt signalling (through overexpression of dAkt or dPI3K) leads to inhibition of dFOXO activity. Similar results were obtained when overexpressing (mFoxo1) (Figure 4 I-L), indicating that the regulatory mechanisms between these two proteins are conserved and that they are functionally interchangeable.

Growth effects of dPI3K and dAkt are masked by expression of mFoxo1-AA.

The constitutively active mFoxo1-AA construct [45] was also expressed in the developing eye. Expression of this construct causes a phenotype similar to that of dFOXO and mFoxo1, with characteristic lack of ommatidia and mechanosensory bristles (Figure 4M). When mFoxo1-AA is co-expressed with dPI3K-DN the eye is nearly

obliterated (Figure 4N), as seen with dFOXO and mFoxo1 (Figures 4F and 4J). Co-expression of mFoxo1-AA with dPI3K leads to a partial rescue of the phenotype, with still an obvious lack of ommatidia and mechanosensory bristles (Figure 4O). In contrast, co-expression of mFoxo1-AA with dAkt does not cause rescue of the ommatidia or mechanosensory bristles (Figure 4P), indicating that this construct is not responsive to dAkt signalling. The partial rescue of the dFOXO phenotype by dPI3K appears to be mediated through alterations in cell size (Figure 5) rather than cell number, as there is still an obvious lack of ommatidia and mechanosensory bristles (Figure 4O). These data indicate that inactivation of dFOXO is required for the full effects of growth mediated by dPI3K and dAkt.

dPI3K can increase cell size in the presence of constitutively active Foxo

To examine the effect of dFOXO overexpression on cell size we measured the area of the ommatidia. Expression of dFOXO, mFoxo1, and mFoxo1-AA caused a significant reduction in the area of the ommatidia ($p=0.001$) (Figure 5). Expression of dPI3K caused a significant increase in ommatidia size over wild type ($p=0.001$) (Figure 5). This result is consistent with previous studies showing that dPI3K affects cell size in a cell autonomous manner [46]. Co-expression of dFOXO, mFoxo1, and mFoxo1-AA with dPI3K had no significant effect on the enlarged ommatidia ($p=0.001$) (Figure 5). Thus, it appears that FOXO proteins have a very minimal effect on cell size in the presence of high levels of dPI3K. Surprisingly, this is the case even with the mFoxo1-AA construct, which is only partially responsive to PI3K signalling [45]. This indicates

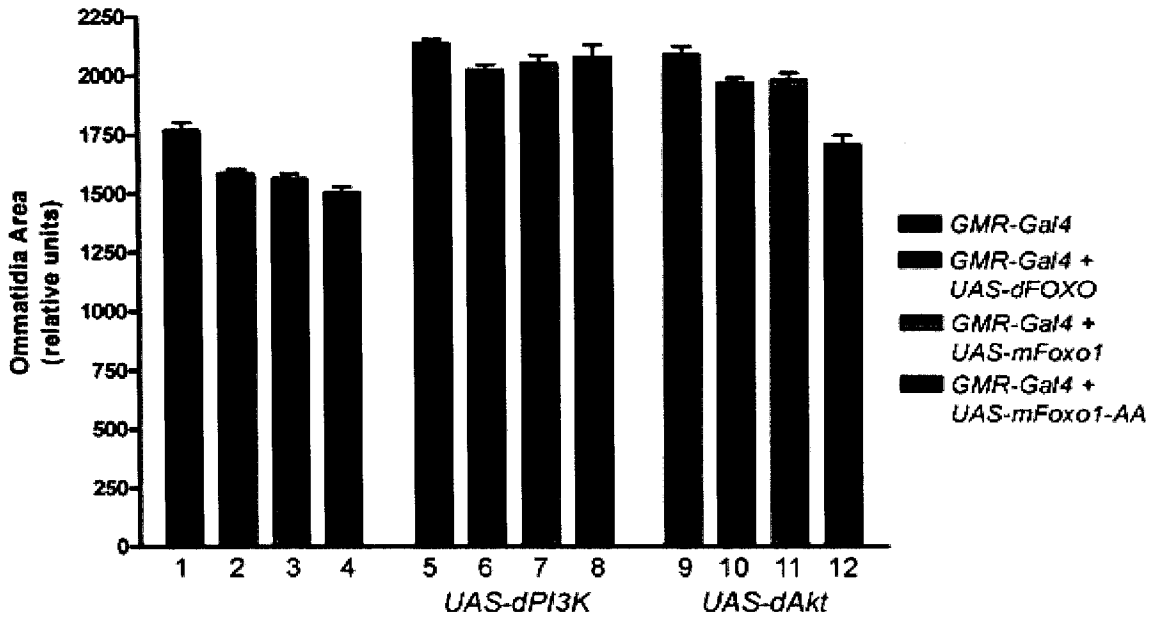

Figure 5: dFOXO inactivation is essential for dAkt-, but not dPI3K-mediated increases in cell size. Ommatidia area was measured as a means to determine the effect of Foxo overexpression on cell size. Expression of dFOXO (bar 2), mFoxo1 (bar 3), and mFoxo1-AA (bar 4) under the control of *GMR-Gal4* causes a significant decrease in ommatidia area when compared to the expression of Gal4 alone (bar 1). In addition, *GMR-Gal4* was used to drive the expression of *dPI3K* (bars 5-8), and *UAS-dAkt* (bars 9-12), either alone (grey bars), or in the presence of *UAS-dFOXO* (red bars), *UAS-mFoxo1* (light green bars), or *UAS-mFoxo1-AA* (dark green bars). Two sided t-tests were preformed to determine statistical significance (p=0.001). Genotypes are: (1) *w; GMR-Gal4/+*, (2) *w; GMR-Gal4/+; UAS-dFOXO/+*, (3) *w; GMR-Gal4, UAS-mFoxo1/+*, (4) *w, UAS-mFoxo1-AA/w; GMR-Gal4/+*, (5) *w; UAS-dPI3K/GMR-Gal4*, (6) *w; UAS-dPI3K/ GMR-Gal4; UAS-dFOXO/+*, (7) *w; GMR-Gal4, UAS-mFoxo1/UAS-dPI3K*, (8) *w, UAS-mFoxo1-AA/w; GMR-Gal4/UAS-dPI3K*, (9) *w; UAS-dAkt/GMR-Gal4*, (10) *w; UAS-dAkt/GMR-Gal4; UAS-dFOXO/+* (11) *w; GMR-Gal4, UAS-mFoxo1/UAS-dAkt* (12) *w, UAS-mFoxo1-AA/w; GMR-Gal4/UAS-dAkt.*

that the dPI3K mediated increase in cell size can occur through dAkt independent mechanisms.

Expression of dAkt in the developing eye caused a significant increase in ommatidia size, similar to that seen with dPI3K (p=0.001) (Figure 5). Co-expression of dAkt with either dFOXO or mFoxo1, caused a slight, but insignificant decrease in the size of the enlarged ommatidia (Figure 5). However, co-expression of dAkt with mFoxo1-AA resulted in ommatidia that were approximately the same size as the

ommatidia in eyes expressing Gal4 alone (Figure 5), and significantly smaller than the ommatidia in eyes expressing dAkt alone (p=0.001) (Figure 5). This indicates that the deactivation of FOXO by dAkt is essential for dAkt to induce an increase in cell size.

dFOXO may reduce cell number through inhibition of the cell cycle and not apoptosis.

The lack of ommatidia and mechanosensory bristles caused by dFOXO expression suggests a reduction in cell number during eye development (Figure 6A). Reduction of cell number can occur through either increased cell death, or decreased cell proliferation. The Drosophila inhibitors of apoptosis, Diap1 and Diap2 (data not shown), and the baculovirus inhibitor of apoptosis, p35 (Figure 6B), were unable to rescue the phenotype caused by dFOXO expression. In addition, acridine orange staining of eye imaginal discs expressing dFOXO showed no increase in apoptosis when compared to controls (data not shown). Drosophila epidermal growth factor receptor (dEGFR) signalling acts to protect differentiated cells from death during eye development [51]. We initially thought that the pro-survival effects of dEGFR may be sufficient to suppress the phenotype caused by dFOXO overexpression. Co-expression of dEGFR with dFOXO, however, does not rescue the dFOXO phenotype as ommatidia and bristles are clearly still missing (Figure 6D). Conversely, dFOXO does not appear to affect the phenotype of dEGFR overexpression as the general disorganization of the ommatidia appears to be the same (Figure 6C). Thus, it appears that these two mechanisms are acting independently. Taken together, these results suggest that dFOXO overexpression does not cause cell death during eye development, as direct inhibitors of the apoptotic

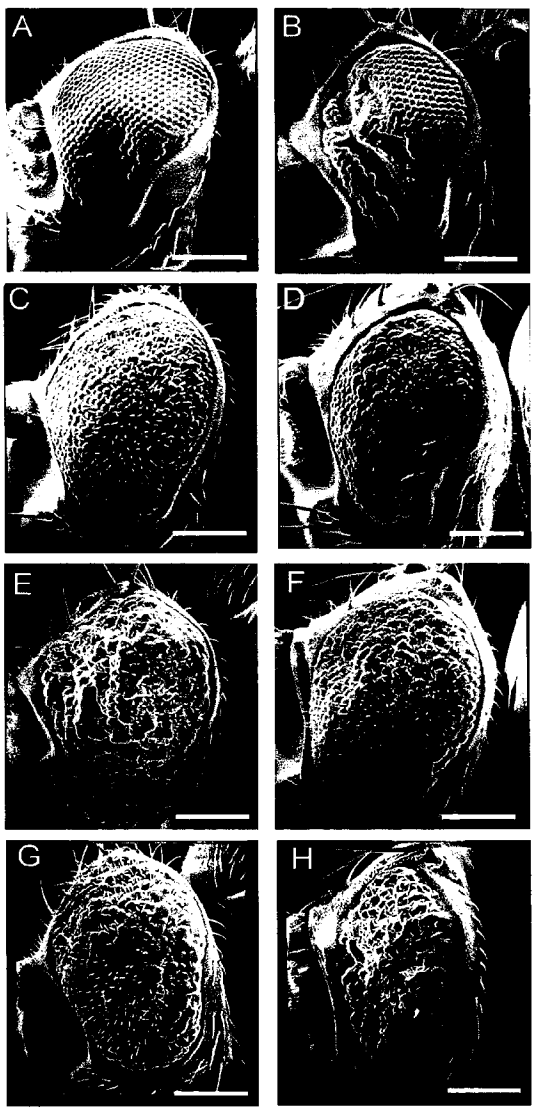

Figure 6: dFOXO responds to dRas2 signaling, but not to inhibitors of apoptosis. *GMR-Gal4* was used to drive the expression of *UAS-dFOXO* (A) alone, and in the presence of (B) *UAS-p35*, (D) *UAS-dEGFR*, (F) *UAS-Ras2^{V14}*. *UAS-Ras2^{V14}* was also expressed in combination with *UAS-mFoxo1* (G) and *UAS-mFoxo1-AA* (H). Scale bars equal 150 m. Genotypes are: (A) *w; GMR-Gal4/+; UAS-dFOXO/+*, (B) *w; GMR-Gal4/UAS-p35; UAS-dFOXO/+*, (C) *w; GMR-Gal4/UAS-dEGFR*, (D) *w; GMR-Gal4/UAS-dEGFR; UAS-dFOXO/+*, (E) *w; GMR-Gal4/UAS-Ras^{V14}*, (F) *w; GMR-Gal4/ UAS-Ras2^{V14}; UAS-dFOXO/+*, (G) *w; GMR-Gal4, UAS-mFoxo1/ UAS-Ras2^{V14}*, and (H) *w, UAS-mFoxo1-AA/w;GMR-Gal4/ UAS-Ras2^{V14}*.

machinery (p35 and Diap1/2) and a known cell survival factor (dEGFR) were unable to rescue the dFOXO-induced phenotype.

Since inhibition of apoptosis could not rescue the phenotype caused by dFOXO overexpression in the eye, we examined if activating the cell cycle could inhibit the phenotype. Expression of the E2F and Dp transcription factors has been shown to promote cell proliferation in the wing imaginal disc [4]. Co-expression of E2F and Dp with dFOXO was not sufficient to rescue the dFOXO phenotype (data not shown). Overexpression of constitutively active dRas1 ($dRas1^{V12}$) has been shown to induce ectopic cell proliferation [52] and G1/S progression in the Drosophila wing disc [53, 54]. Co-expression of $dRas1^{V12}$ with dFOXO was lethal, so we used a constitutively active version of dRas2 ($dRas2^{V14}$). Although dRas2 has not been characterized for its role in cell cycle control, it is possible that it has a similar function to dRas1. Expression of UAS-$dRas2^{V14}$ under the control of GMR-Gal4 led to extreme overgrowth of the eye, lack of ommatidial organization, and the formation of huge ommatidia (Figure 6E). Co-expression of $dRas2^{V14}$ with dFOXO was sufficient to restore many of the ommatidia and mechanosensory bristles lost through overexpression of dFOXO alone (Figure 6A and 6F). A similar effect was observed upon co-expression of $dRas2^{V14}$ with mFoxo1 (Figure 6G). In contrast, the loss of ommatidia and bristles seen upon over expression of mFoxo1-AA was not rescued by $dRas2^{V14}$ (Figure 6H). This suggests that $dRas2^{V14}$ inhibits dFOXO through a dAkt phosphorylation-dependent mechanism.

1.2.4 - Discussion

For the most part, the genetic mechanisms that control size in multicellular organisms are not well understood [2]. Recently, components of the insulin signalling pathway have been shown to regulate body size in *Drosophila melanogaster* through alterations in cell size and cell number [1, 6]. We have identified dFOXO as a negative controller of growth and organism size that is regulated by components of the Drosophila insulin signalling pathway, dPI3K and dAkt. Through overexpression studies in the developing eye, we have shown that dFOXO is regulated by dPI3K and dAkt in a manner that is consistent with the regulatory mechanisms deduced through studies in *C. elegans* and mammalian cell culture. In addition, overexpression of dFOXO in the larvae reduces larval growth, phenocopies the effects of nutritional stress, and causes alterations in feeding behavior. With this in mind, we propose that dFOXO is involved in the response of Drosophila larvae to nutritional stress.

Conservation of FOXO in Drosophila.

The FOXO homologues appear to play an evolutionarily conserved role in the control of cellular processes under conditions of low levels of insulin signalling [30, 31]. Our experiments provide three lines of evidence supporting the conservation of this mechanism in Drosophila. First, dFOXO shows strong sequence homology to Daf-16 and the human FOXO homologues (Figure 1B). One significant characteristic is the high conservation of the three consensus Akt phosphorylation sites, suggesting that dAkt is most likely able to phosphorylate dFOXO *in vivo*, similarly to that shown in mammalian

FOXO homologues [33-35]. Second, our experiments show that dFOXO and mFoxo1 cause nearly identical phenotypic responses when overexpressed in the developing Drosophila eye (Figure 4, 5 and 6). This suggests that the activity of these proteins is highly conserved as was observed when the *C. elegans* FOXO homologue, Daf-16, is expressed in mammalian cell culture [32]. Third, the phenotypic effects of FOXO overexpression can be modulated by alterations in the insulin signalling pathway. Reduced PI3K signalling leads to a drastic enhancement of the phenotype that results from expression of FOXO factors (Figure 4). In contrast, increased PI3K/Akt signalling tends to mask these phenotypes, in a manner that is dependent on the integrity of the Akt phosphorylation sites (Figures 4 and 5). As a result, we believe that regulation of FOXO is conserved in Drosophila and that this will be a very useful system in elucidating the function of FOXO transcription factors in a model organism.

Regulation of size by dFOXO.

Our results show that ectopic dFOXO expression can mediate reduction in cell size and cell number (Figures 3, 4, and 5). However, the mechanisms by which these reductions occur are still unclear. Net reduction in cell number may occur through decreased cell proliferation or increased apoptosis. Insulin and other growth factors that activate PI3K and Akt have been implicated as potent survival factors in mammalian cell culture [10, 11]. They prevent cell death, in part, by inhibiting FOXO transcription factors. It has also been shown that FOXO3a can upregulate expression of the pro-apoptotic protein Bim [55]. In Drosophila, reduction of insulin signalling can lead to

apoptosis in the developing embryo [13, 14, 56, 57]. It is possible that this increase in apoptosis is a result of dFOXO activation, however, when dFOXO is expressed in the developing eye there is no apparent increase in apoptosis, nor is the phenotype suppressed by inhibition of caspases, or by co-expression of a known cell survival factor, dEGFR (Figure 6). These apparent discrepancies may be the result of tissue specific differences. In mammalian cell culture, induction of cell death by FOXO factors seems to be limited to non-transformed haematopoietic cell lineages [31]. In Drosophila, loss of dAkt function, inhibition of dPI3K, or overexpression of dPTEN, all induce cell death in the embryo [13, 14]. However, in imaginal disc cells lacking PI3K function, there is no increase in apoptosis [58]. Thus, the cells in the embryo and imaginal discs may react differently to reduced levels of PI3K/Akt signalling. Although we do not observe induction of apoptosis upon dFOXO expression, it is possible that increased levels of dFOXO activity (eg. through dominant negative inhibition of PI3K) do cause apoptosis.

Studies in mammalian cell culture have implicated FOXO factors in control of the cell cycle through increased expression of the cyclin dependent kinase inhibitor $p27^{Kip1}$ [59, 60]. It is possible that the reduction in cell number seen upon dFOXO expression is a result of cell cycle inhibition. Co-expression of an activated version of Drosophila Ras2 ($dRas2^{V14}$) was sufficient to increase cell number in the presence of dFOXO (Figure 6). dRas1 has been shown to induce growth in Drosophila imaginal discs [52-54] through activation of dPI3K and the transcription factor dMyc [53]. Although there is very little information available about dRas2, it is possible that the function of dRas2 overlaps with that of dRas1. Expression of $dRas2^{V14}$ in the developing eye does cause a

phenotype that suggests overgrowth of cells (Figure 6E), and the dRas2^{V14} interaction with dFOXO appears to dAkt-like signalling (Figure 6H). This is not surprising considering that dRas1 [53] and mammalian Ras [61] have both been shown to activate PI3K signalling. Interestingly, increasing the cell cycle through overexpression of the transcription factors E2F and Dp did not rescue the cell number deficit seen upon overexpression of dFOXO (unpublished observations). This suggests the possibility that activation of dFOXO may override the function of other growth promoting factors, such as dMyc, which mediates dRas1 induced G1/S progression [53]. Supporting this hypothesis, we have observed that increased dAkt-mediated growth is entirely dependent on the ability dAKT to inactivate dFOXO (Figures 4P and 5). Furthermore, increased dPI3K-mediated growth appears to be dependent on dFOXO inactivation with respect to increased cell number, but not cell size (Figures 4O and 5). In humans, inactivation of FOXO factors may play an important role in tumor suppression by down regulating expression of D-type cyclins, thus inhibiting cell cycle progression and transformation [62]. It would be interesting to test the interactions between dFOXO and other cell cycle promoters to determine the extent of dFOXO dominance over cell proliferation.

In addition to its effect on cell number, dFOXO is able to control cell size (Figures 3 and 5). The ability of dAkt to increase cell size is dependent on dFOXO inactivation, however, dPI3K does not need to inactivate dFOXO to increase cell size (Figure 5). The difference between dPI3K and dAkt might be attributed to greater activity of the *UAS-dPI3K* transgene. However, expression of these constructs individually yields very

similar results (Figures 4 and 5) indicating that this is probably not the case. These results suggest that dPI3K may control size through dAkt-independent mechanisms.

PI3K/Akt signaling and stress response.

Studies in *C. elegans* indicate that insulin signalling is a critical mediator of longevity and stress resistance [63, 64]. One of the most well-studied stress responses is the Daf-16-mediated formation of the dauer larvae under conditions of starvation and/or crowding. Several lines of evidence indicate that dFOXO may play a similar role in Drosophila larvae. When Drosophila larvae are deprived of food prior to 70 hours after hatching (AH), they live in a state of developmental arrest for several days before they eventually die. However, when starved after 70 hours AH, the larvae are able to develop into adults that are reduced in size. This alteration in developmental response has been termed the '70 hour change' and is likely determined by the minimum size required for a Drosophila larvae to enter pupation [16]. We have mimicked the phenotypes resulting from starvation before and after the '70 hour change' through overexpression of dFOXO at different stages of larval development (Figures 2 and 3). Ubiquitously high levels of dFOXO expression in the early larvae (i.e. before 70 hours AH) leads to developmental arrest, whereas heat shock-induced expression of dFOXO during the third instar (i.e. after 70 hours AH) leads to the development of small adults. The normal development of starved larvae can resume upon the acquisition of food. Similarly, developmental arrest caused by expression of dFOXO prior to the "70 hour change" can be reversed if dFOXO expression is discontinued (Figure 2). Developmental arrest caused by expression of

mFoxo1-AA before the "70 hour change" is not reversible suggesting a constitutive starvation type response as seen in *C. elegans* when Daf-16 phosphorylation sites are mutated [49]. Interestingly, the reversibility of FOXO-induced arrest has also been observed in mammalian cell culture [65]. Under conditions of poor nutrition or crowding, larval development does not cease, but the larval period is extended and small adults are produced [15]. We have replicated this effect through low-level expression of dFOXO during the course of development (Figure 3). Finally, feeding behavior is drastically altered in larvae expressing dFOXO (Figure 2), causing them to wander away from their food. These larvae are often found crawling on the sides and lids of Petri dishes. This response may provide a selective advantage in the search for food as seen in *C. elegans* dauer larvae, which often crawl up to the highest point possible in hopes of attaching to passing organisms that could move the larvae to new locations with better food supply [19]. Taken together, these results suggest that dFOXO activity may act to promote survival during times of nutritional stress in a manner that recapitulates the formation of dauer larvae in *C. elegans*. It is tempting to speculate that dFOXO plays a role in the response to other forms of stress, as observed with Daf-16 [63, 64]. Mammalian FOXO factors have been implicated in the protective response to oxidative stress [66-68] and FOXO factors are upregulated in response to caloric restriction in rat skeletal muscle [69]. Thus, it is possible that FOXO factors provide an evolutionarily conserved switch, by which an organism can alter its developmental program in order to promote survival under harsh conditions.

Insulin signalling and feeding behavior.

Previously, it was observed that activation of PI3K/Akt signalling caused larvae to wander away from their food [18]. We have observed a similar effect through overexpression of dFOXO, which acts in opposition to PI3K. Thus, two opposing signaling mechanisms produce the same effect. It is possible that exogenous activation of PI3K/Akt signalling may lead to depletion of the haemolymph by increasing the cellular uptake of nutrients [18]. This would lead to increased hunger and cause the larvae to wander in search of food. In contrast, exogenous activation of dFOXO could alter the 'transcriptional profile' of the cell to be similar to that experienced under conditions of starvation. This altered transcriptional profile could contribute to larval wandering. Thus, dFOXO could induce larval wandering through expression of a sub-set of genes that are normally active during starvation, whereas exogenous activation of PI3K/Akt signalling could induce larval wandering by causing physiological changes that lead to a false sense of starvation.

1.2.5 - Conclusions

We have shown that dFOXO is conserved in sequence and regulatory mechanisms when compared to homologues from mammals and *C. elegans*. *Drosophila melanogaster* provides a powerful tool for the analysis of genes in a whole organism. Thus, future studies in this organism should provide new insights into the biological function of the FOXO transcription factors. This may have implications to the study of cancer and diseases related to insulin, such as diabetes and obesity. Our data, taken

together with that of others, suggests that dFOXO plays a protective role in the developmental response of Drosophila larvae to nutritional stress. Thus, it is possible that dFOXO plays a functional role in response to multiple forms of stress.

1.2.6 - Methods

Identification and sequence analysis of dFOXO.

The human *FOXO4* gene was used to search the National Center for Biotechnology Information genomic data bank for Drosophila homologues. Drosophila genomic sequences with high homology to *FOXO4* were identified and used to search the Berkeley Drosophila Genome Project for homologous cDNAs. This procedure allowed us to identify the clone, LD05569, which was sub-cloned and sent for sequencing to Cortec DNA Laboratories, Inc., Kingston, Ontario. Restriction mapping and sequencing revealed a cDNA of approximately 3.6 kb translating into a theoretical protein sequence of 463 amino acids (Fig 1B). Note that there are two other potential start codons that may act as sites for translation initiation, and are located slightly upstream of the start site we have identified.

Creation of transgenic Drosophila lines and overexpression studies.

mFoxo1, and *mFoxo1-T24A/S253A* (AA) clones were generously provided by Dr. William H. Biggs III [45] and the *dFOXO* cDNA, LD05569, was obtained from Research Genetics. These cDNA were ligated into the p[PUAST] expression vector for use of the UAS/Gal4 ectopic expression system [43]. Transgenic flies were created by injecting

p[PUAST]-FOXO constructs in to w^{1118} Drosophila embryos. Driver lines, *GMR-Gal4* [50], *heat shock-Gal4 (hsGal4)*[47], and *Act5C-Gal4 (ActGal4)* [44] were obtained from the Bloomington stock center, as were the UAS lines *UAS-dEGFR*, *UAS-dRas2^{V12}*, *UAS-E2F*, *UAS-Dp*, *UAS-p35*, *UAS-Diap1*, and *UAS-Diap2*. *UAS-dPI3K* and *UAS-dPI3K-DN* (*UAS-dp110^{D954A}*) were generously provided by Dr. Sally Leavers. Heat shock treatment was conducted in a 37°C water bath.

Phenotypic analysis.

All experiments were performed at 25°C unless otherwise stated. For scanning electron micrographs, flies were desiccated overnight and coated in gold. Ommatidia area was measured using NIHimage 6.2 and each value shown is the mean of 9 measurements, taken from 3 individual eyes. Due to the low survival rate of males expressing dFOXO, only females were included in the analysis of wings and body weight. Flies were raised under non-crowded conditions and a minimum of 12 flies were weighed individually to determine average body weight. Wing area was measured using ImageJ 1.28u, from the National Institute of Health. Cell size and cell number were calculated as previously described [70]. A minimum of 10 wings were analyzed per genotype. For all data, two-sided t-tests were performed to determine significant differences at $p=0.05$ and standard error of the mean is represented by error bars on graphs.

Feeding behavior and phenocopy of starvation using ActGal4.

The Gal4 driver line *w; ActGal4/CyO* was crossed to w^{1118}, *w; UAS-dFOXO/UAS-dFOXO*, *w,UAS- mFoxo1-AA/w, UAS-mFoxo1-AA*, and *yw; UAS-dPI3K-DN/UAS-dPI3K-DN* . Since the *ActGal4* insertion is not homozygous, we assumed that only half of the hatched larvae contained the insertion. This assumption was supported by observation of the adults arising from each cross. For *w; ActGal4/CyO* X w^{1118} the number of adults produced was nearly equal to the number of hatched embryos, with approximately half bearing the CyO balancer chromosome. For *w; ActGal4/CyO* X *w; UAS-dFOXO/UAS-dFOXO* and *w; ActGal4/CyO* X *UAS-mFoxo1-AA/w, UAS-mFoxo1-AA* only flies bearing the *CyO* chromosome survived and the number of adults was approximately half the number of the total hatched larvae. Small, wandering larvae were observed only for *w; ActGal4/CyO* X *w; UAS-dFOXO/UAS-dFOXO* and *w; ActGal4/CyO* X *UAS-mFoxo1-AA/w, UAS-mFoxo1-AA*, and in these crosses, only the larvae present in the food were growing. Thus, we assumed that small, wandering larvae were of the genotypes *w; ActGal4/+; UAS-dFOXO/+*, and *w, UAS-mFoxo1-AA/w; ActGal4/+*.

For the feeding behavior assay, embryos were collected on apple juice agar over ~2 hour time periods, counted, and transferred to a Petri dish with filter paper that was soaked in 20% sucrose in PBS. In the center of the Petri dish was a small piece of standard Drosophila food. At 48 hours AEL the number of hatched eggs was counted to account for unfertilized embryos. At both 48 hours and 72 hours AEL the number of larvae not on the food were counted. The percent wandering larvae was calculated based

on the number of larvae off the food, the number of hatched eggs, and the assumption that only half of the total larvae contained the *ActGal4* transgene. The results presented are the average from three separate trials. Statistical significance was determined using a two-sided t-test at p=0.05 and the standard error of the mean is represented by error bars on graphs. Individual values were taken from analysis of approximately 50 larvae.

1.2.7 - Authors' Contributions

JK conducted all genetic experiments and drafted the manuscript, as well as playing a partial role in sequence analysis of *dFOXO* and the creation of transgenic fly lines. JD was responsible for the sub-cloning and sequence analysis of *LD05569*. JL participated in the creation of transgenic fly lines. BES initiated investigation of the *dFOXO* gene, and created and initiated characterization of *UAS-dFOXO* transgenics, as well as acting as supervisor and primary investigator.

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1.2.9 - References

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Chapter 1.3

Drosophila FOXO is required for the early larval response to starvation and is regulated by nutrient availability.

A version of this chapter is submitted as:

Kramer, J.M., J.D. Slade, and B.E. Staveley. Drosophila FOXO is required during amino acid starvation and is regulated by nutrient availability.
BMC Dev. Biol.

1.3.1 - Abstract

The insulin signalling pathway is an evolutionarily conserved mechanism used by metazoan animals to regulate growth, metabolism, and behaviour in response to environmental cues. The FOXO transcription factors regulate cellular function in the absence of insulin signalling. Insulin levels are normally reduced during starvation, thus, FOXO may mediate the transcription of genes required for adaptive changes, such as altered metabolism and growth. We show that loss of FOXO in first and second instar Drosophila larvae causes starvation sensitivity. During starvation, we observed an increase in dFOXO-mediated transcription of a luciferase reporter gene. Transcription of the luciferase transgene was inhibited upon reintroduction of the larvae to food. FOXO was not required for the response of third instar larvae to starvation. However, loss of FOXO resulted in a decreased height of pupation, which may indicate a role for FOXO in larval wandering. In addition, dFOXO transcriptional activity increased dramatically during the wandering stage of the larval life cycle. Although FOXO is not required for development of Drosophila under laboratory conditions, it may be essential in nature where a consistent food source is not always available. This work provides the first evidence of starvation sensitivity due to loss of FOXO in Drosophila and highlights the importance of the insulin signalling pathway in the response of metazoan animals to fluctuations in nutrient availability.

1.3.2 - Introduction

The growth of metazoan animals is regulated by developmental signals and the availability of nutrients. The insulin/insulin-like growth factor (IGF) signalling (IIS) pathway is an evolutionarily conserved signalling system that regulates growth and metabolism in response to nutritional cues in mammals and model organisms such as *Caenorhaebditis elegans* and *Drosophila melanogaster* [1-3]. In mammals, circulating insulin levels are reduced in response to starvation and this leads to decreased IIS activity in the affected tissues, resulting in alterations in growth, metabolism, and feeding behaviour [3-5]. When *C. elegans* are raised under conditions of nutrient limitation, they enter an alternate developmental stage called the dauer larvae. The dauer stage is characterized by arrest of growth at a sexually immature stage along with altered metabolism to increase the storage of fat and increased food searching behaviour [6]. Genetic studies have linked the components of the IIS pathway in *C. elegans* to the formation of dauer larvae [7-11]. Drosophila larvae also undergo developmental alterations in response to nutritional signals that may be mediated by insulin-like peptides [12, 13]. Nearly 80 years ago, Beadle determined that starvation of young Drosophila larvae leads to developmental arrest that can last for several days before the larvae dies [14]. This developmental arrest can be relieved upon acquisition of food and normal development will resume [14-16]. In contrast, larvae that are starved in the latter stages of development survive to be adults, albeit reduced in size [14, 16]. This change in the developmental response to starvation occurs approximately 70 hours after hatching (AH), and has been coined the "70 hour change" [14]. There are seven Drosophila insulin-like

peptide (dILPs), all of which promote growth when overexpressed during larval development [17]. dILPs 2, 3, and 5 are produced in the median neurosecretory cells (m-NSCs) in the larval brain. Expression of dILPs 3 and 5 in these cells is dependent on the availability of nutrients [13]. m-NSC cells have direct connections to the circulatory system so that it is likely that levels of dILPs 3 and 5 are reduced during starvation and that this may cause reduced growth in developing Drosophila larvae. In support of this hypothesis, reducing dILP production by ablation of m-NSC cells in the larval brain leads to the generation of small adults [13]. Thus, it appears that the IIS pathway has an evolutionarily conserved role in nutrient sensing.

Insulin affects growth and other cellular activities by binding to receptor tyrosine kinases in the cell membrane. These receptors are activated through autophosphorylation of tyrosine residues within the intracellular domain of the receptor [18]. This leads to the recruitment of adaptor proteins from the insulin receptor substrate (IRS) family, that bind phosphotyrosine residues through their phosphotyrosine binding domain (PTB) [2, 19, 20]. IRSs provide a docking site for phosphoinositide 3'-kinase (PI3K), which phosphorylates inositol lipids on the inner surface of the cell membrane, converting phosphatidylinositol-4,5-bisphosphate (PIP_2) to phosphatidylinositol-3,4,5-trisphosphate (PIP_3) [21]. Generation of PIP_3 is negatively regulated by the tumor suppressor, PTEN (phosphatase and tensin homologue on chromosome 10), which removes a phosphate converting PIP_3 back to PIP_2 [22]. PIP_3 promotes signalling by binding to proteins that have a pleckstrin homology (PH) domain. The main downstream effector of PIP_3 is Akt (also called protein kinase B), a serine/threonine kinase that contains a PH domain and

becomes activated following localization to PIP_3 residues at the cell membrane. After activation, Akt moves into the cytoplasm and nucleus where it phosphorylates targets involved in cell growth, cell survival, and metabolism [23-26]. The primary targets of Akt include the forkhead box subgroup 'O' (FOXO) family of transcription factors [27-30]. Akt phosphorylates these transcription factors at three sites, which causes them to be sequestered in the cytoplasm. When IIS is diminished, Akt is down-regulated and FOXO factors are able to move into the nucleus and induce transcription of genes involved in cell death, cell cycle arrest, metabolism, and oxidative stress response [27-29]. These alterations in gene transcription may be critical to activate the cellular response to low levels of IIS activity.

Manipulation of IIS components in Drosophila has revealed phenotypes that bear a striking resemblance to the effects of starvation. Mutations that lead to the complete or partial loss-of-function of the Drosophila insulin receptor (*dInR*), the Drosophila insulin receptor substrate (*chico*), and Drosophila Akt (*dAkt*), lead to the generation of small adults [17, 31-33], a phenotype that is also typical when larvae are starved after the '70 hour change'. Inhibition of IIS during early larval development through overexpression of PTEN or dominant negative versions of PI3K leads to developmental arrest similar to that seen during starvation in first instar larvae [12]. Forced activation of IIS through overexpression of PI3K or dInr in young Drosophila larvae causes starvation sensitivity, suggesting that reduced IIS activity is required for maximal survival during starvation [12]. Drosophila FOXO (dFOXO) also causes developmental arrest when expressed at high levels in first instar larvae [34]. Low levels of expression of dFOXO during larval

development or overexpression of dFOXO at high levels during the early third instar (i.e. after the '70 hour change') leads to the formation of small adults [34]. Thus, the missexpression of dFOXO produces age-dependent phenotypes that copy the effects of starvation with respect to the "70 hour change". Furthermore, starvation increases the effects of dFOXO overexpression in the developing Drosophila eye, suggesting that nutrients can inhibit dFOXO activity [35]. These studies support a model in which reduced IIS activity leads to activation of dFOXO, resulting in alterations in gene transcription that lead to reduced growth and developmental arrest in nutrient-deprived larvae.

dFOXO overexpression does closely mimic the effects of starvation, however it is possible that these effects are caused by unusually high expression levels, and are not representative of naturally occurring processes. We set out to determine the true relevance of dFOXO in mediating the effect of low IIS during amino acid starvation by examining dFOXO loss-of-function mutants and by measuring endogenous dFOXO activity during starvation.

1.3.3 - Materials and Methods

Fly Stocks and Culture

FOXO^{21} and *FOXO*^{25} flies were obtained from Martin Junger and Ernst Hafen of the University of Zurich [35]. dFOXO mutants were balanced over the *TM6C, Tb* balancer chromosome containing the *Tubby (Tb)* marker to aid in selection of specific larval genotypes. To create dFOXO mutant heterozygotes and transhomozygotes, *w*;

dFOXO^{21}*/TM6C* and *w*; *dFOXO*^{25}*/TM6C* flies were crossed together, and to *w*; *dFOXO*$^{+}$*/dFOXO*$^{+}$ flies. The resulting larval progeny were selected for analysis based on the altered body shape resulting from the *Tb* marker. As it is difficult to distinguish between *Tb* and non-*Tb* larvae in the first instar, we waited until 48 hours after hatching (AH) before attempting to separate these genotypes.

FRE-Luc flies were created using the *8xFK1tkLuc* construct provided by William Biggs III [36]. The *8xFK1tkLuc* construct contains the firefly luciferase gene under the control of a herpes simplex virus thymidine kinase minimal promoter and eight direct repeats of a FOXO1 (also known as FKHR1) enhancer sequence, which is referred to here as the FOXO recognition element (FRE). This construct was cloned into the *pP:{CaSpeR-1}* transformation vector and injected into the developing germ line of *white*$^{-/-}$ embryos. Transgenic flies were identified based on red eye colour and a stable insertion was isolated on the second chromosome.

Phenotypic analysis

All experiments were performed at approximately 25°C. Average body weight was determined by weighing individual flies. Developmental delay and pupation height were determined from multiple vials, containing 20 larvae, which were collected at the age of 48 hours (AH). Pupation height was measured from the top of the food source, which was approximately equal in each vial. Each vial was considered an experimental unit for the determination of the standard error of the mean, which is represented by error bars on graphs.

Starvation Assay

Flies were allowed to lay eggs on apple juice agar overnight. In the morning, plates were collected and hatched larvae were removed. Within the next six hours newly hatched larvae were transferred to 10 cm Petri dishes containing standard Drosophila media (cornmeal/molasses/yeast extract/agar). A maximum of 150 larvae were kept per plate to avoid crowded conditions. Since all larvae collected are hatched within a six hour time frame the age after hatching is calculated from the mid-point of the six hour time frame ± three hours. At the appropriate age, larvae were rinsed with a sucrose solution and placed into 5-6 mL of 20% sucrose in phosphate buffered saline (pH 7.4) in a 6 cm Petri dish as described by Britton *et al.* [15]. 30-40 larvae were placed in each dish and the media was changed every one to two days. Each dish was considered an experimental unit for the determination of the standard error of the mean, which is represented by error bars on graphs.

Luciferase Assay

Luciferase assays were done using the Luciferase Assay System produced by the Promega Corporation. Larvae were collected in samples of ten and frozen in a -70°C ethanol bath. Protein extracts were performed according to the Promega Luciferase Assay System manual. Frozen larvae were thawed in 100 µL, or 200 µL for larvae older than 72 hours AH, of Promega 'cell culture lysis reagent' (CCLR) and ground with a pestle. Samples were frozen and thawed 3 times in a -70°C ethanol bath and a 37°C water bath and then centrifuged for 5-10 min. The supernatant was transferred to a new

tube and the pellet was ground again in an equal volume of CCLR. The sample was then recentrifuged and the resulting supernatant was combined with the first supernatant to be stored at -70°C.

The total protein concentration of extracts was determined using the Biorad D_C Protein Assay Kit that is based on the Lowry assay [37]. Absorbance was read at 750 nm using a Spectramax 190 microplate reader (Molecular Devices) and protein concentration was estimated in µg/mL from absorbance readings using a standard curve derived from known concentrations of BSA dissolved in CCLR. Protein extracts with absorbance readings that were higher than those within the range of the standard curve were diluted 10-fold in order to accurately estimate protein concentration. Luciferase activity of larval protein extracts was measured using a Top Count NXT microplate scintillation and luminescence counter. 100 µL of Promega Luciferase Assay Reagent was added to 20 µL of larval extract and light production was measured in relative light units (RLU) emitted over a 10 second time period. Final values on all graphs are normalized to the protein concentration and presented as RLU/µg of protein, with the standard error of the mean represented by error bars.

1.3.4 - Results

dFOXO loss-of-function mutants are developmentally delayed and sensitive to amino acid starvation.

Previously, we observed that overexpression of dFOXO in young Drosophila larvae results in a phenotype that is similar to that observed in larvae undergoing

starvation [34]. This suggests that dFOXO may play a role in the response of Drosophila larvae to amino acid starvation. As overexpression of dFOXO may not reflect a physiological response we examined the phenotypes associated with loss of *dFOXO* activity. $dFOXO^{21/25}$ transhomozygous mutants were analysed to avoid the effects of any possible second site mutations that may have arisen during the EMS mutagenesis procedure used in the creation of the $dFOXO^{21}$ and $dFOXO^{25}$ alleles [35]. $dFOXO^{21/25}$ mutants survive to adulthood, but show defects in growth control and are sensitive to oxidative stress [35]. In addition, $dFOXO^{21/25}$ mutants are developmentally delayed by approximately one day when compared to $dFOXO^{21/+}$, $dFOXO^{25/+}$, and $dFOXO^{+/+}$ controls (Figure 1A).

To determine the importance of $dFOXO^{21/25}$ during the larval response to starvation, we monitored the survival of *dFOXO* mutants under conditions of amino acid starvation. $dFOXO^{21/25}$ larvae are sensitive to amino acid starvation when compared to *dFOXO* heterozygotes and $dFOXO^{+/+}$ control larvae (Figure 1B). $dFOXO^{25/+}$, $dFOXO^{21/+}$, and $dFOXO^{+/+}$ larvae all had comparable survival curves during amino acid starvation and reached 50% mortality between days 7 and 8 when starved at the age of 48 hours AH. In contrast, $dFOXO^{21/25}$ mutants reached 50% mortality at approximately three days under the same conditions. Due to the apparent developmental delay observed with these mutants, we were concerned that the difference in survival may be due to differences in developmental stage rather than the loss of *dFOXO*. To test this, we examined $dFOXO^{+/+}$ control larvae that were transferred to starvation medium at 24 hours AH and found that they reached 50% mortality at approximately 5 days of

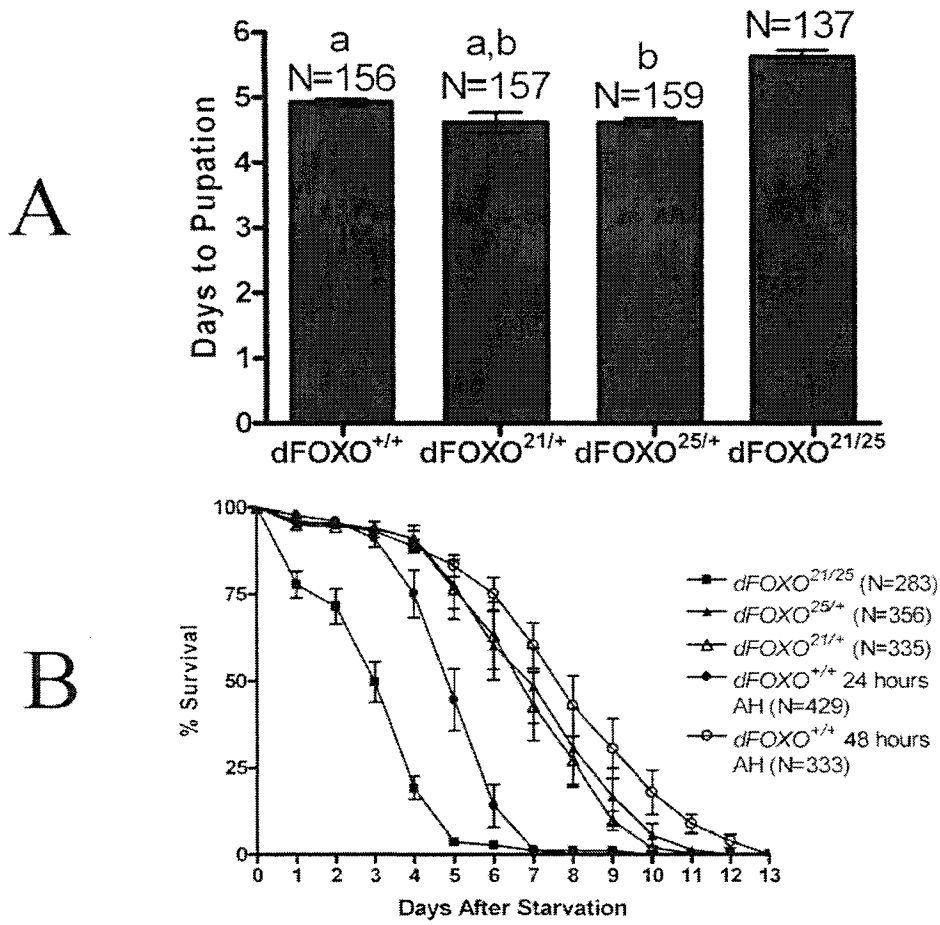

Figure 1: dFOXO mutant larvae are developmentally delayed and show sensitivity to amino acid starvation. A) The number of days for larvae to reach pupation was determined from the time of larval hatching for the genotypes: *w*; $dFOXO^{+/+}$, *w*; $dFOXO^{21/+}$, *w*; $dFOXO^{25/+}$, and *w*; $dFOXO^{21/25}$. $dFOXO^{21/25}$ mutant larvae take significantly longer (about 1 day) to reach pupation than do *dFOXO* heterozygotes and $dFOXO^{+/+}$ larvae. Common letters indicate a lack of significant difference as judged by a t-test (p=0.05) B) Larvae of the genotypes *w*; $dFOXO^{+/+}$, *w*; $dFOXO^{21/+}$, *w*; $dFOXO^{25/+}$, *w*; $dFOXO^{21/25}$ were collected at 48 hours AH and starved of amino acids in a 20% sucrose solution. $dFOXO^{21/25}$ mutants (squares) died earlier than the $dFOXO^{21/+}$ larvae (open triangles), $dFOXO^{25/+}$ larvae (closed triangles), and the 48 hour old $dFOXO^{+/+}$ controls (open circles). To account for differences in developmental time $dFOXO^{+/+}$ larvae were collected and starved at 24 hours AH (closed circles). 24 hour old larvae die sooner than the 48 hour old controls, but lived longer than the $dFOXO^{21/25}$ mutant larvae.

amino acid deprivation (Figure 1B). The 24 hour AH $dFOXO^{+/+}$ control larvae appeared to be approximately the same size as the 48 hour AH $dFOXO^{21/25}$ larvae, to indicate that these two groups were likely at a similar stage of development. The 24 hour AH $dFOXO^{+/+}$ larvae did have a reduced length of survival during amino acid starvation, compared to the older control larvae, yet they lived approximately 40% longer than the $dFOXO^{21/25}$ mutants (Figure 1B). This data suggests that dFOXO plays an important role in the ability of young Drosophila larvae to withstand the stress of nutritional deprivation.

dFOXO transcriptional activity is increased during starvation.

The sensitivity of $dFOXO^{21/25}$ mutants to amino acid starvation suggests that dFOXO is required to mediate transcription of genes that promote survival during starvation. To monitor dFOXO-mediated transcription, we used a FOXO recognition element (FRE) in conjunction with the firefly *luciferase* gene as a detectable marker [36]. To verify that dFOXO is involved in mediating transcription from the *FRE-Luc* transgene we tested luciferase activity in protein extracts of $dFOXO^{21/25}$ larvae (Figure 2D). Protein extracts from $dFOXO^{21/25}$ larvae had reduced luciferase activity in response to 24 hours of amino acid starvation when compared to $dFOXO^{+/+}$ controls (24 and 48 hours AH) and the $dFOXO^{+/-}$ heterozygotes (Figure 2D). These differences were statistically significant ($p=0.0007$, 0.0260, 0.0055) except for $dFOXO^{25/+}$ ($p=0.080$), which had a similar mean to the other controls but showed high variability. The residual luciferase activity seen in the absence of dFOXO may be due to maternally inherited dFOXO or to other factors that

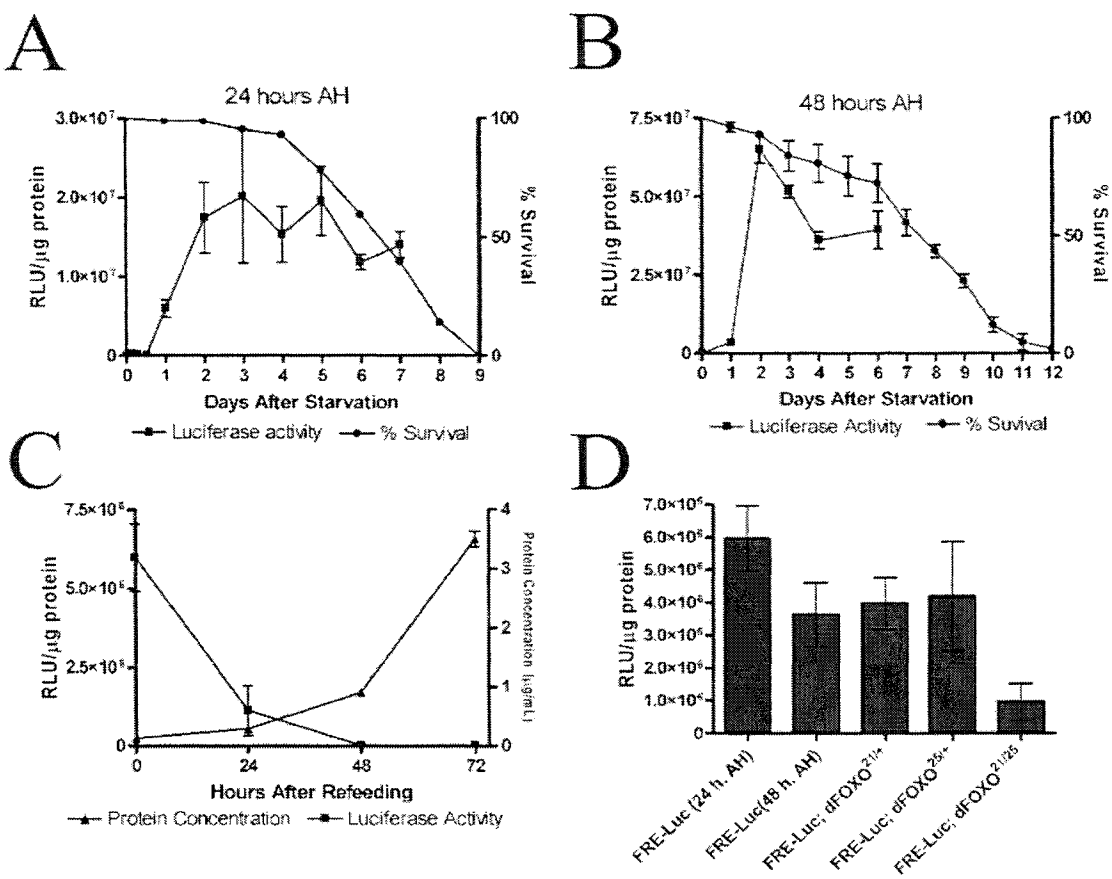

Figure 2: dFOXO transcriptional activity is controlled by amino acid availability. *FRE-Luc* larvae (genotype: *w*; *FRE-Luc/FRE-Luc*) were subjected to amino acid starvation at (A) 24 and (B) 48 hours AH. (A-B) These larvae survived for several days (circles-right axis) and were analysed for luciferase activity on a daily basis during this time. Luciferase activity increased dramatically in response to amino acid withdrawal (squares-left axis). (C) Luciferase activity is decreased after the return of starving larvae to food (squares-left axis). Growth resumed after the placement on food as indicated by an increase in protein concentration in larval extracts (triangles-right axis). Luciferase activity decreased significantly by 24 hours after refeeding and was completely abolished within 48 hours. (D) *dFOXO* is required for the full response of the *FRE-Luc* transgene to starvation. Larvae of genotypes, *w*; *FRE-Luc*; $dFOXO^{-/-}$, *w*; *FRE-Luc*; $dFOXO^{21/+}$, *w*; *FRE-Luc*; $dFOXO^{25/+}$, and *w*; *FRE-Luc*; $dFOXO^{21/25}$ were starved for 24 hours. $dFOXO^{21/25}$ larvae had significantly less luciferase activity than $dFOXO^{21/+}$ larvae (p=0.0055), and $dFOXO^{-/-}$ larvae that were starved at 24 hours AH (p=0.0007) and 48 hours AH (p=0.026). $dFOXO^{25/+}$ larvae did show higher luciferase activity than the $dFOXO^{21/25}$ mutants, however, this difference was not statistically significant (p=0.080).

can influence gene expression from the FRE in response to amino acid withdrawal. However, *dFOXO* is clearly required for the full transcriptional activation of the *FRE-Luc* construct, as luciferase activity is 75-85% greater in the presence of two copies of the intact *dFOXO* gene.

FRE-Luc larvae were subjected to amino acid starvation at 24 hours AH (Figure 2A) and 48 hours AH (Figure 2B), and reached 50% survival at approximately 6 and 7 days AH respectively, which is comparable to controls examined above. During amino acid starvation, luciferase activity increased dramatically in protein extracts of *FRE-Luc* larvae (Figure 2A,B). Significant increases were seen as early as one day post amino acid withdrawal (Figure 2A,B,D) and appeared to plateau after 2 days (Figure 2A,B). This suggests that dFOXO actively promotes transcription during amino acid starvation.

The developmental arrest of Drosophila larvae in response to starvation is reversible upon acquisition of a suitable food source [14, 16]. Correspondingly, the observed increase in dFOXO activity upon amino acid withdrawal is also reversible (Figure 2C). Larvae were fed for 24 hours, and then transferred to starvation media for two days before being returned to food. Within 2 days of the return to complete medium, larval growth resumed, and is reflected by an increase in the protein concentration of larval extracts (Figure 2C). During this period of growth initiation, luciferase activity dropped dramatically, to reach basal levels within two days (Figure 2C). The responsiveness of the *FRE-Luc* transgene to the availability of amino acids suggests that dFOXO transcriptional activity is modulated in response to nutritional cues.

dFOXO does not affect the survival of larvae starved after the '70 hour change'.

We demonstrated that forced expression of transgenic *dFOXO* after the '70 hour change' does not cause developmental arrest but leads to the generation of small adults [34]. This phenotype is similar to that observed upon starvation of larvae after 70 hours of development (Figure 3A) [16] and through loss of IIS components such as *chico*, *dAkt*, and *dInR* [31-33]. *FRE-Luc* flies that were starved at 80 hours AH showed a significant reduction in size when compared to controls that were not starved (Figure 3A) and showed a large increase in luciferase activity in protein extracts taken 24 hours after the onset of starvation (Figure 3C). This suggests that dFOXO is active during starvation in the later stages of larval development, however, $dFOXO^{21/25}$ mutants do not appear to be sensitive to starvation at this age (Figure 3B). Between 80% and 90% of control larvae ($dFOXO^{+/+}$, *FRE-Luc*; $dFOXO^{+/+}$, $dFOXO^{21/+}$, $dFOXO^{25/+}$) undergo pupation after amino acid starvation at 80 hours AH (Figure 3B). Only 40% of $dFOXO^{21/25}$ larvae reached pupation when starved at 80 hours AH, however these larvae were smaller than the controls due to developmental delay. At 96 hours AH, pupation of $dFOXO^{21/25}$ larvae reached 94%, suggesting that the reduced pupation at 80 hours AH is indeed a result of developmental delay. Although dFOXO appears to be activated in response to starvation after the '70 hour change' (Figure 2C), it is not required for the adaptive responses observed in larvae at this age (Figure 3B).

Larval crowding affects adult size but not dFOXO activity.

Low expression levels of dFOXO during larval development lead to reduction in

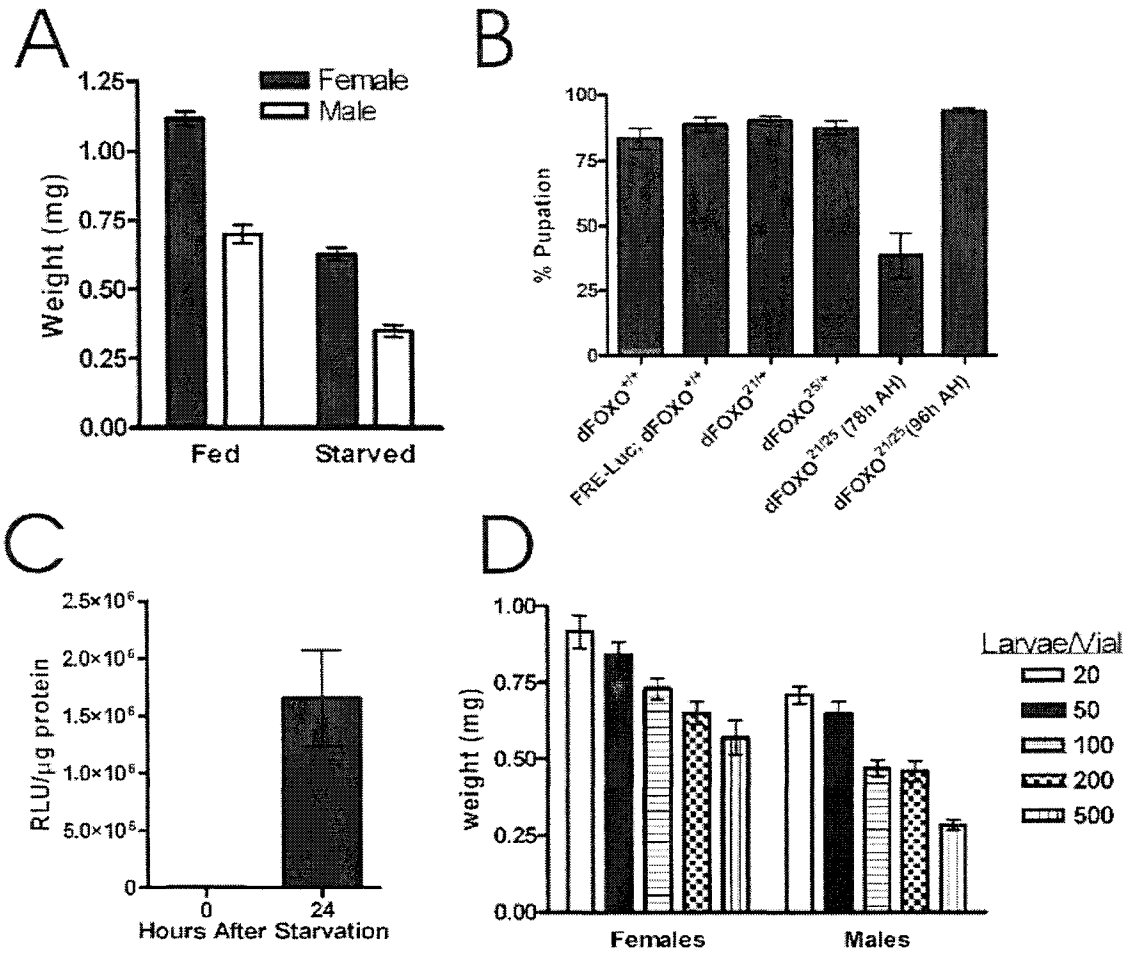

Figure 3: dFOXO is active during amino acid starvation after the 70 hour change, but is not required for maximal survival. (A) The weight of male and female *w*; *FRE-Luc* adults was measured for fed larvae and larvae that were starved at 80 hours AH. (B) The percentage of larvae undergoing pupation after starvation at 80 hours AH was measured for the genotypes, *w*; $dFOXO^{-/-}$, *w*; *FRE-Luc*; $dFOXO^{-/-}$, *w*; $dFOXO^{21/+}$, *w*; $dFOXO^{25/+}$, and *w*; $dFOXO^{21/25}$. $dFOXO^{21/25}$ larvae showed reduced pupation at 80 hours AH, but not at 96 hour AH, suggesting that the reduction at 80 hours AH is due to developmental delay. (C) Luciferase activity was calculated in protein extracts from *w*; *FRE-Luc* larvae immediately before the onset of starvation (0 hours) and 24 hours after initiation of starvation at 80 hours AH. (D) Body weight of male and female *w*; *FRE-Luc* adults was determined from larvae that had been raised under variable conditions of crowding with 20 (clear), 50 (black), 100 (horizontal lines), 200 (checkers), and 500 (vertical lines) larvae per vial. Body weight is reduced as the number of larvae per vial increases.

the size of adults [34]. We speculated that this low level of expression may reflect a situation such as larval crowding, which also causes the generation of small adults (Figure 3D). We found that *FRE-Luc* flies were significantly reduced in size when the number of larvae per vial was increased from 20 to 100, 200, and 500 (Figure 3D). However, we did not notice an increase in luciferase activity in larvae that were crowded at 200 larvae per vial (data not shown) suggesting that other factors may be responsible for the reduction in larval growth during crowded conditions.

dFOXO activity is increased in the wandering third instar larvae and may influence larval wandering.

We examined the luciferase activity in protein extracts from fed *FRE-Luc* larvae over the course of larval development and noticed that there was a dramatic increase in dFOXO activity during the wandering stage of the third instar larvae, at 102 hours AH (Figure 4A). We examined luciferase activity in protein extracts from wandering $dFOXO^{21/25}$ mutant larvae and found that it was significantly reduced when compared to $dFOXO^{+/+}$ larvae (Figure 4B). Due to the variability in development time in these lines, larvae were selected for comparison based on advancement into the wandering stage rather than selection at 102 hours AH. Thus, it appears that dFOXO is required for the full activation of the *FRE-Luc* transgene in the wandering third instar larvae. In addition, $dFOXO^{21/25}$ mutants had a lower height of pupation than $dFOXO^{+/+}$ larvae (Figure 4C), suggesting that dFOXO may influence wandering behaviour during the late third instar.

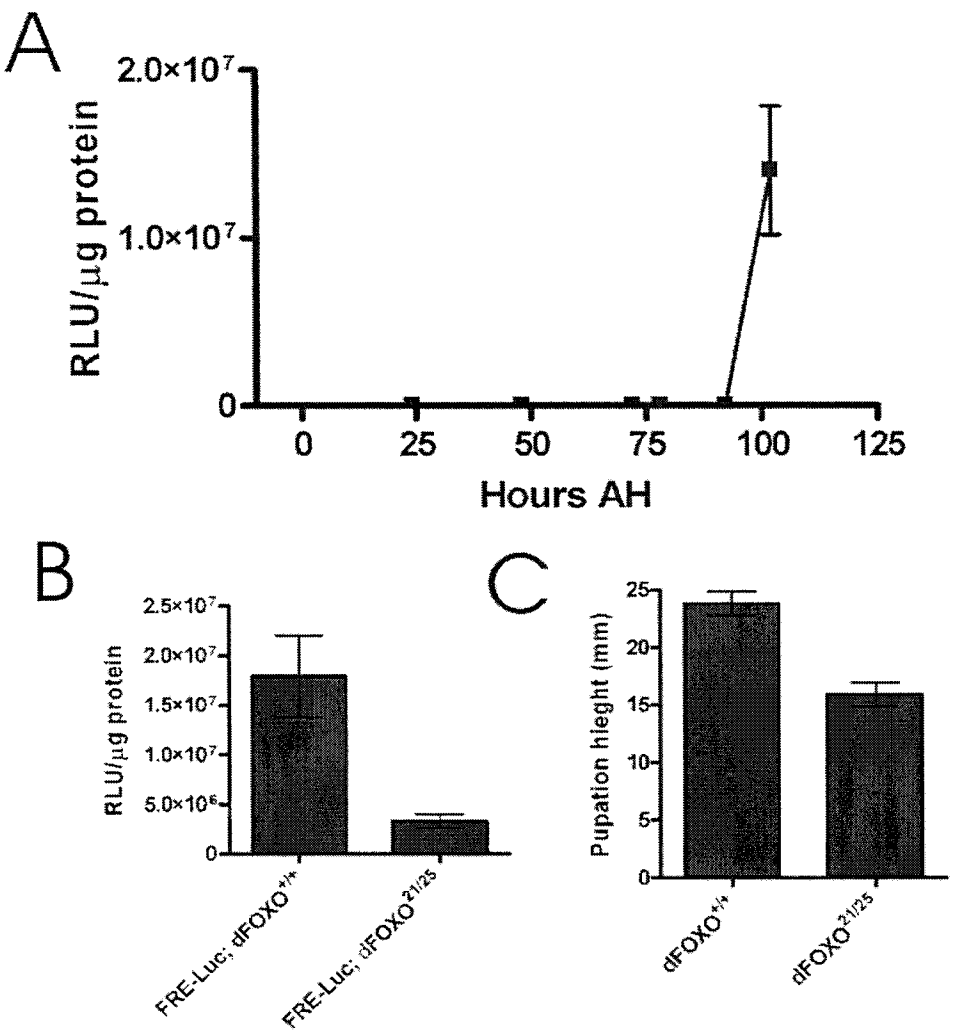

Figure 4: dFOXO is active during larval wandering and may effect wandering behaviour. (A) Luciferase activity was determined in *w*; *FRE-Luc* larvae at 24, 48, 72, 78, 96 and 102 hours AH. Luciferase activity remained at the basal level until 102 hour AH during the onset of larval wandering. (B) Luciferase activity was measure in wandering stage larvae of genotypes, *w*; *FRE-Luc* and *w*; *FRE-Luc*; $dFOXO^{21/25}$. The luciferase activity observed during wandering was reduced in a $dFOXO^{21/25}$ mutant background when compared to *w*; *FRE-Luc*; $dFOXO^{+/+}$ controls. (C) Pupation height was measured as the distance traveled from the top of the food in vials containing 20 larvae of the genotypes *w*; $dFOXO^{+/+}$, and *w*; $dFOXO^{21/25}$. $dFOXO^{21/25}$ larvae climbed significantly less than the controls larvae ($p<0.0001$).

1.3.5 Discussion

The growth of metazoan animals during development is controlled by an array of signalling events that are influenced by cellular and environmental factors. The availability of nutrients is one of the key factors influencing growth, yet we are only beginning to unravel the signalling mechanisms involved in the relay of dietary information to individual cells. Our work in Drosophila has revealed a FOXO-mediated transcriptional response to starvation that suggests an evolutionarily conserved role for the IIS pathway in the relay of nutritional signals.

The role of dFOXO in the response of young Drosophila larvae to starvation.

We have shown that *dFOXO* is required for maximal survival and enhances transcription through FOXO recognition elements during periods of starvation in first and second instar larvae (Figures 1, 2). There are several mechanisms through which dFOXO-mediated transcription could enhance the survival of starving larvae. One possibility is that dFOXO induces cell cycle arrest in larval endoreplicative tissues, thus allowing stored nutrients to be used to favor the maintenance of adult forming tissues such as the central nervous system and the imaginal discs. Several lines of evidence support this. First, forced activation of insulin signalling in starved larvae bypasses the cell cycle arrest that is normally observed and leads to starvation sensitivity similar to that seen in *dFOXO* mutants [12, 15] (Figure 1B). Second, overexpression of dFOXO in first instar larvae causes developmental arrest that is very similar to that observed in starved Drosophila larvae [34]. Third, mammalian cell culture studies have suggested a

role for the FOXO homologues in transcription of genes involved in cell cycle arrest. Expression of mammalian FOXO in cultivated cells causes G1 cell cycle arrest by inducing expression of *$p27^{KIP1}$* [38-41], and possibly by promoting the stability of $p27^{KIP1}$ post-translationally [42]. Mammalian FOXO homologues have also been implicated in the expression of *p130*, thought to contribute to cell senescence [43], and *cyclin G2*, a marker for G_0 that inhibits cell cycle entry [44]. In addition, FOXO3a can promote G2 cell cycle arrest through transcription of *GADD45* in response to oxidative stress [45]. FOXO also may inhibit the cell cycle by down-regulating the expression of *cyclins D1* and *D2*, both required for cell cycle progression [46, 47]. dFOXO has not been directly implicated in the control of cell cycle in Drosophila, however, it is highly conserved in sequence and has been implicated in growth reduction [34, 35, 48]. Thus, dFOXO may reduce growth by increasing transcription of cell cycle inhibitors in Drosophila, such as the *$p27^{KIP1}$* homologue, *dacapo* [49].

dFOXO is activated during starvation after the '70 hour change' but is not required for survival.

We found that *dFOXO* is not required for the survival of larvae that are starved after the '70 hour change', despite an increase in transcription from the FRE promoter (Figure 3). Our previous studies showed that overexpression of dFOXO reduced the growth of larvae after the '70 hour change' leading to the formation of small adults [34]. While the phenotype resulting from dFOXO overexpression in these experiments does

highlight a role for dFOXO in the reduction of growth, our data suggests that this is not the mechanism used for starvation-induced reductions in adult size (Figure 3).

Why is *dFOXO* required for starvation resistance before, and not after the '70 hour change'? The answer may lie in the tissue specific modulation of signalling pathways in larval endoreplicative tissues, which do not form adult structures, and larval imaginal tissues, which eventually develop into adult structures (Figure 5). The larval fat body is thought to be a nutrient sensing organ in Drosophila larvae [15, 50]. In response to amino acid uptake, the larval fat body produces ALS (acid labile subunit), which enters into circulation and may act to stabilize dILPs and increase IIS activation in peripheral tissues [50]. Both starvation and inhibition of amino acid uptake in the fat body cause a decrease in expression of *ALS*. This leads to inhibition of IIS activity in larval tissues, but not in the adult forming tissues, such as the imaginal discs or central nervous system (CNS) [50]. IIS signalling may be maintained in the adult forming tissues through nutrient-independent expression of dILP2, known to be expressed in the imaginal discs and the CNS [17]. The response of young Drosophila larvae to starvation requires inhibition of cell cycle progression in larval tissues, thus, the loss of *dFOXO* may lead to starvation sensitivity at this stage of development. In contrast, starvation of older larvae results primarily in a reduction in size of adults. Since dFOXO is likely inhibited in adult forming tissues during starvation, its absence would have minimal effect on the survival activity in crowded larvae (Figure 3D) and why dFOXO null mutants show no visible growth defects under normal conditions [35]. An alternative mechanism that may be involved in the regulation of adult size in response to starvation or crowding is the TOR

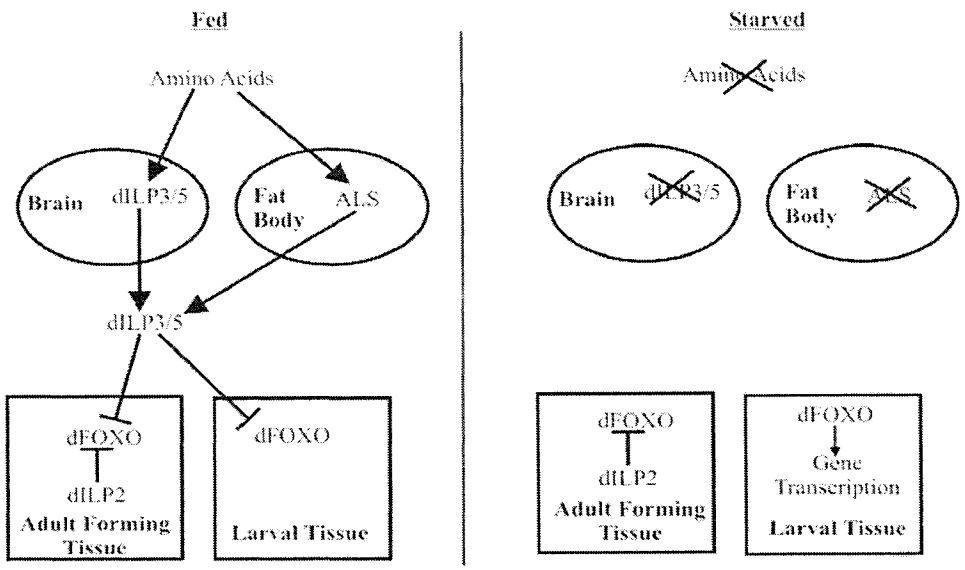

Figure 5: Schematic diagram showing tissue specific regulation of dFOXO. See text for details.

pathway. The TOR pathway is an evolutionarily conserved nutrient sensing system that is controlled by IIS and/or the availability of amino acids [51]. Thus, the IIS system may use multiple downstream targets, including dFOXO and TOR, to regulate tissue specific growth control in response to nutrient deprivation.

The role of dFOXO in larval wandering

We observed a large increase in dFOXO activity during the wandering stage of third instar larvae (Figure 4A,B). The survival of dFOXO mutants to adulthood suggests that dFOXO plays a minimal role during the process of wandering and pupation [35]. However, it is possible that dFOXO contributes to these processes in a minor or redundant manner, as $dFOXO^{21/25}$ mutants show a decrease in wandering distance whole

searching for a pupation site (Figure 4C). In addition, overexpression of dFOXO in first instar larvae causes them to wander away from their food, suggesting that a high level of *dFOXO* expression is sufficient to induce wandering [34]. Although dFOXO is not required for wandering, it may contribute to the ability or desire of larvae to wander by mediating alterations in metabolism or neuronal signalling. As FOXO family members have been implicated in the transcription of metabolic genes [52], the loss of dFOXO may result in inefficient metabolism and a decrease in the amount of energy available for wandering. Alternatively, dFOXO may become active in neuronal cells to produce direct effects upon behaviour in the CNS. The ability of dFOXO to promote wandering may result from an evolutionarily conserved mechanism allowing organisms to seek out alternate food sources in response to low nutrient intake.

Cell signalling pathways are important for the response of metazoan animals to environmental factors such as nutrient availability. Our data supports a model in which the IIS pathway is reduced during starvation leading to alterations in development and behaviour that are mediated in part by the FOXO family of transcription factors.

1.3.5 - Acknowledgments

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Chapter 1.4: Summary and Conclusions

The FOXO transcription factors are central regulators of cell function in the absence of insulin signalling. These factors interact with other transcription factors to regulate the expression of genes involved in growth, metabolism, longevity, oxidative stress resistance, and starvation response. The previous chapters, 1.2 and 1.3, describe the analysis of the FOXO family of transcription factors in *Drosophila melanogaster*. This work has established Drosophila as a suitable model for the analysis of FOXO family members and has provided novel insight into the role of dFOXO in the regulation of starvation response and larval wandering.

The identification of dFOXO using bioinformatics served as a starting point for this research, providing verification that dFOXO is the only homologue of FOXO in Drosophila, and that it retains the forkhead box DNA binding domain and Akt phosphorylation sites observed in mammalian homologues. To further analyze the function and regulation of these transcription factors in Drosophila, we have employed three main experimental strategies; overexpression, analysis of endogenous dFOXO activity, and loss of function analysis. Taken together, these experiments have demonstrated that dFOXO; 1) regulates growth through PI3K/Akt-dependent signalling, 2) is involved in the regulation of starvation response, and 3) promotes larval wandering. The characterization of dFOXO provides a basis for future research aimed at identification of the physiological mediators of FOXO function.

Overexpression of dFOXO and mFoxo1 in the developing eye of Drosophila produces a distinct phenotype that is characterized by a loss of ommatidia and mechanosensory bristles. This phenotype is rescued by coexpression of PI3K and Akt,

and exacerbated by coexpression of a 'dominant negative' mutant of PI3K. PI3K and Akt are unable to fully rescue the phenotype induced by overexpression of a mutant version of mFoxo1 that is constitutively active due to the alteration of its Akt phosphorylation sites by *in vitro* mutagenesis. These experiments verify that Drosophila FOXO is a downstream target of the PI3K/Akt signalling pathway and is inhibited upon phosphorylation by Akt.

There are two main adaptations of Drosophila larvae to starvation and the ubiquitous expression of dFOXO during larval development can mimic the phenotypic consequences of these adaptations. First and second instar larvae undergo developmental arrest during amino acid starvation that is reversible upon the acquisition of food. When third instar larvae are starved, they continue through the stages of development, but give rise to adults that are reduced in size. dFOXO overexpression in young larvae causes developmental arrest, while dFOXO overexpression in third instar larvae gives rise to adults that are reduced in size. Analysis of the transcriptional activity of a reporter gene directed by FOXO recognition elements revealed that dFOXO transactivation of gene expression is increased during amino acid starvation and is decreased when larvae are re-fed. Analysis of dFOXO loss of function demonstrated that dFOXO is required for the survival of larvae to starvation in the first instar, but not the third instar. Although dFOXO loss of function during the starvation of first instar larvae causes a 40 to 60 percent reduction in the duration of survival, the loss of dFOXO function in the third instar has no effect on survival. The effect of dFOXO loss-of-function on body size after starvation in the third instar has not been measured. This effect should be measured to

get a full understanding of the importance of dFOXO during starvation after the '70 hour change'.

Nutrient withdrawal is known to affect food searching behaviour in Drosophila. For instance, when Drosophila larvae deplete a food source, they tend to disperse. Interestingly, overexpression of dFOXO causes larvae to disperse when food is plentiful, to suggest that dFOXO induces larval wandering. Interestingly, endogenous dFOXO activity is increased dramatically during the wandering stage, near the conclusion of larval development. Loss of function of dFOXO does not eliminate larval wandering, but does result in reduction of the vertical distance traveled in search of site for pupation. Taken together, these results suggest that dFOXO expression can promote larval wandering, through an undetermined mechanism.

In conclusion, Chapters 1.2 and 1.3 contribute to the growing body of evidence demonstrating the evolutionary importance of the IIS signalling pathway in the response of metazoan animals to fluctuating nutrient availability. These experiments provide a basis for future research examining genes that mediate the physiological functions of dFOXO. One candidate is 4EBP, a transcriptional target of dFOXO that mediates the effects of dFOXO in promoting resistance to oxidative stress [1]. It would be interesting to learn if 4EBP is important in mediating the effect of dFOXO on starvation resistance and growth. dFOXO has also been implicated in the extension of lifespan [2, 3] and can delay aging related defects of the heart [4]. Is 4EBP involved in all of the functions of dFOXO or are there other important targets? Most likely there are many important targets. Novel targets could be identified through large scale genetic screens looking for

modifiers of dFOXO phenotypes. A screen looking for modifiers of the dFOXO eye overexpression phenotype presented in Chapter 2.2 could reveal novel targets of dFOXO that are involved in important processes such as aging and growth and stress resistance. Now that dFOXO has been characterized in this thesis [5, 6] and in the work of others [2-4, 7-9] it is possible to use the true power of Drosophila genetics to advance knowledge of the cellular control of aging, stress resistance, and diseases such as obesity and diabetes.

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Chapter 2

Toxic Effects of Gal4 Expression in Drosophila

Chapter 2.1

Introduction and Overview

The UAS/Gal4 ectopic expression system is an important tool for the overexpression of genes in *Drosophila melanogaster* [1, 2] (Figure 1). This system utilizes the yeast transcription factor Gal4, which activates transcription of genes through an upstream activation sequence (UAS) in the promoter region of selected genes. Conveniently, the UAS consensus sequence (CGGAGTACTGTCCTCC) is not contained in the genome of *D. melanogaster* (Berkeley Drosophila Genome Project, personal communication). In theory, this allows for the expression of *Gal4* in flies with little effect. Since the introduction of the UAS/Gal4 system, many transgenic Drosophila strains, called 'driver lines', have been created that express *Gal4* under the control of Drosophila promoters. *Gal4* expression profiles have been characterized by reporter gene analysis and *in situ* hybridization, and in many cases, expression is regulated both spatially and temporally [1, 2]. In tandem, conditionally responsive transgenic flies called 'UAS lines' can be generated that contain specific sequences under the control of UAS containing promoters [2]. Thus, tissue specific overexpression of genes in Drosophila can be achieved by simply crossing 'driver lines' with 'UAS lines'. Currently, there are hundreds of different 'UAS lines' and Gal4 'driver lines' that are publicly available in the Bloomington Drosophila Stock center at Indiana University. The relative simplicity of the UAS/Gal4 system, combined with the free availability of pre-existing stocks, makes this a popular and powerful technique to employ for genetic experiments.

In Chapter 1.2 we made extensive use of the *GMR-Gal4* driver [3] line to express dFOXO and other signalling genes in the developing eye [4]. Since cell culture studies

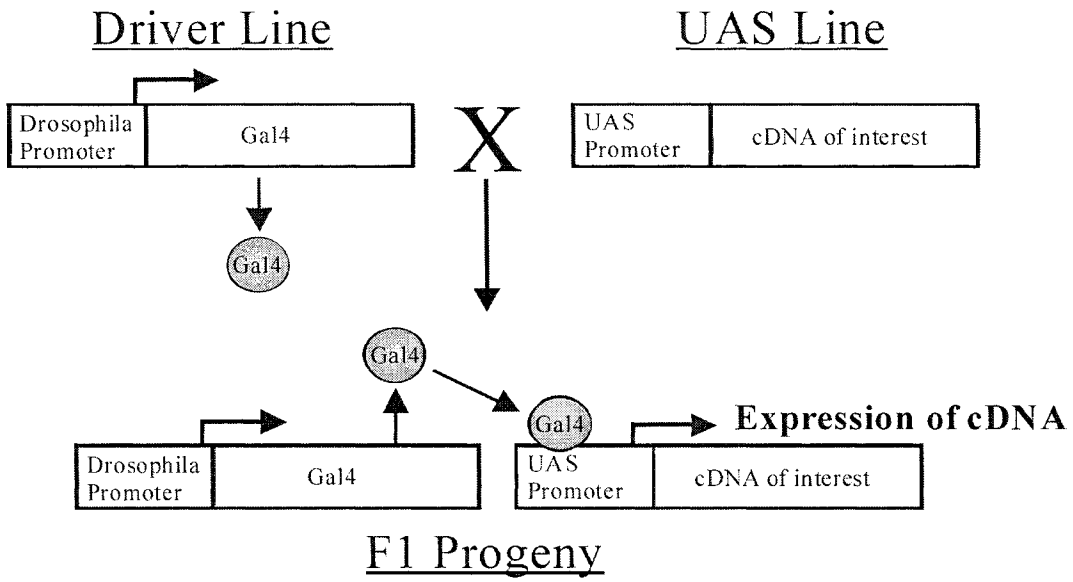

Figure 1: Schematic diagram of the UAS/Gal4 ectopic expression system. See text for details.

have implicated FOXO factors in the expression of genes involved in apoptosis [5-7], we searched for apoptosis in the eye imaginal discs of GMR-Gal4/UAS-dFOXO flies [4]. Upon examining the controls for these experiments, we noticed larvae with two copies of the *GMR-Gal4* transgene had a great increase in apoptosis in the region of the imaginal disc posterior to the morphogenic furrow, where Gal4 is expressed [3, 8]. This inspired us to do a more thorough analysis of the effects of *GMR-Gal4* on eye development, revealing temperature-sensitive effects of Gal4 on the formation of a rough eye phenotype and increased apoptosis in the eye imaginal discs (Chapter 2.2) [8]. In addition, we have shown that the rough eye phenotype and apoptosis that are induced by Gal4 can be suppressed by p35, an inhibitor of caspase mediated apoptosis (Chapter 2.3) [9]. As a result, we conclude that Gal4 acts as a toxic protein with the capability to activate the cellular apoptotic machinery when expressed at high levels.

As of September 2005, Flybase lists 238 references that utilize the *GMR-Gal4* line, yet our report appears to be unique in our attempt to address the potential problems with this line. The activation of cell death in GMR-Gal4 flies indicates that Gal4 could cause apoptosis in other 'driver lines'. Experiments in our lab have shown that Gal4 has detrimental effects on lifespan when expressed in the dopaminergic neurons with or without a responsive gene target [10]. Reduced lifespan in these flies may be a result of Gal4-induced neuronal cell death. Thus, the experiments presented in chapters 2.2 and 2.3 provide important information that should be taken into consideration during the planning and execution of any experiment using the Gal4 system.

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Chapter 2.2

GAL4 causes developmental defects and apoptosis when expressed in the developing eye of *Drosophila melanogaster.*

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2.2.1 - Abstract

The UAS/GAL4 ectopic expression system is widely used in *Drosophila melanogaster* for the overexpression of transgenes. This system operates under the assumption that the yeast transcription factor, GAL4, is inactive in Drosophila. Thus, GAL4 can be expressed using Drosophila-specific promoters with little effect on the organism. We have shown that expression of GAL4 in the developing eye using the *glass multiple reporter (GMR)* promoter element can cause defects in eye development. Although flies with one copy of the *GMR-GAL4* transgene appear normal when raised at 25°C, those with two copies have a highly disorganized ommatidial array. In addition, the levels of apoptosis in the third instar eye imaginal disc (where GAL4 is being expressed) are slightly higher in *GMR-GAL4* heterozygotes, and much higher in *GMR-GAL4* homozygotes when compared to wild type discs. The morphological eye defects caused by *GMR-GAL4* are significantly enhanced when flies are raised at 29°C (presumably due to the higher activity of GAL4 at this temperature). Taken together, this data suggests that GAL4 can have adverse effects on Drosophila development, especially at high expression levels. In addition, GAL4 appears to induce apoptosis even in the absence of any visible morphological defects.

2.2.2 - Introduction

The UAS/GAL4 ectopic expression system [1, 2] has become a widely used and extremely valuable tool for the overexpression of transgenes in *Drosophila melanogaster*. This bipartite expression system utilizes the yeast transcription factor, GAL4, and its

target sequence, UAS (upstream activation sequence), to which GAL4 binds in order to activate gene transcription. GAL4 can be expressed in many different patterns by placing it under the control of various Drosophila promoter sequences. Since UAS promoter sequences (CGGAGTACTGTCCTCC) are not found in Drosophila (Berkeley Drosophila Genome Project, personal communication), it is assumed that GAL4 is inactive. This is very useful for the overexpression of transgenes from a UAS promoter because GAL4 will drive expression of the transgene while not otherwise affecting the cells.

Many overexpression studies in Drosophila have been carried out in the developing eye using the *glass multiple reporter (GMR)-GAL4* driver line [3]. Flybase currently lists 111 articles which have used the construct since its creation in 1996. The *GMR* promoter element causes high-level expression of Gal4 in the eye imaginal discs in cells posterior to the morphogenetic furrow. We have shown that *GMR-GAL4* causes developmental defects and increased apoptosis in the eye, thus contradicting the notion that GAL4 is inactive in Drosophila.

2.2.3 - Materials and Methods

Fly stocks and culture.

GMR-GAL4 flies [3] were obtained from the Bloomington Stock Center and w^{1118} was obtained from Dr. Howard Clipsheets. *GMR-GAL4* homozygous females were crossed to w^{1118} males and cultured on standard media at 25°C and 29°C. All fly stocks were transferred to a new vial every 2 days to avoid overcrowding.

Analysis of adult eyes and imaginal discs.

For scanning electron microscopy, 2 day old females were desiccated overnight and coated in gold before photography with a Hitachi S570 scanning electron microscope (SEM). Acridine orange was used to visualize apoptotic cells in the eye imaginal discs, as described [4]. Third instar larvae were dissected in phosphate buffered saline (PBS) at pH 7.4 and imaginal discs were incubated for 5 minutes in 5 μg/mL acridine orange solution. Discs were rinsed in PBS and photographed immediately using Nikon eclipse E600 fluorescent microscope.

2.2.4 - Results and Discussion

The development of the *Drosophila* eye is a complex, yet relatively well understood process [5]. As a result, the eye is an excellent model for the study of genes involved in the control of developmental processes such as cell proliferation, differentiation, and apoptosis [6]. The UAS/GAL4 ectopic expression system has made it possible to overexpress genes specifically in the developing eye and test the effects of overexpression on eye development.

The *GMR-GAL4* driver is a commonly used construct for the missexpression of transgenes in the developing eye. It has been reported that *GMR-GAL4* homozygotes have a rough eye phenotype that is visible under the dissecting microscope [3, 7-9]. Upon SEM analysis of flies with two copies of the *GMR-GAL4* transgene raised at 25°C, it was revealed that the ommatidial array was disrupted due to the presence of irregularly sized ommatidia (Fig. 1C). Flies with one copy of the *GMR-GAL4* transgene do not have an obvious phenotype at 25°C (Fig. 1B) when compared to a control (Fig. 1A), indicating

that the effects of GAL4 may be dependent upon the gene dosage. When flies were raised at 29°C the phenotypic effects of *GMR-GAL4* were enhanced (Fig. 1 G-I). At this temperature two copies of the *GMR-GAL4* transgene lead to the development of grossly deformed eyes (Fig. 1I) and one copy lead to slight abnormalities (Fig. 1H), whereas the control eyes still appeared normal (Fig. 1G). The increased phenotypic severity seen at 29°C may be due to the higher activity of GAL4 at this temperature [10]. Thus, we conclude that *GMR-GAL4* causes developmental abnormalities in a dose- and temperature-sensitive manner.

Acridine orange staining of the eye imaginal discs revealed high levels of apoptosis with two copies of the *GMR-GAL4* transgene (Fig. 1 F & L) and intermediate levels with one copy (Fig. 1 E & K), when compared to controls (Fig. 1 D & J). Apoptotic cells are brightly fluorescent and are seen primarily where GAL4 is being expressed, in the region of the eye imaginal disc that is posterior to the morphogenetic furrow. Unlike the phenotype seen in the adult eye, there was no apparent difference in the levels of apoptosis between flies cultured at 25°C and 29°C (Fig.1, compare D-F to J-L). Thus, induction of apoptosis by GAL4 appears to be dose sensitive, but not temperature sensitive, and can occur in the absence of a visible adult phenotype.

The induction of apoptosis by *GMR-GAL4* may be indirect; i.e. apoptosis occurs as a result of developmental confusion caused by high levels of GAL4 and not through direct regulation of genes involved in apoptosis. For example, the *Drosophila* nucleoporin, *members only*, is required for nuclear import of GAL4 [11], thus, it is possible that high levels of GAL4 may block the normal shuttling of molecules in and out

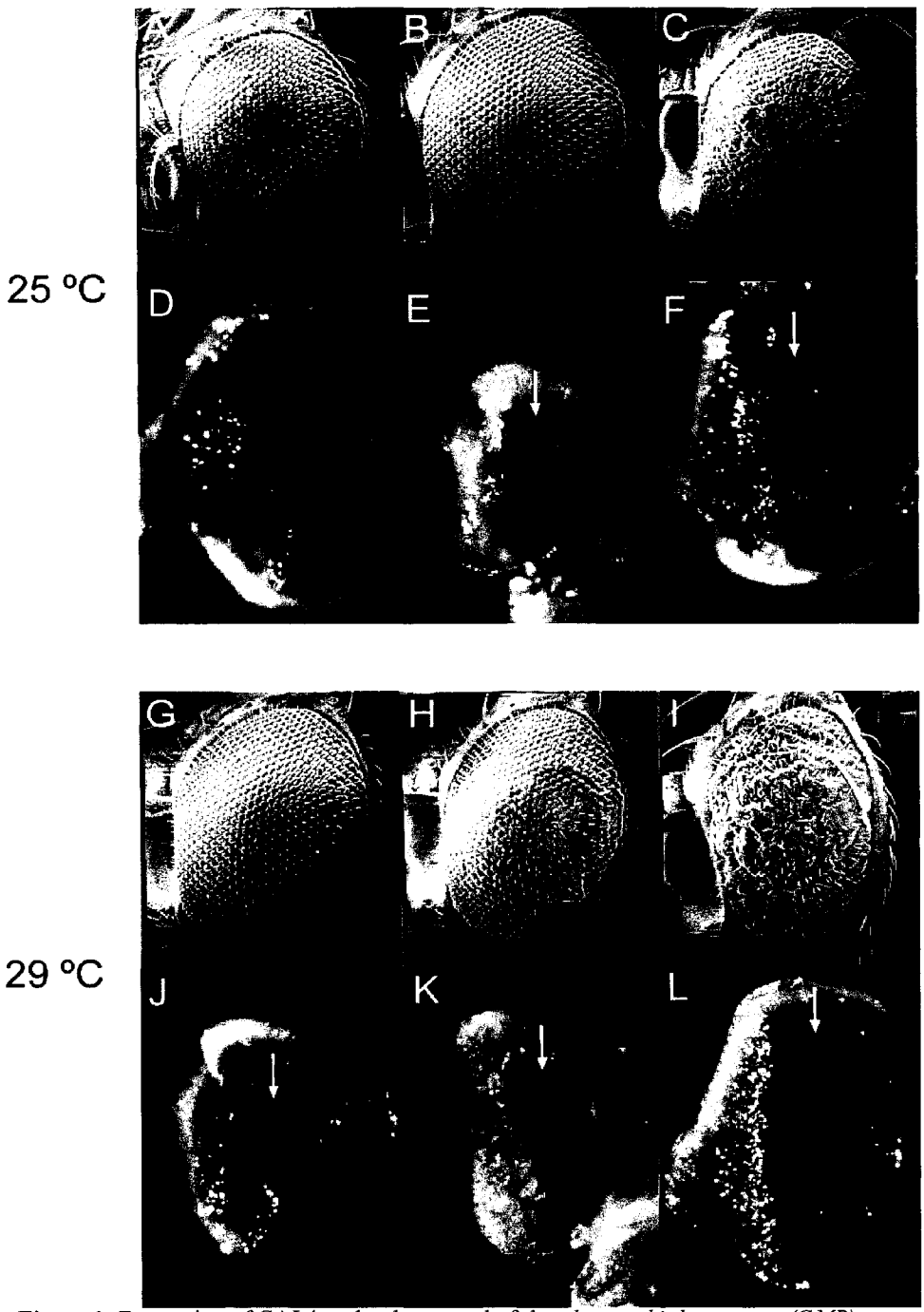

Figure 1: Expression of GAL4 under the control of the *glass multiple reporter (GMR)* promoter element causes developmental defects and apoptosis in the *Drosophila* eye. Scanning electron microscopy reveals the phenotypic effect of *GMR-GAL4* at 25 degrees celcius (A-C), and 29 degrees celcius (G-I). Acridine orange was used to stain apoptotic cells in the third instar eye imaginal discs from larvae raised at 25degrees celcius C (D-F) and 29 degrees celcius (J-L). Genotypes are w^{1118} (A, D, G, J), *w; GMR-GAL4/+* (B, E, H, K), and *w; GMR-GAL4/GMR-GAL4* (C, F, I, L). Arrows indicate the location of the morphogenetic furrow.

of the nucleus. However, the temperature sensitivity of the *GMR-GAL4* rough eye phenotype may indicate that GAL4 is causing direct transcriptional activation of certain genes. If the phenotypic enhancement at 29°C is due to the innate preference of GAL4 for this temperature, then GAL4 must be directly activating gene transcription in order to produce the phenotype. Alternatively, increased temperature may cause increased expression of GAL4 which could enhance the phenotype as well.

Regardless of the mechanism by which GAL4 disrupts eye development, it is apparent that there is an effect, and that apoptosis is increased. Thus, it is imperative that experiments be well controlled and that new GAL4 constructs are well characterized to avoid misinterpretation of results.

2.2.5 - Acknowledgments

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Chapter 2.3

Gal4-induced defects in *Drosophila melanogaster* are prevented by p35.

This data has been submitted as part of a larger manuscript entitled:

Haywood, A.F.M., Kramer, J.M., Sheppard, G.A., and B.E. Staveley, submitted. *parkin* suppresses Gal4-induced cell death.

2.3.1 - Introduction

The UAS/Gal4 ectopic expression system is an important tool for the study of developmental processes in *Drosophila melanogaster*. In the previous chapter it was shown that *Gal4* causes a rough eye phenotype in adult flies when expressed at high levels during eye development. The rough eye phenotype is characterized by a loss of ommatidial organization and is accompanied by a high level of apoptosis in the eye imaginal discs where *Gal4* is expressed. Here it is shown that these phenotypes are essentially eliminated through co-expression of the caspase inhibitor, *p35*. This suggests that Gal4 has the capability to act as a toxic protein with the ability to activate the cellular apoptotic machinery. SEM and acridine orange analysis are done according to the same methods presented in chapter 2.2.3.

2.3.2- Results and Discussion

The *GMR-Gal4* transgene expresses a high level of Gal4 in the eye imaginal discs in cells posterior to the morphogenetic furrow [5]. This expression causes obvious developmental defects in the adult eye (Figure 1B) when compared to control flies (Figure 1A) that do not contain the *GMR-Gal4* transgene. Flies that have two copies of the *GMR-Gal4* transgene also show a high level of apoptosis in the eye imaginal disc in the area posterior to the morphogenetic furrow (Figure 1F) [3]. In contrast, control flies show some, but much less, apoptosis in the eye imaginal disc (Figure 1E). This suggests that Gal4 induces apoptosis during development, which may cause the developmental defects seen in the adults.

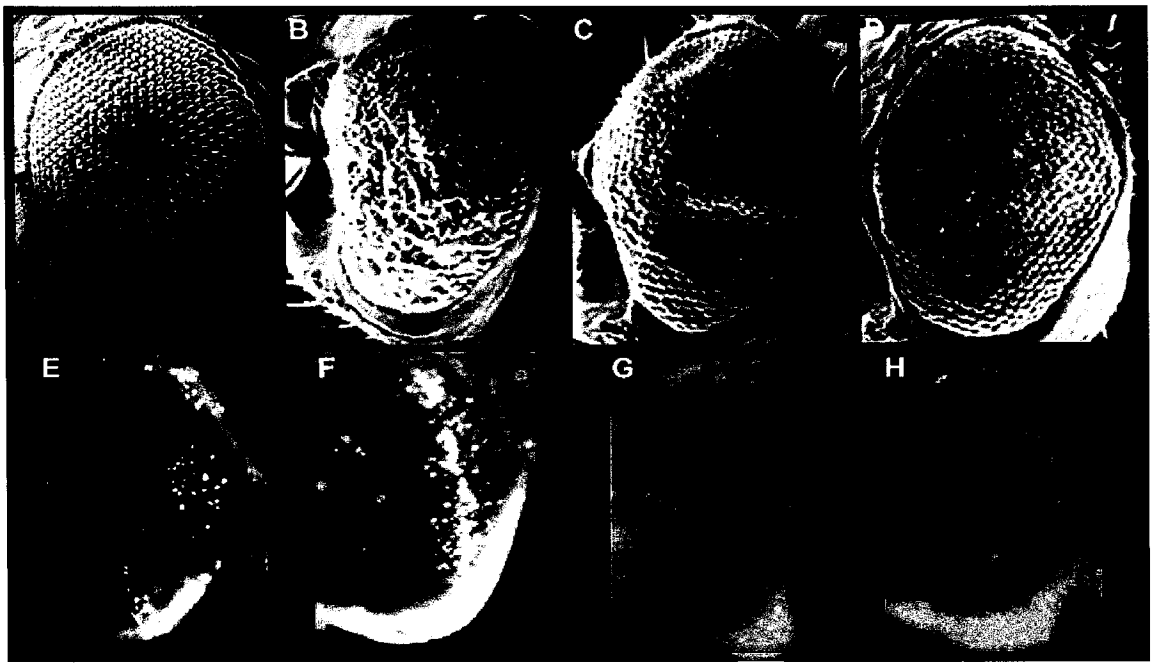

Figure 1: Expression of *p35* inhibits developmental defects and apoptosis in *GMR-Gal4* homozygotes. Scanning electron microscopy (A-D) reveals the rough eye phenotype seen in *GMR-Gal4* homozygotes (B) as compared to a control (A). The rough eye phenotype is inhibited in the presence of one (C) or two (D) copies of the *UAS-p35* transgene. Acridine orange staining (E-H) reveals apoptotic cells in the eye imaginal discs of third instar larvae. The amount of apoptosis is greater in *GMR-Gal4* homozygotes (F) than in control larvae (E). The presence of one (G) or two (H) copies of *UAS-p35* essentially eliminates all apoptosis from the eye imaginal discs. Genotypes are w^{1118} (A, E), *w;GMR-Gal4/GMR-Gal4* (B, F), *w;GMR-Gal4/GMR-Gal4;UAS-p35/TM6B* (C, G), and *w;GMR-Gal4/GMR-Gal4;UAS-p35/UAS-p35*.

To further examine this possibility, we co-expressed the caspase inhibitor, *p35*, along with *Gal4* in the eye imaginal discs. Flies that have two copies of the *GMR-Gal4* transgene and either one (Figure 1G) or two (Figure 1H) copies of the *UAS-p35* transgene show a nearly complete elimination of apoptosis during eye development. The disorganization of the ommatidial array observed with *GMR-Gal4* homozygotes (Figure 1B) is reduced in the presence of one (Figure 1C) and two (Figure 1D) copies of *UAS-p35*. The lack of complete suppression of the rough eye phenotype in the presence of p35 suggest that lack of apoptosis can also disrupt normal eye development, or that Gal4

effects eye development through mechanisms other than apoptosis. Nevertheless, these experiments suggests that Gal4 can cause apoptosis in the developing eye and that this can result in developmental defects.

How does Gal4 cause apoptosis? High levels of Gal4 may cause transcription of genes involved in apoptosis. It seems unlikely that Gal4 causes transcription of the apoptotic machinery as there are no UAS sequences (CGGAGTACTGTCCTCC) found in the *Drosophila melanogaster* genome (Berkeley Drosophila Genome Project, personal communication). Alternatively, Gal4 may act as a toxic protein that can activate an apoptotic cascade. The inhibition of Gal4-mediated apoptosis by the caspase inhibitor p35 (Figure 1) suggests that the toxic effect of Gal4 leads to activation of the cellular apoptotic machinery. This may occur through a similar mechanism used by toxic proteins that cause apoptosis in neurodegenerative diseases [10, 11]. These mechanisms are not well understood, however, further analysis of Gal4-induced cell death may reveal additional mediators of toxic protein induced apoptosis.

In summary, the UAS/Gal4 ectopic expression system can be an excellent tool, when used with the proper controls. However, caution should be used in the selection of promoters through which Gal4 is expressed, as high expression levels can cause caspase-mediated cell death.

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Chapter 3: Conclusions

This thesis describes the analysis of the FOXO family of transcription factors (Chapter 1) and the toxic effects of Gal4 expression (Chapter 2) in *Drosophila melanogaster*. In addition to establishing the existence of dFOXO, our analysis of the FOXO transcription factors has revealed their physiological importance in the adaptations of Drosophila larvae to starvation. Our analysis of Gal4 overexpression has revealed that Gal4 can cause apoptosis in the developing eye. The effects of Gal4 expression are primarily a technical concern. However, further analysis of Gal4-induced apoptosis could provide insight into the general mechanisms controlling toxic protein-induced apoptosis (TPIA).

Initial studies examining dFOXO loss of function in flies show that it is not required for normal development [1], thus raising questions regarding its evolutionary importance. Our studies suggest that dFOXO may provide an adaptive advantage in flies that is not observed under the optimal conditions experienced during laboratory culture, but may be essential for natural populations of Drosophila which are exposed to environmental challenges. Increased dFOXO activity allows larvae to survive nearly twice as long in the absence of food, and may contribute to the control of larval wandering (Chapter 1) [2, 3]. This increased survival time and wandering could significantly increase the chance for individuals to seek out food, and further their development to a reproductive age. Thus, the unnatural conditions of laboratory culture might mask the true importance of dFOXO in development and evolution.

Future Directions

The studies described in this thesis have revealed several novel phenotypes resulting from genetic manipulation of dFOXO [2, 3] and expression of Gal4 [4, 5]. These phenotypes provide a basis for future research that may increase our knowledge of human biology and provide a better understanding of human diseases, such as diabetes, cancer, and neurodegeneration. Indeed, Drosophila is an ideal organism in which to perform genetic screens aimed at identifying novel genes that participate in cellular processes and my contribute to disease symptoms observed in human ailments [6, 7].

Type 2 diabetes is characterised by defects in insulin secretion and/or insulin action [8]. These defects are influenced by environmental factors, such as diet, and genetic factors [8]. In mice, haploinsufficiency at the FOXO1 locus suppresses diabetes, suggesting that aberrant regulation of FOXO1 is partially responsible for the diabetic phenotype [9-11]. Thus, the molecular mechanisms causing this disease could be further clarified by the dissection of genetic components involved in insulin signalling and regulation of the FOXO transcription factors. In Drosophila, loss of insulin producing cells leads to a diabetic phenotype [12] and loss of dFOXO leads to a great sensitivity to starvation in young larvae (Chapter 1.3) [3]. By searching for loss or gain of function mutations that suppress this phenotype, new physiologically relevant targets of FOXO could be identified. Further analysis of these targets in Drosophila, mice, and humans, could provide insight into the molecular basis of diabetes. Thus, by truly understanding the mechanisms of the disease, we can begin to seek new methods of therapy.

Mutations in human FOXO homologues have been observed in patients with alveolar rhabdomyosarcoma [13, 14] and acute lymphocytic leukemia [15-18]. Cancer is a disease characterized by the loss of growth control in cells, thus, FOXO may play an important role in the suppression of cancer through its ability to induce cell cycle arrest [19]. Our experiments show that dFOXO can reduce growth when expressed in the developing eye of Drosophila (Chapter 2.2) [2]. Searching for mutations that suppress or enhance dFOXO-mediated growth in the eye could reveal physiological targets of FOXO involved in the regulation of growth. It is possible that the analysis of these targets could provide information about the molecular basis of cancer, and provide novel targets for chemotherapy and/or gene therapy.

Our analysis of Gal4 expression in the Drosophila eye suggests that Gal4 may act as a toxic protein to activate the cellular apoptotic machinery (Chapters 2.2 and 2.3) [4, 5]. It has been suggested that toxic protein-induced apoptosis (TPIA) may contribute to neurodegenerative disorders, including Parkinson's disease, Huntington's disease, and Alzheimer's disease [20]. It is possible that a genetic screen for mutants that modify the Gal4-induced rough-eye phenotype could reveal important mediators of TPIA. This would contribute to our knowledge of the molecular mechanisms responsible for TPIA, which may help reveal the physiological mediators of progressive neurodegeneration. Ultimately, these studies could lead to new therapeutic approaches to the treatment of neurodegenerative disorders.

Drosophila is a powerful tool for the dissection of genetic pathways. Our analysis of the FOXO family of transcription factors and the toxic effects of Gal4 has revealed

several novel phenotypes [2-5], which could be used as a basis for further experiments aimed at identifying novel genetic components involved in the IIS signalling pathway and in TPIA. In conclusion, this could provide insight into the mechanisms of human diseases, and inspire new strategies for the treatment or prevention of these diseases.

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