Regulation of Growth by *Drosophila* FOXO Transcription Factor

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Jos olemme viisaita,
meidän on erittäin kriitillisesti tutkittava varsinkin niitä käsityksiämme,
joiden epäileminen tuottaa meille suurinta tuskaa.

Bertrand Russell

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications and unpublished data, which are referred to in the text by their Roman numerals I-III.

I Harvey KF, Mattila J, Sofer A, Bennett FC, Ramsey MR, Ellisen LW, Puig O, Hariharan IK. FoxO-regulated transcription restricts overgrowth of *Tsc* mutant organs. *J Cell Biol*. 2008 Feb;180(4):691-696

II Mattila J, Kallijärvi J, Puig O. RNAi screening for kinases and phosphatases identifies FoxO regulators. *Proc Natl Acad Sci USA*. Sep;105(39):14873-14878

III Mattila J, Bremer A, Puig O. Drosophila FoxO regulates organism size and stress resistance through an Adenylate cyclase. (submitted)

ABBREVIATIONS

4EBP	eIF4E binding protein
AC	Adenylate cyclase
AKH	Adipokinetic hormone
Akt	Protein kinase B
AMP	Adenosine monophosphate
AMPK	Adenosine monophosphate activated kinase
ATP	Adenosine triphosphate
CA	Corpus allatum
cAMP	Cyclic adenosine monophosphate
CC	Corpora cardiaca
CDK2	Cyclin dependent kinase 2
cGMP	Cyclic guanoside monophosphate
CGKI	Cyclic guanosine monophosphate regulated kinase I
CK1	Casein kinase 1
CREB	cAMP-response-element-binding protein
DAF-2/16	Abnormal dauer formation 2/16
DILP	*Drosophila* Insulin-like peptide
DR	Dietary restriction
DSB	Double strand break
DYRK1	Dual-specificity tyrosine-phosphorylated and regulated kinase 1
eIF4E	Eukaryotic Initiation factor 4E
FoxO	Forkhead box class “O”
FRE	FoxO recognition element
G6PASE	Glucose-6-phosphatase
GSK3β	Glucose and serum regulated kinase 3β
IGF	Insulin-like growth factor
IKKβ	Ikappaβ kinase
INR	Insulin receptor
IPC	Insulin producing cell
IRS-1	Insulin receptor substrate 1
JNK	Jun N-terminal Kinase
MAPK	Mitogen activated protein kinase
MST-1	Mammalian sterile 20-like kinase-1
NLS	Nuclear localization signal
NES	Nuclear export signal
PDE	Phosphodiesterase
PDK1	Phosphoinositide-dependent kinase 1
PEPCK	Phosphoenolpyruvate carboxykinase
PH	Plecstrin homology domain
PI3K	Phosphatidylinositol 3’ –kinase
$PIP_{2/3}$	phosphatidylinositol phosphate
PKA	Protein kinase A
PKB	Protein kinase B

PKC	Protein kinase C
PP2A	Protein phosphatase 2A
PTEN	Phosphatase with tensin homology
ROS	Reactive oxygen species
SER	Serine
SGK	Serum and glucocorticoid inducible kinase
TF	Transcription factor
TFIIB	Transcription factor IIB
TGF-β	Transforming growth factor β
THR	Threonine
TOR	Target of rapamycin
TORC	Transducer of regulated CREB activity
TORC1/2	Target of rapamycin complex 1/2
TSC1/2	Tuberous sclerosis complex 1/2
UV	Ultraviolet

ABSTRACT

Forkhead box class "O" (FoxO) transcription factors are members of the forkhead box transcription factor superfamily, with orthologues in various species such as human, worm and fly. FoxO proteins are key regulators of growth, metabolism, stress resistance and, consequently, life span. FoxOs integrate signals from different pathways, e.g. the growth controlling Insulin-TOR signaling pathway and the stress induced JNK and Hippo signaling pathways. FoxO proteins have evolved to guide the cellular response to varying energy and stress conditions by inducing the expression of genes involved in the regulation of growth and metabolism. This work has aimed to deepen the understanding of how FoxO executes its biological functions. A particular emphasis has been laid to its role in growth control. Specifically, evidence is presented indicating that FoxO restricts tissue growth in a situation when TOR signaling is high. This finding can have implications in a human condition called Tuberous sclerosis, manifested by multiple benign tumors. Further, it is shown that FoxO directly binds to the promoter and regulates the expression of a *Drosophila* Adenylate cyclase gene, *ac76e*, which in turn modulates the fly's development and growth systemically. These results strengthen FoxOs position among central size regulators as it is able to operate at the level of individual cells as well as in the whole organism. Finally, an attempt to reveal the regulatory network upstream of FoxO has been carried out. Several putative FoxO activity regulators were identified in an RNAi screen of *Drosophila* kinases and phosphatases. The results underscore that FoxO is regulated through an elaborate network, ensuring the correct execution of key cellular processes in metabolism and response to stress. Overall, the evidence provided in this study strengthens our view of FoxO as a key integrator of growth and stress signals.

INTRODUCTION

Shortly after their discovery and characterization as transcription factors it became clear that FoxO proteins possess functions of exceptional interest in regard to organism well being. Consequently, FoxO mediated cellular processes have drawn a great deal of attention among researchers and after some fifteen years of intense investigation, it is now known that FoxOs are a family of conserved multifunctional transducers of various growth and stress signals having implications in devastating diseases such as cancer and diabetes. Due to the combined effort from research in different model organisms it has become evident that FoxO proteins hold key roles in regulating processes such as growth (Junger et al. 2003, Puig et al. 2003), energy homeostasis (Zhang et al. 2006), protection from DNA-damage (Huang et al. 2006), cell cycle (Alvarez et al. 2001), cellular differentiation (Bois & Grosveld 2003) and life span (Hwangbo et al. 2004, Giannakou et al. 2004). It is the wide range of biological functions that has made FoxO particularly attractive as well as a challenging research object.

Growth is a fundamental biological process regulated through various means. FoxO proteins are known to exert their growth regulatory function by participating in the well conserved nutritional perception machinery regulated by the Insulin-Glucagon axis. Their role in this process is to mediate responses to energy deprivation. In mammals, upon hypoglycemia and subsequent attenuation of circulating Insulin, hepatic FoxO1 is localized in the nucleus where it activates a pattern of gene expression devoted to gluconeogenesis and lipid catabolism (Zhang et al. 2006). This mechanism, among others, ensures the sustaining of correct blood glucose level and, consequently, cell growth. On the other hand, FoxO has a well defined role in slowing down growth in the peripheral tissues, i.e. muscle and adipose tissue, as a transducer of the Insulin/TOR signaling pathway (Brunet et al. 1999). For example, upon fasting FoxO is activated and induces expression of genes such as $p27^{kip1}$ and $p21^{cip1}$ which inhibit the cell cycle progress (Medema et al. 2000, Seoane et al. 2004). Hence, FoxO is acting in a cell autonomous and non-autonomous manner in adapting growth to the prevailing nutritional condition. The action of FoxO is therefore closely linked to the complex system of growth and metabolism and the understanding of these processes is pivotal in prevention and curing associated diseases such as diabetes and cancer.

Mechanisms behind FoxO regulated processes can be understood by functional analysis of its target genes. For example, the role of FoxO in stress resistance and life span regulation (Giannakou et al. 2004, Hwangbo et al. 2004, Wang et al. 2005), is achieved by ordered spatiotemporal activation of specific gene expression targets. Essential to understanding the regulation of these processes is to uncover which of the FoxO targets are involved and in which tissue the regulation takes place. For instance, a systems biology approach was recently applied to demonstrate the key role that FoxO holds in shifting the gene expression pattern upon changes in energy balance (Gershman et al. 2007). Whereas the role of some of the target genes is obvious, such as enzymes devoted to gluconeogenesis, a number of target genes still possess unknown functions. Hence, a more specific analysis of the target gene biology is needed to complement the findings of the system biology approach and to understand FoxO biology in detail.

Given the interesting range of cellular and physiological processes where FoxO participates, particular interest has been addressed to its upstream regulatory factors. To date, several signaling pathways are known to act through FoxO transcription factors, regulating their stability, intracellular localization and transactivation property, and the number of new regulators is rapidly increasing (Huang & Tindall 2007). The general opinion is that FoxO is inhibited by growth factor-induced phosphorylation and nuclear exclusion (Brunet et al. 1999). However, further studies have demonstrated this to be a simplified view since its transcriptional activity can be modulated with or without growth factors (Alvarez et al. 2001, Luong et al. 2006). It is therefore likely that our knowledge of the regulatory network around FoxO is still limited. At present, there has not been any report of a systemic screen aiming to find novel FoxO modulators.

This work has focused on the role of FoxO in growth control. Evidence is presented increasing our knowledge of how FoxO regulates growth at the level of an individual cell and the whole organism. In summary, we demonstrate that FoxO induces the expression of a negative growth regulator *scylla*/*redd1* upon an elevated TOR signaling pathway, presumably protecting tissues from overproliferation. Further, we establish the role of a novel *Drosophila* FoxO target, *adenylate cyclase 76e*, in systemic growth regulation. These results provide insights into how FoxO exerts its growth regulatory function. Finally, through an RNAi-based screen we show that FoxO is regulated by a complex network of kinases, which allows for an exquisitely balanced transcriptional activity necessary to adjust metabolic and growth responses to the prevailing nutritional conditions. Overall, our results underscore the complexity of processes regulated by FoxO transcription factors, which reflects their primary role, the coordination of organism growth in response to the nutrient availability and stress.

REVIEW OF THE LITERATURE

Cellular signaling

In order to survive, an organism needs to react to an alternating environment and adjust its behaviour to meet the prevailing conditions. The reaction of a metazoan animal is a result of a perception event which is followed by a coordinated response of various specialized cell types. This response ultimately leads to a change in animal behaviour. This coordinated action of metazoan operations relies on communication between cells. Hence, individual cells are faced with the same requirements as the organism as a whole: they need to receive information and respond in an appropriate manner. For the purpose of cellular communication, cells have evolved signaling mechanisms, depending on protein-protein interaction, to elicit and receive information (Bhattachayya et al. 2006). These mechanisms, i.e. cellular signaling pathways, collect and integrate information about extracellular conditions important for survival, growth and differentiation.

By collecting information from its external milieu a cell is able to respond to changes in hormone, nutrient and growth factor composition and abundance by modulating its protein post-translational modifications and, ultimately, its transcriptional profile. These alterations can lead to responses such as regulation of metabolism (Marshall 2006), cell differentiation (Pires-Da Silva & Sommer 2003), cell division (Jones & Kazlauskas 2001), cell death (Jin & El-Deiry 2005), modulation of cellular cytoskeleton (Sinha & Yang 2008), cell movements (Condeelis et al. 2001, Guvakova 2007) and changes in action potential (Barnett & Larkman 2007). The response of a given cell to a given signal is dictated by its competence, i.e. the repertoire of molecular signal transduction mechanisms. These mechanisms, which reflect the cells developmental history, consist of cell surface or intracellular receptors, intracellular signaling pathway components, transcription factors and other co-factors. The concept of competence explains why a given signal might result in different responses, depending on the cell type it stimulates. In addition, the outcome of a signaling event is also dependent on its strength and duration (Pawson & Nash 2001).

Core components of a classical signaling cascade are a ligand and its cognate receptor positioned either in the cell membrane or within a cell for small membrane permeable ligands. In its simplest form, a ligand is able to modify gene expression directly by binding to its receptor which in turn functions as a transcription factor. Such signaling is known to occur for many lipophilic steroid hormones such as estrogen and progesterone (Levin 2008). However, a number of more elegant systems consisting of several intracellular transducer and effector molecules have evolved in metazoans to allow a refined regulation of signal transduction. Common features in these signal transduction pathways are (1) specific protein-protein interactions which transmit the signal to one direction, (2) signal amplification, (3) signal diversification and/or integration and (4) multiple points of control (Weng et al. 1999, Pawson & Nash 2001).

Signaling pathway components

Signaling molecules, i.e. ligands, can vary from single ions to large multi-protein complexes. Roughly, ligands can be classified as either cell-membrane permeable or non-permeable. The former includes lipid soluble cholesterol, tyrosine and vitamin

A derivatives also known as steroid hormones, thyroid hormones and retinoids, respectively (Cheskis 2004, Zhang et al. 2000, Fields et al. 2007). Due to the insolubility to the aqueous environment, these ligands can not diffuse freely within a cell and are therefore attached to soluble carrier proteins (Schroeder et al. 2007). Ligands which can not penetrate passively through the cell membrane include ions (K^+, Na^+, Ca^{2+} and Cl^-), organic compounds such as glycine and acetylcholine, nucleotide derivatives such as cAMP and cGMP, and finally, the large group of peptides and proteins. The heterogeneous group of protein ligands include growth factor families, for example Insulin-like growth factors and the Transforming growth factor β family (TGF-β) (Grimberg & Cohen 2000, Massague 2000). The action of the soluble ligands is transmitted through the cell membrane by specific ligand gated ion channels or transmembrane receptors. Transmembrane receptors, such as Receptor tyrosine kinases (Hubbard & Miller 2007), possess intrinsic enzymatic activity in their intracellular catalytic domain. The ligand-receptor interaction triggers an excitatory signal elicited by the enzymatic activity of this catalytic domain. Alternatively, receptors are coupled with proteins possessing enzymatic activity (Shindler et al. 2007). Another, large group of receptors is the G-protein coupled receptors which transmit the signal through G-proteins, enzymes with GTPase activity (Oldham & Hamm 2008). Whatever the exact mode of action, a common theme is that the binding of a ligand to the extracellular domain of a transmembrane-receptor triggers the production/recruitment of signaling molecules, i.e. second messengers and signaling proteins, into the plasma membrane where an excitatory activity takes place. As a result, the signal is carried onward within the cell.

A signaling pathway can consist of several intracellular transducers. These are typically kinases and phosphatases which by incorporating or removing a phosphate group alter the physiochemical properties of a substrate protein or a second messenger such as inositol-lipids (Daves & Krebs 1999, Vanhaesebroeck et al. 2001). A signaling pathway may consist of several kinases which sequentially phosphorylate each other in a specified order. Classical examples are the mitogen activated protein kinase (MAPK) signaling pathways, having at least three sequentially activated protein kinases (Pimienta & Pascual 2007). A characteristic consequence of a protein phosphorylation event is a conformational change in its three dimensional structure, which exposes its catalytic cleft to interact with its substrates (Shi et al. 2006). Good examples of such an allosteric regulation are the activation of the ABC kinase family members Protein kinase C (PKC) and Protein kinase B (PKB/Akt) (Dutil & Newton 2000, Calleja et al. 2007). In addition to kinases and phosphatases a signaling pathway may contain several other adapter proteins which may facilitate protein-protein interactions and enzymatic activity (Pratt et al. 2008). For example, Akt is known to associate with more then ten different proteins having roles in its stabilization, kinase activity and intracellular transport (Du & Tsichlis 2005).

Most, if not all, signaling events result in changes in the transcriptional activity of the cell. These changes are mediated by inducible transcription factors (TF) which are sequence specific DNA binding factors regulating the initiation of gene expression. TFs bind to the so called response elements, i.e. short consensus DNA sequences, with their DNA binding domain and facilitate the initiation of transcription by directly contacting and stabilizing the basal transcription apparatus (Becket 2001, Marmorstein & Fitzgerald

2002). TFs are grouped into super-families based on their DNA binding domains. These include, for example, the helix-turn-helix or the leucine zipper domain (Pabo & Sauer 1992). Finally, the activity of TFs is modulated by a heterogeneous group of co-factors which by themselves do not bind to DNA, but regulate the TF-DNA complex stability (Featherstone 2002).

Cellular signaling regulating growth and metabolism

One of the major, still incompletely understood problems in biology is how the final size and organ proportions of an organism are achieved (Mirth & Riddiford 2007). The highly ordered and synchronized tissue growth requires crosstalk between the animal's energy sensing/producing and energy consuming tissues. Many signaling pathways are involved in this communication. These include MAPK, TGF-β, Insulin/TOR, cAMP and PKC signaling. Below are briefly introduced central signaling systems involved in the regulation of growth and metabolism important for this work.

Insulin signaling

Important solution for systemic growth regulation in metazoan organisms are Insulin and an Insulin like growth factor (IGF) which have evolved to be regulators of metabolism and growth, respectively (Oldham & Hafen 2003). These signaling molecules transmit nutritional information to cells and guide their growth and division rate to meet the environmental conditions. Proper functioning of the Insulin signaling pathway is pivotal to organism homeostasis and failure to regulate its activity can lead to diseases such as diabetes and cancer (Vivanco & Sawyers 2002).

Insulin binding to its cognate receptor leads to an ordered, well defined cascade of signaling molecule interactions (Figure 1). Briefly, activation of the Insulin receptor (InR) by ligand binding is followed by the production of a membrane tethered lipid second messenger phosphatidylinositol (3,4,5) triphosphate (PIP_3) by Phosphatidylinositol 3' –kinase (PI3K) (Engelman et al. 2006). PIP_3 attracts proteins with a pleckstrin homology (PH) domain to the plasma membrane and brings these proteins into proximity with each other to interact (DiNitto et al. 2003). A protein phosphatase PTEN counteracts the function of PI3K by dephosphorylating PIP_3 to PIP_2 thereby releasing proteins from the membrane and disrupting the pathway (Maehama et al. 1998). One of the PH domain-containing proteins is Akt which, once it is tethered to the membrane, becomes phosphorylated and activated by Phosphoinositide-dependent kinase 1 (PDK1) and Target of rapamycin complex 2 (TORC2) (Franke et al. 1997, Klippel et al. 1997, Alessi et al. 1997, Sarbassov et al. 2005). The activity of Akt is known to be directly inhibited by several phosphatases. These include Protein phosphatase 2A (PP2A) (Ugi et al. 2004, Van Kanegan et al. 2005, Padmanabhan et al. 2009) and PH domain leucine-rich repeat protein phosphatase (Gao et al. 2005). Akt is a key player in promoting cell survival, growth and division having more than 100 known substrates (Manning & Cantley 2007). The best known substrates directly involved in growth control include Glucose and serum regulated kinase 3 α and β (GSK3) (Cross et al. 1995), Tuberous sclerosis complex 2 (TSC2) (Inoki et al. 2002, Manning et al. 2002, Potter et al. 2002) and FoxO transcription factors (see p.13). The inhibition of the TSC1/2 complex by Akt makes a connection to another well defined nutrient regulated pathway, the TOR signaling

pathway. The insulin-TOR signaling pathways form a network of interactions intricately balanced by nutrient availability, regulating processes such as energy metabolism and translation (Wullschleger et al. 2006). The coordinated activity of this network is the main growth regulator of a metazoan organism. In figure 1 the best known interactions within the Insulin and TOR signaling network are summarized.

The main physiological consequences of Insulin signaling activation are (1) cellular glucose, amino acid and fatty acid uptake, (2) production of energy reserves in the form of glycogen and fat and (3) releasing the cell from a cell cycle and translational arrest allowing cellular growth and division. In the latter, a main role is played by the regulation of the Forkhead box transcription factors (Figure 1 and see below).

Cyclic adenosine monophosphate signaling pathway

Cyclic AMP (cAMP) signaling is a well characterized nutrient and hormone regulated signaling pathway. Its role for transmitting the action of hormones, for example Glucagon, Epinephrine, Vasopressin and Corticotrophin, has been known for decades (Major & Kilpatrick 1972). Cyclic AMP is a ubiquitous intracellular second messenger

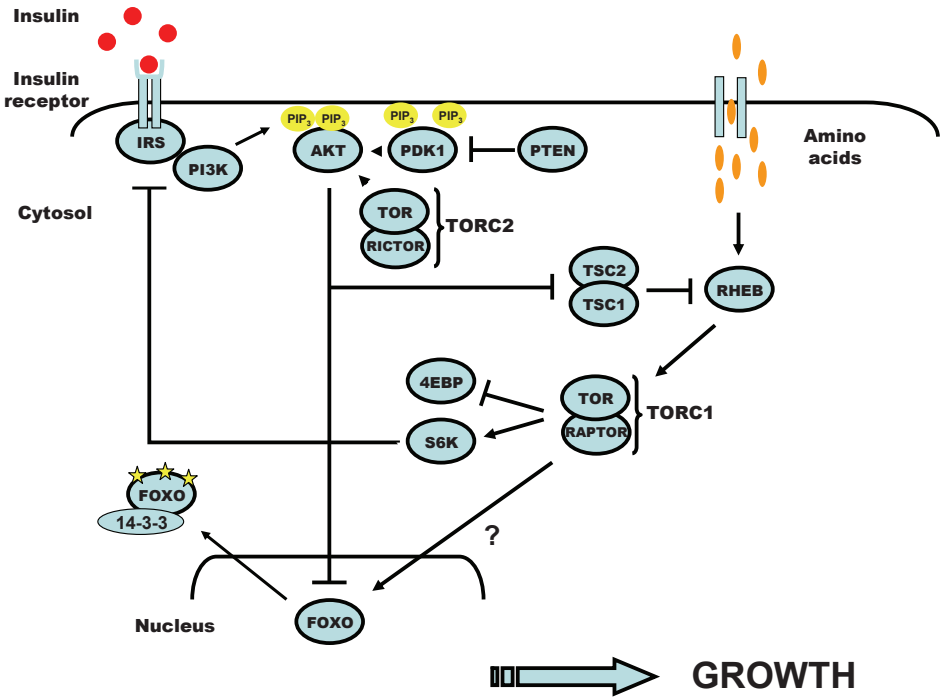

Figure 1. The Insulin/TOR signaling network.

The balanced activity of this network regulates growth to meet the prevailing nutritional condition. An important consequence of the activation of the Insulin signaling is the sequestering of the FoxO transcription factors into the cytosol. The connection from TOR/Raptor complex to FoxO can be direct or through IRS (see discussion p.32). For the sake of simplicity only the best known interactions are illustrated.

in all animal cells. It is synthesized from ATP by an enzyme Adenylate cyclase (AC) and it is degraded by a Phosphodiesterase (PDE) which hydrolyzes it to AMP. The binding of a hormone to its cognate receptor results in production of active GTP-bound G-protein α-subunit which in turn binds to and activates the AC. In addition, the activity of AC is modulated through phosphorylation by PKC and binding to Ca^{2+}/Calmodulin (Hurley 1999). The formation of cAMP can then stimulate multiple different processes which include gluconeogenesis, secretory processes, apoptosis and growth control (Houslay & Milligan 1997). Hence, the pathway is a general signaling mechanism used in all cells and the outcome of its stimulation depends on various factors associated with cell type and signal strength.

The classical outcome of increasing cellular cAMP levels is the activation of the cAMP dependent protein kinase A (PKA) which is a tetrameric holoenzyme consisting of catalytic and regulatory sub-units. The binding of cAMP releases PKA from its regulatory sub-units yielding an active enzyme (Skålhegg & Tasken 2000). The signal generated by cAMP is then transmitted to the nucleus by cAMP-response-element-binding protein (CREB) phosphorylated and activated by PKA (Gonzales & Montminy 1989, Sands & Palmer 2008). CREB acts as a transcription factor by binding to the core cAMP response element (CRE) sequence TGACGTCA (Carlezon et al. 2005) (Figure 2). In the liver, Glucagon stimulates the activation of CREB which acts in concert with

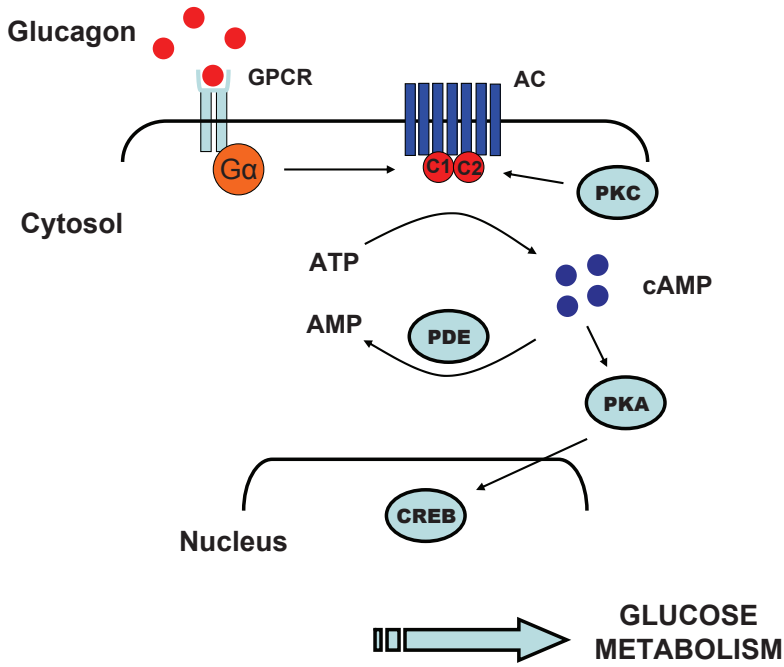

Figure 2. cAMP signaling pathway.
The activation of the G-protein coupled receptor (GPCR) by Glucagon leads to the activation of Adenylate cyclase (AC) and production of cAMP. cAMP activates PKA and CREB transcription factor leading to the activation of transcriptional program of gluconeogenesis.

coactivators Peroxisome-proliferation-activated receptor-γ coactivator-1 (PGC-1α), CREB binding protein (CBP), Transducer of regulated CREB activity 2 (TORC2) and FoxO1 to induce the program of gluconeogenesis under fasting conditions (Chrivia et al. 1993, Herzig et al. 2001, Puigserver et al. 2003, Koo et al. 2005). This program of gene expression is inhibited by Insulin demonstrating the intimate link between the cAMP and Insulin signaling pathways (Zhou et al. 2004). In summary, in regard to metabolism and growth, the cAMP signaling pathway acts as a starvation signal activated by Glucagon, resulting in the release of energy from the liver.

Protein kinase C signaling

Protein kinase C (PKC) is a close relative of PKB/Akt and PKA which form the family of ABC kinases (Newton 2003). PKCs itself form a family of kinases divided into the conventional (cPKC), atypical (aPKC) and novel isoforms (nPKC), classified on the basis of their domain structure and co-factor regulation (Newton 2001). PKCs are important mediators of the Insulin signaling having both inductive and repressive functions depending on the cell type and activated isoform. Overall, six out of the ten known mammalian PKC isoforms are implicated in Insulin signaling (Sampson & Cooper 2006). However, their role as negative regulators of Insulin signaling and, consequently, enhancers of Insulin resistance is more profoundly understood. Especially, their involvement in the free fatty acid (FFA) induced Insulin resistance is well established (Schmitz-Peiffer et al. 1997, Letiges et al. 2002, Yu et al. 2002, Puljak et al. 2005). Some PKC isoforms are also connected to the MAPK pathways in various cell types having direct effects on proliferation and cancer (Ueda et al. 1996, Skaletz-Rorowski et al. 2005, Cozzi et al. 2006). As mentioned above, PKC is also known to be involved in the cAMP signaling by regulating the activity of the Adenylate cyclases. These examples of interactions demonstrate how intimately the pathways regulating metabolism and growth are linked.

PKCs are activated through various growth factor receptors and by second messengers. These include (1) diacylglycerol (DAG), a glycerolipid derivative, (2) Ca^{2+}/calmodulin and (3) phosphatidylserine (PS), a membrane phospholipid. Specifically, there are two requirements to fully activate PKC; phosphorylation and the presence of a subfamily-specific second messengers acting as a cofactor. PKC goes through three sequential phosphorylations to take its mature form. First, it is phosphorylated at a residue in its activation loop catalyzed by PDK1 (similar to PKB and PKA) and second, it goes through two autophosphorylation events which enable the enzyme to adopt a catalytically competent confirmation and allows it to be released into the cytosol. The three phosphorylation events result in a number of key conformational rearrangements that lock PKC into a more thermally stable, protease and phosphatase resistant conformation (Cazaubon et al. 1994, Orr & Newton 1994, Keränen et al. 1995, Le Good et al. 1998).

Upon activation and release into the cytosol from the cell membrane, PKC can phosphorylate its substrates. PKC isoforms are known to have multiple targets for phosphorylation such as other kinases, signaling adapter proteins and transcripton factors (Hurov et al. 2004, del Rincon et al. 2004, Wang et al. 2005, Holden et al. 2008, Yamasaki et al. 2009). An important PKC substrate is the Insulin receptor substrate 1

(IRS-1) which is an adapter protein linking Insulin receptor and PI3K (Ogawa et al. 1998). The levels of IRS-1 are regulated by a proteasome dependent mechanism and deregulation of this process is known to be associated with Insulin resistance in humans and rodent diabetes models (Saad et al. 1992, Tamemoto et al. 1994, Rondinone et al. 1997, Zhande et al. 2002). It has been shown that PKC inhibits insulin signaling through IRS-1 phosphorylation (Leitges et al. 2002). In addition, del Rincon et al. (2004) demonstrated that IRS-1 is a direct substrate of a nPKC isoform, PKCδ, having implications in IRS-1 proteosomal degradation. It has been suggested that PKC serves as a physiological feedback mechanism to inhibit elevated Insulin signaling (Leitges et al. 2002).

From the above considerations it becomes evident how complicated network of signaling events are involved in the regulation of the metabolism and growth of a metazoan organism. It is noteworthy that in the course of mammalian evolution most of the signaling molecules have gone through several duplication events. These homologues have nowadays redundant and independent functions making the investigation of a signaling pathway extremely challenging. Importantly, in lower eukaryotes gene duplication events have been rather rare, leaving the signaling pathways easier to interpret. For example, comparison of the Insulin signaling pathway between mammals and *Drosophila* reveals a lack of redundancy in many pathway components in the latter (Figure 3). Hence, the employment of model organisms such as *Drosophila melanogaster* can have a profound impact in the understanding of cellular signaling.

Forkhead box class "O" transcription factors

Forkhead box class "O" (FoxO) transcription factors are a family of conserved proteins belonging to the large superfamily of forkhead box, or winged-helix, transcription factors. The superfamily consists of 19 sub-classes termed from FoxA to FoxS having at

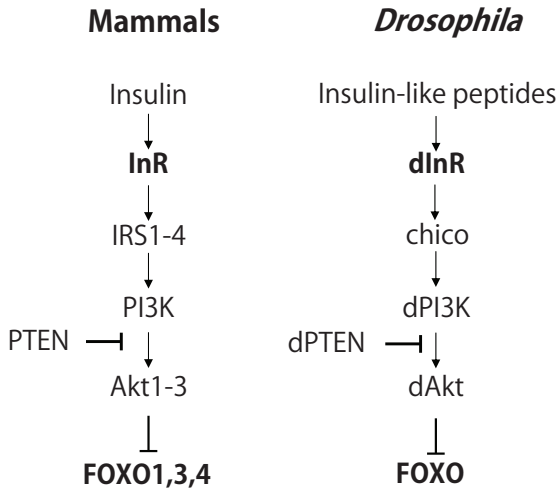

Figure 3. The Insulin signaling pathway in mammals and *Drosophila*

present more then 100 known family members throughout the animal kingdom. These proteins are classified solely based on the existence of the approximately 100-amino acid long forkhead DNA binding domain. The position of the domain can vary greatly within the family and not all of the members are considered to be transcriptional activators. For example, FoxP is thought to be a transcriptional repressor since it lacks a transactivation domain characteristic to transcriptional activators (Wang et al. 2003). FoxO proteins are unique in having a five amino acid insertion (GDSNS) in their DNA binding domain (Weigelt et al. 2001). The function of this short stretch of amino acids is not known. The members of the forkhead box family are commonly known to associate with metazoan development but lately a variety of different biological functions have been associated with these proteins.

Short historic perspective

The first forkhead family member was characterized from a genetic screen looking for defects in embryogenesis in the fruit fly *Drosophila melanogaster* (Jurgens & Weigel 1988). Mutations in this gene caused a homeotic transformation of the embryo's posterior segments into head-like structures. The gene responsible for this "forked head" embryo was named after its phenotype and cloning of the gene soon followed (Weigel et al. 1989). The gene was found to contain a putative DNA binding domain unknown at that time. Subsequently, *fork head* has been classified as a member of the forkhead box class "A" family. The sequence of the *fork head* DNA binding domain has been then used for characterization of other members of the forkhead box superfamily in diverse species. The crystal structure of the forkhead domain in complex with DNA was published already in 1993 by Clark et al.

To date, four functional *foxo* genes are known in mammals. The first FoxO-encoding gene was reported in humans in 1993 (Galili et al. 1993). It was found in the breakpoint of a chromosomal fusion in chromosome t(2;13)(q35;q14) associated with alveolar rhabdomyosarcoma, a type of cancer formed in the skeletal muscle at early ages. The gene was named as *fkhr* (forkhead in rhabdomyosarcoma) but was later renamed as *foxo1* by a winged helix/forkhead nomenclature committee (Kaestner et al. 2000). Since the identification of *foxo1*, three other FoxO members have been found in the human genome; *foxo3* (Anderson et al. 1997, Hillion et al. 1997), *foxo4* (Parry et al. 1994, Borkhardt, et al. 1997) and *foxo6* (Jacobs et al. 2003).The genes *foxo3* and *foxo4* were also found in chromosomal translocation sites, both associated with acute leukaemia. FoxO6 is sometimes not included in the family since it has distinct regulatory properties from other FoxO proteins. Initially, *foxo3* was identified independently by two groups and was for a short time thought to be two different genes. Hence, *foxo2* was later omitted from the nomenclature. For *foxo1*, and *foxo3*, additional homologous open reading frames have been found that contain a stop codon preventing the synthesis of full length proteins (Anderson et al. 1997). These are thought to represent pseudogenes and are sometimes referred as *foxo1b* and *foxo3b*.

Parallel to the findings in humans, FoxO members were cloned in other organisms as well. Biggs et al. (2001) cloned three mouse genes highly homologous to human *foxo1*, *foxo3* and *foxo4*. In the same study, single orthologues from the chick (*Gallus gallus*) and zebrafish (*Danio rerio*) were found. The mouse *foxo6* was later cloned parallel to

the human orthologue by Jacobs et al. in 2003. The sole nematode worm *Caenorhabditis elegans* FoxO member, *daf-16*, was identified by Ogg et al. as early as 1997. Since then, studies with *C. elegans* have provided much of the knowledge of the biological functions of FoxO proteins. The single *Drosophila melanogaster* FoxO ortholog (dFoxO) was reported independently by three different groups in 2003 (Junger et al. 2003, Kramer et al. 2003, Puig et al. 2003). Subsequently, *Drosophila* has become the animal model most widely used to discern different properties of FoxO biology.

Molecular function, structure and conservation of FoxO proteins

FoxO proteins are sequence-specific DNA binding proteins with transcription factor activity. The core DNA binding sequence of FoxO proteins, TTGTTTAC, has been determined (Furuyama et al. 2000). FoxO proteins possess the characteristic domain structure of transcription factors (Figure 4). Roughly, the protein can be divided into two parts; the DNA binding domain and the transactivation domain. The DNA binding forkhead domain, also called as the winged-helix domain, contains a helix-turn-helix core of three α-helices flanked by two large loops or wings. In this conformation, helix 3 is responsible for most of the direct contact with DNA (Clark et al. 1993). The structure of the human FoxO4 DNA binding domain has also been determined. It was found that the five amino acid insertion characteristic of FoxO proteins, adds a small loop between helix 1 and 2 but has little effect on the overall structure (Weigelt et al. 2001). Therefore the target gene specificity could not be attributed to the forkhead domain and remains elusive. Interestingly, similar topology is used by several other DNA binding proteins with a completely unrelated amino acid sequence representing a nice example of convergent evolution at the molecular level (Carlsson & Mahlapuu 2002). Within the forkhead domain there is a short stretch of sequence rich in basic amino acids, denoted as nuclear localization signal (NLS) (Qian & Costa 1995). This motif is responsible for its nuclear localization, and in FoxO proteins it is one of the main sites targeted by FoxO regulators (Biggs et al. 1999, Brunet et al. 1999). Additional to the NLS, two leucine rich nuclear export signals (NES) are present in the carboxy terminal sequence of FoxO proteins (Biggs et al. 1999, Brunet et al. 2002).

The FoxO family share very little similarity at the protein sequence level, with the exception of the DNA binding forkhead domain where human FoxO4 and *Drosophila* dFoxO share 45% overall identity and 85% identity in the region of the three α-helices (Puig et al. 2003). Apart from the forkhead domain, FoxO and other forkhead box

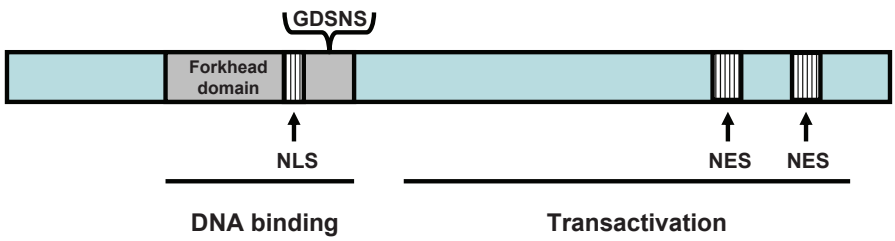

Figure 4. Schematic representation of the characteristic domain structure of a FoxO protein.

family member proteins differ significantly in their sequence. Little is known how the putative transactivation domain contributes to the interaction with the transcriptional apparatus or to target gene specificity. Additionally, the exact position of this domain within the protein varies significantly in literature. Only a few studies have addressed the position of the so called activation or repression domains in different forkhead proteins responsible for protein-protein interactions. For example, Hellqvist et al. (1998) showed the existence of two activation domains in human Forkhead-related activator 2 protein that are responsible for contacts with the general transcription factors TATA-binding protein and TFIIB. At present, little is known about these domains in FoxO proteins.

Regulation of FoxO activity

The metazoan gene expression machinery is highlighted by its multiple level of regulation. For example, expression of a given gene is dependent on factors such as chromatin structure and the availability and activity of regulatory trans-activating proteins. These inducible transcription factors are present, or their activity is modulated, in temporal and spatial patterns and are responsible for most of the gene expression specificity in a given tissue or developmental process. Studies of FoxO activity regulation have been concentrated on the post-translational level. At present little is known how the expressions of *foxo* genes are regulated. Interestingly, a recent study by Essaghir et al. (2009) demonstrated the presence of FoxO recognition elements (FRE) in the FoxO1 gene. It was further shown that FoxO1 and FoxO3 regulate their own expression by binding to these sequences in human fibroblasts. The prevalence of such a regulation in other cell types and organisms is yet to be determined.

During *Drosophila* development *dfoxo* is expressed ubiquitously throughout the embryo (J. Mattila, unpublished observation). A similar observation has been made in mice where different FoxO isoforms occupy different tissues during development resulting in nearly uniform expression (Furuyama et al. 2000). Furthermore, critical FoxO functions such as stress and metabolism response, as well as cell cycle regulation (see below), require rapid changes in activity. This implies that most of the regulation during development as well as in maintaining cellular homeostasis is achieved at the post-translational level. Indeed, FoxO is known to be regulated through phosphorylation, acetylation, methylation and ubiquitylation.

In principal, FoxO activity can be modulated in three different ways: (1) by sub-cellular localization between nucleus and cytoplasm, (2) by regulated protein stabilization/destabilization and (3) by post-translational modifications altering its DNA and/or transcriptional cofactor binding capacity. All of these mechanisms have been shown to play a role in regulating FoxO activity. All the known FoxO proteins, except FoxO6, are regulated through phosphorylation by Protein kinase B (PKB/Akt) or a redundant Serum and glucocorticoid inducible kinase (SGK), which modulates transport between the nucleus and cytoplasm (see below). Protein localization and degradation are coupled since in the cytoplasm FoxO is ubiquitynated and targeted for degradation to the proteasome (Matsuzaki et al. 2003, Plas & Thompson 2003). Furthermore, FoxO activity can also be modulated while in the nucleus. Luong et al. (2006) have shown that nuclear dFoxO activity is at least partially dependent on the Target of rapamycin (TOR) kinase. Additionally, Tsai et al. (2003) demonstrated that nuclear exclusion is not necessary for

Akt mediated inhibition of FoxO1. In addition, FoxO1 is shown to be methylated by protein arginine methyltransferase PRMT1 which protects it from the negative regulation by Akt (Yamagata et al. 2008). Finally, acetylation by histone acetyltransferases such as CREB-binding protein and p300 can weaken the DNA binding of FoxO (van der Horst & Burgering 2007).

Multiple signaling pathways are known to affect FoxO activity (Huang & Tindall 2007). At present, emphasis is put on the regulatory phosphorylation events by various kinases. Below are reviewed the best known interactions between FoxO and its regulatory kinases.

Regulation of FoxO by Akt

The first indication that Insulin signaling and FoxO interact, came from observations in *C. elegans* where a long lived *daf-2* mutant (abnormal dauer formation-2, worm orthologue of InR) was suppressed by a mutation in a *daf-16* gene (Gottlieb & Ruvkun 1994, Larsen et al. 1995). The *daf-16* gene was soon after characterized to represent the worm FoxO transcription factor ortholog (Ogg et al. 1997). Only two years later, it was directly demonstrated by a combined effort of six different groups that Insulin signaling inhibited mammalian FoxO activity by Akt mediated phosphorylation in three serine/threonine residues (Thr32, Ser253 and Ser315 in FoxO3 sequence) (Biggs et al. 1999, Brunet et al. 1999, Kops et al. 1999, Nakae et al. 1999, Rena et al. 1999, Tang et al. 1999). Interestingly, FoxO6 lacks these phosphorylation sites and it is therefore regulated independently of Akt, remaining predominantly nuclear (Jacobs et al. 2003). The phosphorylation events were shown to result in the association of FoxO with the 14-3-3 chaperone proteins leading to subsequent cytoplasmic retention and transcriptional inactivation (Brunet et al. 1999). The mechanism by which 14-3-3 facilitates the nuclear exclusion of FoxO is not fully understood. However, it has been shown that both phosphorylation of FoxO by Akt and interaction with 14-3-3 are necessary requirements for FoxO nuclear exclusion (Brunet et al. 2002). One possible explanation is that these events lead to a conformational change and exposure of the NES signals to the nuclear export machinery (Brunet et al. 2002). An alternative view is presented by Brownawell et al. (2001) who suggest that Akt mediated phosphorylation attenuates nuclear import rather then augments export. Whatever the exact mechanism, it has been shown to be a conserved feature in FoxO regulation. The consensus Akt phosphorylation sites are also present in dFoxO and conversion of these residues (T44, S190 and S259 in dFoxO sequence) to alanines results in constitutively nuclear FoxO protein (Puig et al. 2003). These mutant forms are widely exploited in FoxO research nowadays and sometimes inaccurately referred to as constitutively active.

Regulatory network around FoxO

Besides Akt/SGK, FoxO is known to be phosphorylated by at least eight different kinases (Table 1). To date, only one FoxO specific phosphatase, PP2A, has been identified (Yan et al. 2008). Interestingly, PP2A has also been found to directly regulate Akt activity (Padmanabhan et al. 2009). Many of the phosphorylation events are associated with facilitated nuclear exclusion and transcriptional inactivation. These include Casein kinase 1 (CK1) (Rena et al. 2002), dual-specificity tyrosine-phosphorylated and regulated

kinase 1A (DYRK1) (Woods et al. 2001), Ikappaβ kinase (IKKβ) (Hu et al. 2004) and Cyclin dependent kinase 2 (CDK2) (Huang et al. 2006). In addition, members of the mitogen activated protein kinase Extracellular signal-regulated kinase and p38 have been shown to phosphorylate and inactivate FoxO by a localization-independent mechanism (Asada et al. 2007). Phosphorylation can also lead to enhanced nuclear localization and transcriptional activation as it has been shown with Jun N-terminal kinase (JNK) (Essers et al. 2004, Oh et al. 2005, Wang et al. 2005) and Mammalian sterile 20-like kinase-1 (MST-1) (Lehtinen et al. 2006). Furthermore, the AMP-activated protein kinase (AMPK) phosphorylates and activates FoxO without modulating its sub-cellular localization (Greer et al. 2007).

Table 1. Kinases known to phosphorylate FoxO.

Kinase	Reference
Akt/SGK	Biggs et al. 1999, Brunet et al. 1999, Kops et al. 1999, Nakae et al. 1999, Rena et al. 1999, Tang et al. 1999
cGKI	Bois et al. 2005
IKKβ	Hu et al. 2004
DYRK	Woods et al. 2001
CDK2/1	Huang et al. 2006, Yuan et al. 2008
JNK	Essers et al. 2004
MST-1	Lehtinen et al. 2006, Yuan et al. 2009
CK1	Rena et al. 2002
AMPK	Greer et al. 2007

As with any other molecular interaction data, critical evaluation of the reported information is necessary. In other words, probability that an interaction represents biological significance is enhanced when (1) a regulatory event and/or site in a protein is conserved among species and (2) interruption of the interaction results in a phenotype *in vivo*. Based on this classification, convincing evidence as FoxO regulators has been presented for Akt, IKKβ, CDK2, JNK, AMPK and MST-1. These interactions and their consequence *in vivo* are discussed below.

IKKβ

The IKKβ kinase is an important positive regulator of cell survival and proliferation by activating members of the NF-kB transcription factors. The IKKβ - NF-kB interaction has known implications in inflammation and cancer (Schmid & Birbach 2008). Hu et al. (2004) demonstrated beautifully how IKKβ contributes to the pathogenesis of cancer by regulating FoxO3 activity. Their study is an example of excellent characterization of a molecular interaction. The initial finding was that in a significant number of breast cancer specimens, FoxO3 was localized to cytoplasm even in the absence of active Akt. The authors found that cytoplasmic FoxO3 correlated with Akt and IKKβ staining in most of the breast cancer samples as well as in lung, liver and stomach carcinomas. It was then further shown by biochemical analysis that IKKβ modulates the FoxO3 transcriptional activity by phosphorylation and nuclear exclusion. The seminal finding

from the study was that cytoplasmic FoxO3 correlates with Akt and/or IKKβ activity and that this condition yields poor prognosis among breast cancer patients.

The findings of Hu et al. (2004) emphasize the importance of developing anti-cancer treatment with both Akt and IKKβ inhibitory function, since inhibition of Akt alone is not sufficient to restore FoxO3 activity. Accordingly, IKKβ inhibitory drugs are currently a target of intense development and some molecules are already in clinical trials (Schmid & Birbach 2008). However, the relevance of this interaction in regard to normal cell growth and/or development is still to be elucidated since at present no studies address it in untransformed cells.

CDK2

CDK2 is a critical player of the cell cycle machinery by controlling the entrance and progress of the S-phase together with E and A-type of cyclins, respectively. Given its role in driving cells to the replication cycle and maintenance of DNA synthesis in S-phase, it is a key target of inactivation upon induction of G_1 or intra S-phase checkpoint by double strand break (DSB) (Huang & Tindall 2007). DSB can arise from by-products of innate metabolism (reactive oxygen species, ROS) or from environmental genotoxic agents such as UV-light. The DSB checkpoint ensures slowing down DNA replication giving time for the repair machinery to correct the DNA damage or to drive the cell to apoptosis (Bartek et al. 2004). Huang et al. (2006) demonstrated that an important regulatory mechanism upon DSB is the relief of FoxO1 transcription factor, but not FoxO3 or FoxO4, from CDK2 mediated inhibition. They showed that CDK2 physically interact and phosphorylate a Ser^{249} residue on FoxO1 resulting into its cytoplasmic localization. Blocking of the phosphorylation by a Ser^{249} conversion to alanine leads to elevated apoptosis. The critical observation was, however, that the CDK2 mediated phosphorylation of FoxO1 could be reversed under genotoxic stress. This finding demonstrated that the interaction is biologically meaningful and suggest that FoxO1 has an important role in protecting the organism from DNA damage and cancer. Interestingly, Liu et al. (2008) have demonstrated that CDK1 is also inactivating FoxO1 by directly phosphorylating the Ser^{249} residue in prostate cancer cells. In addition, the same mechanism was found to operate in postmitotic neurons and proliferating cells, but with completely opposite outcome (Yuan et al. 2008). Phosphorylation of FoxO1 at Ser^{249} by CDK1 resulted in its nuclear accumulation and induction of FoxO1 dependent gene expression. These opposing results demonstrate how cellular signaling can be disrupted in cancer cells. In addition, it raises the question how the phosphorylation of the same residue by separate kinases can result in completely different outcome in regard to FoxO activation. As a conclusion, the results presented above imply that a more general interaction between Cyclin dependent kinases and FoxO exists.

JNK

Jun N-terminal kinase (JNK) is a member of the well defined, conserved family of mitogen-activated protein kinases (MAPK). The JNK pathway is one of the most studied signaling cascades and has been implicated in numerous processes (Lin 2003, Nishina et al. 2004). It is considered as a general intracellular transduction mechanism whose outcome is dependent on the type of activating stimulus and cellular constituents. For

example, it is well known for its apoptosis stimulating function upon cellular stress caused by ROS and reactive nitrogen species (RNS) (Shen & Liu 2006). On the other hand, it has been shown to be involved in the regulation of the complex process of tissue regeneration (Mattila et al. 2005). The first evidence that some JNK regulated processes are mediated through FoxO came from the studies of Essers et al. (2004). They showed in mammalian cell culture that JNK induces nuclear localization and transcriptional activation of FoxO4 upon increased ROS levels. They also demonstrated that the interaction between FoxO4 and JNK is direct; JNK phosphorylates the threonine 447 and 451 of FoxO4. Interestingly, Essers et al. (2004) could not reveal the mechanism of the sub-cellular translocation event since FoxO4 binding to the 14-3-3 chaperone was not affected by the phosphorylation event. Hence, it would be very interesting to elucidate how these modifications change FoxO localization, especially since those residues are located at the C-terminal transactivation domain. Sunayama et al. (2005) came up with a suggestion to the problem by showing that JNK actually phosphorylates 14-3-3 proteins too, and this modification dissociates it from its substrates.

In 2005 two groups working with the model organisms *C. elegans* (Oh et al. 2005) and *D. melanogaster* (Wang et al. 2005) demonstrated the physiological significance of the interaction between JNK and FoxO *in vivo*. Their work elegantly showed that the JNK-FoxO interface is critical in mediating organism homeostasis and defects in this regulation lead to premature death. Wang et al. (2005) took the mechanistic explanation even further by showing that JNK extends its function by a cell non-autonomous manner. This is achieved by activating dFoxO in the Insulin producing cells (IPC) thereby inhibiting *Drosophila* Insulin like peptide (DILP) release. Remarkably, flies overexpressing activated Hemipterous, a JNK activating kinase in the IPC cells, are smaller then the controls. This effect was dFoxO dependent since in the *dfoxo* null background the phenotype was reversed. These results suggest an interesting possibility that both Insulin and JNK signaling converge on FoxO having the opposite effects. An important downstream effect of this regulation would be the production of the DILPs, which guide the growth and metabolism of peripheral tissue. Accordingly, Insulin and JNK signaling are balancing the organism growth by perceiving and transmitting information about energy homeostasis and oxidative stress state, respectively.

AMPK

The AMP activated protein kinase (AMPK) is an energy sensing heterotrimeric (α, β and γ subunits) protein complex integrating the cellular AMP/ATP ratio into a response aiming to maintain energy homeostasis. AMPK is activated by high levels of 5'AMP which is antagonized by the increase in ATP concentration (Hardie & Sakamoto 2006). AMPK has both systemic and cell autonomous functions. In hypothalamus, AMPK regulates the production of neuropeptides involved in food intake and growth (Minokoshi et al. 2004). One important downstream target of AMPK is Tuberous sclerosis complex 2 (TSC2) (Inoki et al. 2003). By phosphorylating TSC2, AMPK inhibits the TOR pathway and has therefore an important impact on cellular growth. Given that FoxO is involved in cellular energy homeostasis by activating the mechanisms releasing energy from cell reserves it was not surprising that Greer et al. (2007a) found an interaction between AMPK and FoxO. The authors demonstrated that at least two novel FoxO3

sites, Ser^{413} and Ser^{588}, were phosphorylated by AMPK in mammalian cell culture. These sites are present in the transactivation domain and their phosphorylation confers the ability of FoxO to activate transcription but does not affect its sub-cellular localization or affinity to DNA. Interestingly, by using a combination of mutated FoxO3 protein and microarray profiling, Greer et al. (2007a) found a subset of FoxO3 regulated genes that were specifically up-regulated by AMPK through FoxO3. This experimental approach was able to unambiguously demonstrate that FoxO gene specificity is achieved upon differential phosphorylation by upstream activating kinases. Since the affinity to DNA was not affected by AMPK mediated phosphorylation, it is likely that the gene specificity is achieved by interaction with co-activators within the transcriptional apparatus. An intriguing possibility would be to use the AMPK insensitive FoxO3 mutant to identify these factors by comparing the binding partners of WT and mutant FoxO3. Importantly, the same group was able to show the conservation and biological significance of the AMPK-FoxO pathway in *C. elegans* (Greer et al. 2007b). By using genetic experiments the authors showed that life span increase induced by dietary restriction (DR) is mediated by the AMPK-FoxO interaction.

Taken together, the results of Greer et al (2007a, 2007b) suggest that upon attenuated cellular energy levels, AMPK becomes activated and further activates FoxO to mediate a transcriptional response. FoxO activated transcription then, in turn, enhances cellular mechanisms to protect cells from energy deprivation and stress. A Defect in this regulatory mechanism lead to premature death upon DR. Related to this topic is the possible interaction between AMPK and Insulin signaling. Activation of AMPK and increased Insulin sensitivity are known to correlate in muscle cells (Fisher et al. 2002). Both, elevated AMPK activity and Insulin sensitivity can be achieved by, for example, increased exercise. In addition, anti-diabetic drugs rosiglitazone and metformin activate AMPK in muscle cells (Fryer et al. 2002). Insulin receptor substrate 1 (IRS-1) is known to be phosphorylated by AMPK in cell culture and this leads to elevated signaling activity (Jacobsen et al. 2001). However, whether this activation is sufficient for the enhanced Insulin sensitivity is not known. It is tempting to speculate that FoxO might play a role in this setting through transcriptional feedback regulation of InR (Puig & Tjian 2005). In fact, FoxO gain of function has been shown to enhance Insulin sensitivity in the liver (Matsumoto et al. 2006). Further studies are necessary to elucidate a mechanism for these observations.

MST-1

MST-1 belongs to the so-called Hippo pathway, named after its first identified member in *Drosophila*, the Hippo-kinase. The pathway is known to restrict organ growth in *Drosophila* and mammals and dysregulation of the pathway leads to tumorigenesis (Dong et al. 2007). An elegant study of Lehtinen et al. (2006), representing an excellent characterization of protein-protein interaction, demonstrated the conserved interplay and biological function of the MST-1-FoxO proteins. In this paper the authors observed neuronal death induced by MST-1 mediated oxidative stress and found a transducer, FoxO3, responsible for the phenomenon. Not only they showed that MST-1 directly phosphorylate and activate FoxO3, but the authors were also able to illustrate the mechanism behind it; MST-1-dependent phosphorylation of four serines in the forkhead

domain dissociates FoxO3 from the 14-3-3 proteins and reallocates it to the nucleus. Finally, Lehtinen et al. (2006) took their findings into the model organism *C. elegans* to reveal the biological significance of the interaction. Their experiments demonstrated that the worm orthologue of MST-1 modulates life span in both loss- and gain-of-function conditions. In the case of long lived worms upon MST-1 overexpression, loss-of-function in *foxo* orthologue was able to suppress this phenotype. The same group also demonstrated similar interaction between MST-1 and FoxO1 in the in primary rat cerebellar granule neurons where the MST-1 induced neuron death was FoxO1 dependent (Yuan et al. 2009).

Biological functions of FoxO

Two common themes arise from the considerations described above on the regulation of FoxO activity. These are growth and protection from sub-optimal conditions such as energy deprivation and oxidative stress. Indeed, most of the reported FoxO functions are somehow related to these biologically essential and interconnected processes explaining the evolutionary conservation of FoxO between distant phyla. In the following section, the participation of FoxO in these processes and the consequences to organism well being are discussed. It has to be kept in mind that many, if not all, of the biological processes where FoxO is involved, are somehow linked and should be therefore considered as a whole. For example, the role of FoxO as a stress response agent upon DNA damage or nutrient shortage can not be separated from its function in cell cycle and energy metabolism and ultimately, in life span regulation.

Cell differentiation

Hribal et al. 2003 showed that myogenic differentiation, i.e. formation of multinuclear fusion cells, requires FoxO1 inhibition in mouse C_2C_{12} myoblast cells. This observation was in agreement with previous findings that IGF/PI3K/Akt signaling is required for the execution of myogenic program in these cells (Lawlor & Rotwein, 2000). Interestingly, FoxO1's role in this process seems to be the repression of certain differentiation inducing genes, such as *myogenin* (Hribal et al. 2003). According to the authors, relief of this repression by Akt mediated FoxO1 phosphorylation then induces cell differentiation. Remarkably, a completely opposite mode of action has been reported by Bois & Grosveld (2003). The authors demonstrate that FoxO1 nuclear localization and activity correspond and is required for mouse myotube fusion and differentiation. In addition, this regulation was independent of PI3K/Akt signaling. The striking contrast between the studies of Hribal et al. (2003) and Bois & Grosveld (2003) is most likely reflecting the differences in experimental systems which induced the differentiation. The myoblast fusion can be induced by either growing the cells into confluence or by sudden serum withdrawal, the latter being the commonly used method. These two *in vitro* differentiating procedures have opposite effects to FoxO1 localization and activity. This was later demonstrated by Bois et al. (2005). It is interesting to note how very different experimental approaches can result in similar outcome, in this case cellular differentiation. It emphasizes the critical evaluation of widely employed methodology. Nonetheless, in an elaborate study by Bois et al. (2005) the role of FoxO1 upon confluence induced differentiation was demonstrated. The authors showed that FoxO1 activity is required in the early steps

of myoblast fusion but it induces its own inhibition by induction of the expression of Cyclic GMP-dependent kinase I (cGKI). The cGKI was shown to phosphorylate and inhibit FoxO1. Both, early activity and later inhibition are required for proper myogenic differentiation. Similar to the myoblasts, modulation of FoxO1 activity has also been shown to be correlated with adipocyte differentiation (Nakae et al. 2003). In these experiments differentiation is induced *in vitro* by addition of Insulin into the culture medium. How accurately these differentiation methods reflect the situation of myoblast/adipocyte differentiation *in vivo* is yet to be clarified.

Growth

Growth is fundamental in maintaining and reproducing all forms of life. Growth can be separated into two distinct entities: (1) cellular growth, i.e. an increase of cell mass or volume achieved by accumulation of biomolecules, such as proteins and lipids, and (2) tissue/organism growth through cell proliferation, i.e. coordinated division of individual cells within a tissue or organism. Cellular growth and cell division are coupled since a cell needs to reach a critical size before its separation into two daughters can take place. Cellular growth and cell division rate within an organism varies significantly depending on several parameters such as developmental stage, cell type and prevailing nutritional conditions, and it is regulated through various signaling pathways (Jorgensen & Tyers 2004). Further, cell growth and division are regulated in a cell autonomous and non-autonomous manner, the latter being modulated, for example, by humoral signals elicited from nutrient sensing organs such as pancreatic beta and alpha cells. FoxO has a well characterized role in cell autonomous regulation of cell division as a player in the complex Insulin/TOR signaling network (Figure 1). Defects in this regulatory circuit are known to associate with a plethora of human carcinomas (Altomare & Testa 2005). In addition, recent advances in understanding *Drosophila* growth regulation through non-autonomous hormonal signals in the organism level have proposed a central role for FoxO in this process too.

Cell autonomous growth regulation

The finding that FoxO is situated in the location of chromosomal breakpoint associated with cancer by Galili et al (1993) gave the first indication of its involvement in cell proliferation control. In spite of significant progress in the field since then, it was not until recently that Paik et al. (2007) directly demonstrated that FoxO is indeed a tumour suppressor. The authors developed a conditional triple knockout mouse (*foxo1, foxo3* and *foxo4*) and found that this genotype developed aggressive lymphoblastic thymic lymphomas in addition to the age-progressive hamartomatous phenotype. However, since *foxo1* null mice are embryonic lethal, the most comprehensive evidence of its growth regulatory function *in vivo* comes from the studies of *Drosophila* pioneered by Puig et al. (2003) and Junger et al. (2003). Their work demonstrated that overexpression of dFoxO inhibits proliferation, but not cell growth, in cell culture as well as in the fly eye and wing. At least part of the tissue size reduction was achieved by necrotic cell death, caused by the dFoxO overexpression above physiological levels. Interestingly, the phenotype was found to be enhanced by reduced Insulin signaling consistent with its role as an inhibitor of FoxO activity (Junger et al. 2003). A concurrent study by Kramer

et al. (2003) reported also attenuated proliferation *in vivo*, but in contrast to previous observations, it was accompanied by reduced cell size. It must be noted that the cloned dFoxO protein used by Kramer et al. (2003) was truncated containing ten exons instead of eleven, and therefore possibly retained only partial activity. This is apparent from their overexpression studies where mild ubiquitous overexpression of the truncated dFoxO protein yielded viable, small flies whereas overexpression of the full length protein is lethal (Puig et al. 2003). Although difficult to explain, the results of Kramer et al. (2003) might reflect altered FoxO activity and/or gene specificity and should be interpreted with caution.

The observation that cell size is not affected *in vivo* was at first surprising since Puig et al. (2003) and Junger et al. (2003) identified the eIF4E-binding protein (4EBP) as a direct transcriptional dFoxO target. 4EBP binds to the eukaryotic Initiation factor 4E (eIF4E) and inhibits its binding to mRNA cap structure and initiation of translation (Mamane et al. 2006). The role of 4EBP as a negative growth regulator and as a target of TOR signaling has been well characterized in cell culture (Wullschleger et al. 2006). However, the general view about 4EBP is now being challenged by *in vivo* studies in *Drosophila*. Teleman et al. (2005) demonstrated that 4EBP is dispensable during normal growth but instead has a surprising function in fat metabolism. In fact, a new role of 4EBP as regulator of energy metabolism upon nutrient deprivation has emerged (see below). These results highlight the importance of *in vivo* models to complement and verify the cell culture studies.

The function of FoxO as a regulator of proliferation was compromised by the finding that homozygous null *dfoxo* alleles were viable and manifested no obvious size phenotype (Junger et al. 2003). Actually, these flies have a slightly reduced wing size which is the opposite of what would be expected. However, it was found that the null *dfoxo* allele suppressed the growth phenotype of reduced Insulin signaling, characterized by smaller and fewer cells. Interestingly, only the cell number phenotype was suppressed whereas cell size was unaffected, suggesting that FoxO indeed mediates only the proliferative function of Insulin signaling (Junger et al. 2003). Taken together, the results from gain- and loss-of-function experiments are in favour of a view, that FoxO is dispensable during normal tissue growth but is necessary to hinder the growth rate whenever Insulin signaling is low, i.e. upon lack of nutrition or in conditions of stress. Therefore, under physiological growing conditions Insulin signaling is sufficient to keep FoxO phosphorylated and inactive. Several studies in mammalian cell culture support this hypothesis (Collado et al. 2000, Medema et al. 2000, Nakamura et al. 2000, Kops et al. 2002). An interesting, still unresolved question is how FoxO is released from its inhibitory state? Is Insulin signaling always shut off when FoxO recovers its activity or is there a mechanism to overcome this inhibition without affecting the Akt activity?

Cell non-autonomous growth regulation

Growth in metazoan organisms is coordinated by signals, i.e. growth factors and hormones, emanating from endocrine tissues such as the pancreas and hypothalamus. In *Drosophila*, a major growth organizer at organism level is the Insulin-Adipokinetic hormone (Akh) axis. Insulin and Akh are synthesized from neuroendocrine cells of the bilateral pars intercerebralis in the central nervous system (i.e. Insulin producing cells,

IPCs) and of a subset of corpora cardiaca (CC) in the ring gland, respectively (Rulifson et al. 2002). The hormones are carried through axon projections to the heart from where they are released to the haemolymph (Rulifson et al. 2002). The system operating in the fly is considered functionally analogous to the Insulin-Glucagon hormone control of energy metabolism and growth operating in mammals. The idea is supported by several studies showing defects in growth as well as lipid and carbohydrate metabolism in IPC ablated flies (Rulifson et al. 2002, Ikeya et al. 2002, Broughton et al. 2005). The analogy was recently formally demonstrated by Buch et al. (2008) whose results for the first time identified a downstream target gene for the fly Insulin in the peripheral tissues, encoding an alpha Glucosidase enzyme. The IPC cells produce three of the seven *Drosophila* Insulin like peptides (DILPs 2, 3 and 5), which are structurally similar to the human preproInsulin (Brogiolo et al. 2001). Interestingly, the expression of these peptides is modulated by dFoxO. Overexpression of dFoxO in the fly head fat body by a conditional driver caused a threefold decrease of *dilp2* expression, whereas expression of *dilp3* and *dilp5* were only slightly affected (Hwangbo et al. 2004). In addition, Wang et al. (2005) showed by loss-of-function studies that dFoxO is modulating *dilp2* expression in the IPCs and that it has direct consequences to the fly growth. These studies indicate that dFoxO is suppressing the Insulin production in the fly thereby inhibiting growth in a systemic fashion. Whether this interaction is direct or not is still unresolved. FoxO is also known to have systemic growth regulatory function in mammals by modulating pancreatic β cell proliferation and indirectly, through Pancreatic and duodenal homeobox factor-1, Insulin production (Gross et al. 2008). In addition, another function has been addressed to FoxO as a regulator of mammalian food intake through neuropeptide Y in the hypothalamus (Kim et al. 2006). Similar neuropeptide dependent growth regulation exists in *Drosophila* although the role of FoxO in this setting has not yet been addressed (Lee et al. 2004).

Cell cycle

The growth inhibitory function of FoxO requires a mechanistic explanation at the level of cell cycle regulation. Insight to this problem came from the studies of Medema et al. (2000) and Seoane et al (2004) who found that FoxO regulates the expression of $p27^{kip1}$ and $p21^{cip1}$ in several mammalian cell lines. The function of these proteins arrests the cell cycle in G_1. The $p27^{kip1}$ and $p21^{cip1}$ proteins are important CDK inhibitors that interact with various cyclin-CDK complexes. One of the cell division promoting functions of the Insulin signaling is the phosphorylation and inhibition of $p27^{kip1}$ through Akt (Fujita et al. 2002). Hence, carcinomas with hyperactive Insulin signaling suffer from the loss of $p27^{kip1}$ expression and from inhibition of its activity by elevated phosphorylation. Another important cell cycle regulator, Cyclin D1/2 was shown to be repressed by FoxO (Ramaswamy et al. 2002). This down-regulation also results in G_1 arrest. The exact mechanism of repression is yet to be clarified, however, since it is possibly an indirect interaction. In addition to G_1 arrest, Tran et al. (2002) have presented evidence suggesting that FoxO also has a role in regulating the entrance into mitosis at the G_2 DNA integrity checkpoint. Their data show that FoxO3 activity delays the G_2 progression and induces DNA repair mechanism upon genotoxic stress. They also identified a candidate FoxO

target gene *growth arrest and DNA damage-inducible protein 45* (*gadd45*) as mediator of the response.

As outlined above, multiple pieces of evidence indicate a role for FoxO as a factor slowing down or blocking completely the progression of the cell cycle. It is therefore understandable that the tumour promoting function of elevated Insulin signaling is at least partly mediated by the lack of FoxO activity. Keeping this in mind, it is surprising that FoxO is necessary for the successful completion of the cell cycle in mammalian cells. This staggering discovery was made by Alvarez et al. (2001). The author's initial finding was that shutting down the PI3K signaling pathway activity was required for a cell to exit mitosis. They discovered that the activity of the pathway was gradually hindered towards the end of G_2 allowing FoxO1 to enter to the nucleus, which in turn, activated *cyclin B* and *polo like kinase* expression. The results of Alvarez et al. (2001) explain why a subset of cells in a growing population always possesses nuclear FoxO, a phenomenon often encountered in the literature.

In summary, FoxO appears to have seemingly opposite functions in the cell cycle regulation depending on the stage that it is activated. In G_0 or G_1 poor growing conditions arrest the cell from committing to the cell cycle. This explains the negative effect of elevated FoxO activity to tissue growth observed in *in vivo* experiments (Puig et al 2003, Junger et al. 2003). On the other hand, oscillation in FoxO activity is a requirement in successful completion of the cell cycle. However, this function only affects a relatively small number of cells already committed to cell cycle progression. In both stages the activity of Insulin signaling inversely correlates with FoxO activity. How this fluctuation in Insulin signaling activity is achieved in the presence of growth factors is a fascinating question. A solution to this problem would definitely open new gates in modulating Insulin signaling activity in pathogenic conditions, such as cancer.

Energy metabolism

The maintenance of correct blood glucose level is pivotal to organism homeostasis. An adaptive regulatory mechanism has evolved in metazoan organisms to respond to the lack of consumed energy between meals or upon prolonged starvation. During fasting, glucose is released from energy supplies stored in the form of glycogen and fat. The process is regulated through the action of Insulin and Glucagon, key metabolic hormones produced by the pancreatic beta and alpha cells, respectively.

FoxO proteins have a well characterized role in lipid and glucose metabolism in various tissues such as skeletal muscle, liver and pancreas. The regulation of organism energy homeostasis by FoxO is achieved by either direct means, through modulation of the expression of several genes involved in gluconeogenesis and triglyceride hydroxylation, or by indirect means by modulating the skeletal muscle and adipocyte tissue mass, the major glucose disposing tissues in humans (Gross et al. 2008). In essence, upon hypoglycaemia and subsequent attenuation of circulating Insulin, hepatic FoxO1 is localized into the nucleus where it activates a pattern of gene expression devoted to gluconeogenesis and lipid catabolism (Zhang et al. 2006). These include the key gluconeogenic enzymes Glucose-6-phosphatase (G6Pase) and Phosphoenolpyruvate carboxykinase (PEPCK) (Schmoll et al. 2000, Nakae et al. 2001, Zhang et al. 2006). As a result, the liver provides energy to the peripheral tissues by releasing glucose into

the bloodstream. In parallel, FoxO induces the utilization of fatty acids in myocytes as an energy source which is a characteristic response to fasting and exercise (Bastie et al. 2005). The metabolic effect of FoxO in the adipose tissue is less well understood. A known FoxO modulator Sirtuin 1 mobilizes fat from the white adipocyte tissue in mice (Picard et al. 2004). Whether or not FoxO is involved in this process is so far unknown. However, the importance of FoxO as a mediator of nutrient responsive gene expression is highlighted by a study of Gershman et al. (2007). The authors used the model *Drosophila* and high throughput gene expression pattern profiling to reveal genes regulated by energy level. An astonishing 28% overlap of genes regulated by nutrients and FoxO was observed.

The physiological outcome of FoxO activation during energy shortage is to promote survival upon starvation, which is achieved by parallel mobilization of energy reserves and slowing down the growth rate. The direct involvement of FoxO in starvation resistance at the organism level has been studied in *Drosophila*, which is an organism amenable to large scale survival experiments. Surprisingly, Junger et al. (2003) found that *foxo* null adult flies were as sensitive to complete lack of nutrients as the controls. However, a more refined study was conducted by Kramer et al. (2008) who surveyed the resistance of *foxo* null larvae and adults to lack of carbohydrates and proteins separately. Indeed, it was found that in a diet consisting solely of carbohydrates the *foxo* null individuals died sooner then the controls. This result suggests a critical role for FoxO in the amino acid metabolism. An indirect indication of FoxO's role in this process has come from studies where flies have experimentally reduced Insulin signaling activity. These flies exhibit increased storage of carbohydrates and lipids and are more resistant to starvation (Broughton et al. 2005). In addition, Tettweiler et al. (2005) demonstrated that protein levels of a FoxO target, 4EBP, are elevated upon complete lack of nutrients in *Drosophila* and this mediates the response to starvation resistance. Similar finding was done by Teleman et al. (2005) who also discovered that *4ebp* null flies burn their fat reserves faster than control flies and are therefore more susceptible to starvation. This result suggests that FoxO has an important role in the adipocyte tissue metabolism. Both groups, Teleman et al. (2005) and Tettweiler et al. (2005) also showed the involvement of FoxO and 4EBP in resistance to oxidative stress. Starvation and oxidative stress resistance are thought to have a common genetic background (Wang et al. 2004).

In spite of the numerous studies, the role of FoxO in protection from nutrient deprivation is still unclear. Our understanding of this problem is incomplete and will require more efforts to gain a clear distinction of the metabolic processes regulated by FoxO. One drawback in designing experiments aiming to resolve this question is the developmental lethality of FoxO overexpressing flies. Hwangbo et al. (2004) and Giannakou et al. (2004) have overcome this problem by utilizing a conditional overexpression system which is induced at the adult stage. However, this system has not yet been employed in starvation resistance studies.

Life span

The life span of an organism is determined by its ability to maintain physiological processes, i.e. genetic stability, telomere shortening, stress resistance and metabolic control, decreasing the probability of death (Katic & Kahn 2005). A well known model

for life span extension is caloric restriction which is shown to increase organism life expectancy in invertebrates and vertebrates including mice (Weindruch & Walford 1982) and nonhuman primates (Lane et al. 2000). In addition, caloric restriction (also known as dietary restriction) seems to have beneficial effects in human health although the experimental evidence is still, for obvious reasons, very limited (Fontana et al. 2004). The mechanisms behind life span increase mediated by caloric restriction are complex and not completely understood. This complexity is highlighted by the intriguing finding of Mair et al. (2005) whose results indicate that calories themselves do not explain the longevity of caloric restricted *Drosophila*, since flies kept on a protein diet lived longer than flies on a carbohydrate diet. Nevertheless, strong evidence points to a role for lowered metabolism associated with decreased oxidative stress, which is in agreement with the generally accepted concept of free-radical theory of aging (Heilbronn & Ravussin 2003, Harman 2006). Caloric restriction is known to modulate multiple physiological processes including Insulin signaling through reduced circulating Insulin and IGF-1 (Heilbronn & Ravussin 2003). It is therefore not surprising that genetically manipulated model organisms with defects in Insulin signaling are long lived (Katic & Kahn 2005). In 1997, two groups using the model organism *C. elegans* demonstrated that the life span extension mediated by Insulin signaling was suppressed by loss of function of FoxO (Lin et al. 1997, Ogg et al. 1997). This result raised the attention towards FoxO as a mediator of long life.

Perhaps the most intriguing property of FoxO biology is its ability to regulate organism life span. The underlying mechanisms and its relationship to dietary restriction are at the moment under intense investigation. Many of the studies are conducted in the fruit fly which has a plethora of genetic and experimental tools suitable for life span experiments. The first demonstration that FoxO directly regulates life span in *Drosophila* came from the pioneering studies of Hwangbo et al (2004) and Giannakou et al. (2004). By overexpressing FoxO in the fat body they were able to increase the fly's median life span by approximately 50%. In addition, Wang et al. (2005) showed that JNK mediated life span extension was also mediated through FoxO in flies. Taken together, the results from model organisms suggested that dietary restricted animals extend their life span by lowering the activity of Insulin signaling and consequently, by increasing the activity of FoxO. This would further lead to increased overall stress resistance and, as a by-product, increased longevity. Surprisingly, a direct experiment conducted by Giannakou et al. (2008) failed to demonstrate the accuracy of this hypothesis. The authors showed that flies with a *foxo* null mutation responded equally as the control flies to reduced calorie uptake. This result raises the possibility that dietary restriction and Insulin signaling mediates longevity through separate routes. More effort and experimental scrutiny is needed, however, to unambiguously demonstrate whether this is the case. For example, significant emphasis should be put into the composition of the diet used in these experiments in order to have comparable results between different labs. It is also important to conduct experiments with males and females separately given the difference in resource allocation between reproduction and cellular maintenance between sexes (a central matter often neglected in the literature). Hence, there is an urgent need for general guidelines for performing calorie restriction experiments in *Drosophila* as well as in other model organisms.

AIMS OF THE STUDY

This work has aimed to deepen the understanding of processes regulated by FoxO transcription factor. The study has involved the upstream regulatory network around FoxO as well as its downstream target genes and their function. The study was motivated by the following findings: (1) Hyperactivated TOR signaling (as a result of *tsc1/2* loss-of-function) results in a relatively modest tissue overgrowth phenotype compared to the *pten* loss-of-function. This controversy is manifested in the benign and malignant nature of the tumours associated with *tsc1/2* and *pten* loss-of-function in humans, respectively. (2) dFoxO overexpression in cell culture results in a very high activation of the cAMP signaling pathway component *adenylate cyclase 76e* (*ac76e*) expression. In addition, a more general system biology approach was applied in order to reveal completely new aspects of the regulatory network upstream of FoxO. Hence, the specific questions addressed in this study where:

(1) What is the role of FoxO in tissue overgrowth phenotype produced by *tsc1* loss-off-function?

(2) What is the biological relevance of the interaction between dFoxO and Adenylate cyclase AC76E in *Drosophila*? What is the nature of this regulation and what are its consequences to fly development and metabolism?

(3) What are the kinases and phosphatases regulating the transcriptional activity of dFoxO?

MATERIALS AND METHODS

Brief explanations of the essential methods and materials used in this study are presented below. For a more detailed description of how a given method or material was applied, see the appropriate article.

Cell culture (I, II, III)
The *Drosophila* cell line used throughout this study was Schneider 2 (S2). The mammalian cell lines were mouse hepatoma cells (HEPA1-6) and human embryonic kidney cells (HEK293). The S2 cells in all experiments were maintained, treated with dsRNA and transfected in M3 (Sigma) supplemented with Insect Medium Supplement (Sigma), 2% FBS, penicillin and streptomycin. All the transfections were performed with either Effectene (Qiagen) or Fugene HD (Roche) transfection reagent according to the manufacturer's protocol. If a metallothionin promoter was utilized to induce protein expression, 600μM $CuSO_4$ was induced into the medium to initiate the gene expression. Additives used in cell culture were Human recombinant Insulin (1-10μg/ml) (Sigma), MG-132 (10-20μM) (Sigma), NH_4Cl (20mM), Forskolin (50μM) (Fluka) or IBMX (100μM) (Fluka).

Luciferase assay (I, II, III)
Luciferase assays were used mainly for two purposes: (1) to measure the FoxO activity by utilizing a known FoxO binding sequence upstream of a Luciferase open reading frame or (2) to narrow down a promoter region necessary for FoxO transcriptional activity. Luciferase assays were performed with Promega luciferase assay reagents. Briefly, cells were collected, washed with PBS and lysed in passive lysis buffer (Promega) for 20-30 minutes at room temperature. After luciferase measurement, the total protein content was measured from the samples by Bradford reagent (Bio-Rad). The luciferase values were normalized to the amount of protein in the sample. If necessary, the lysates were mixed with 3XSDS loading buffer and analyzed by Western blotting.

RNA interference (II, III)
RNAi assays in S2 cells were performed in the following way. Double stranded RNA (dsRNA) molecules were synthesized by *in vitro* transcription (Ambion) from PCR generated templates having a T7 promoter attached to both ends. The length of the RNA molecules ranged from 200 to 1000 base pairs. The RNA molecules were heated to 95°C and slowly cooled down to room temperature to yield correct annealing. The dsRNA was diluted to 1μg/μl in dH_2O and was used in various concentrations in the cell culture medium. Normally, 5μg/ml is sufficient to knock down the gene of interest. The cells were then grown for 2-5 days after which they were processed for downstream applications. The RNAi experiments in the mammalian cell culture were done by transfecting plasmids expressing 29-mer hairpin RNAs (Origene). The knockdown efficiency was estimated with Western blotting with the corresponding antibody, or if such an antibody was not available, by quantitative RT-PCR (qPCR).

Band shift assay (I, III)
Band shift assays were used to assess the binding of recombinant purified dFoxO protein to a certain DNA fragment. These fragments were promoter regions containing putative FoxO recognition elements (FREs). Briefly, Recombinant dFoxO with 6xHis-Tag was recovered from *E. coli* by Ni-NTA metal chelate affinity chromatography. Purity and amount of protein was assessed by SDS-PAGE/ Coomassie Blue staining and Bradford assay. Purified dFoxO (100-200nM) was incubated with radioactively labelled (^{32}P-dCTP) PCR fragment (80nM) containing the putative FRE for 20 minutes at room temperature in reaction conditions described in Coleman and Pugh (1997). The positive and negative controls were fragments of the *dinr* promoter or pBs, respectively. The resulting DNA-protein complexes were resolved by native PAGE and were exposed to phosphor imager plates (Fuji).

Cyclic AMP measurement (III)
The concentrations of the cAMP in cell culture or tissue samples were measured by competitive enzyme immunoassay (Assay Designs). Either cells or tissue samples were lysed in 0.1M HCl after which they were measured by the manufacturer's protocol.

Quantitative RT-PCR (I, II, III)
QPCR was performed using the SYBR green methodology in the ABI Prism 7000 sequence detection platform (Applied Biosystems). The results were analyzed using the comparative CT method and normalized with *Drosophila actin*, *rp49*, *tubulin* or human *actin* genes. The primers used in the qPCR assays were designed using the Vector NTI software (Invitrogen) and were always tested for their specificity and their amplification efficiency.

High throughput microscopy (II)
The Cellomics Arrayscan 4.5 high throughput microscope was utilized. This system was used to measure either the amount of FoxO induced reporter representing its activity or the ratio of FoxO protein between nucleus and cytoplasm. The Arrayscan system can score the intensity of a fluorescent protein from thousands of cells or cell compartments in a reasonable time window giving the possibility to integrate large material for analysis. It is therefore a very useful tool to measure subtle differences in FoxO localization that would have been unnoticed by the traditional "eyespotting" method.

Transgenic animals (III)
The injections of constructs into *Drosophila* embryos to produce transgenic animals were done by the Genetic Services Inc. company (USA).

RNA *in situ* hybridization (III)
RNA *in situ* was performed using either *Drosophila* embryos or larval/adult tissue. Digoxigenin-UTP (Roche) labelled sense or anti-sense probes were utilized. In the case of embryos the vitelline membrane was removed by brief bleach treatment and the tissue was subsequently fixed. The probes were hybridized to fixed embryos or adult/larval tis-

sue for 16h at 55°C. After washing away excess probe the hybridization was visualized by alkaline phosphatase conjugated anti-DIG antibody (1:3000, Roche).

Antibodies used for western blotting (II, III)

Antibody	Dilution	Source
V5	**1:5000**	Invitrogen
Akt	**1:1000**	Cell signaling technology
AktSer505	**1:1000**	Cell signaling technology
α-Tubulin	**1:10000**	Sigma
HA	**1:3000**	Covance Research products
FLAG	**1:1000**	Sigma
dFoxO	**1:1000**	Puig et al. (2003)
GFP	**1:500**	Santa Cruz Biotech.

RESULTS

dFoxO induces the expression of scylla *and* astray *(I)*

Previously, genetic evidence has been presented that activity of dFoxO attenuates tissue growth when dTOR pathway activity is elevated (Junger et al. 2003). TOR pathway activation can be genetically achieved by deleting the *tsc1/2* gene, whose function is to restrict the pathway upon sub-optimal growth conditions. To understand how the observed growth regulation by dFoxO is achieved, the transcriptional profiles of tissues with elevated dTOR signaling, as a result of *tsc1* null mutation, and S2 cells overexpressing dFoxO were compared (Article I, Figure 2). A significant overlap was found, indicating that some of the transcriptional response of *tsc1* null tissue was achieved through the activity of dFoxO. Among the overlapping transcriptional profiles, some genes were already shown to be direct dFoxO targets, such as *4ebp* and *pepck*. However, many others were still putative targets. Hence, to further demonstrate that dFoxO indeed is responsible for the induction of these genes, we decided to further characterize dFoxO binding sites from the promoters of two of those genes, *astray* and *scylla*. Luciferase assays were used to narrow down the region of dFoxO binding, which led to the finding of putative FREs. Therefore, band shift experiments were performed to demonstrate the direct binding of dFoxO to those fragments *in vitro* (Article I, Figure 3). Subsequently, we showed the up-regulation of these genes by dFoxO *in vivo* (Article I, Figure 4). Finally, we demonstrated that similar interaction takes place in the mammalian cell culture too; FoxO1 was shown to regulate the expression of several mammalian orthologues of the identified dFoxO targets. These included the *scylla* and *astray* orthologues *redd1* and *heat shock protein 23* (Article I, Figure 5).

Regulatory network around FoxO (II)

An RNAi screen for kinases and phosphatases regulating dFoxO transcriptional activity was performed. All the known and predicted *Drosophila* kinases (251 proteins) and phosphatases (86 proteins) were screened. A primary screen, based on the activity of a reporter GFP driven from a dFoxO sensitive promoter (4xFRE promoter), was used to narrow down the 337 genes to thirty one putative dFoxO regulators. Importantly, among these genes, several of the already known FoxO regulators were present, confirming the validity of the approach. The thirty one candidates were then further characterized in a secondary screen for their ability to affect dFoxO transcriptional activity, protein abundance and sub-cellular localization. Out of the thirty one candidates, we found twenty one regulating at least one of these processes (Table 2 and Article II, Figure 2), therefore being confirmed as dFoxO regulators.

To explore if the network of regulators is conserved in mammals, we decided to assess the ability of some of our hits to modulate FoxO transcriptional activity in human embryonic fibroblasts (HEK-293) and mouse hepatoma (HEPA1-6) cells. We found that eight hits caused a robust increase in FoxO3 transcriptional activity measured by a luciferase reporter construct (Article II, Figure 4 and S4). Positive hits in the mammalian assay include kinases such as Diacylglycerol kinase δ2 (DGKδ2), Vascular endothelial growth factor receptor 1 (FLT-1) and Glycogen synthase kinase-3β. These results

Table 2. Hits obtained from the screening.

Kinase/Phosphatase	Transcriptional activity	Protein stability	Localization
CG17026	+	-	-
CG7177	+	+	+
CG7597	+	+	-
Cyclin dependent kinase 9	+	+	+
Diacylglycerol kinase d	-	-	+
Four wheel drive	+	-	-
Gilgamesh	+	-	-
Greatwall	+	-	+
Ire-1	+	+	-
Meiotic 41	+	+	-
Neorospecific receptor kinase	-	+	-
NinaC	+	-	-
Pdgf- and vdgf-receptor related	+	-	-
Protein kinase C	+	+	+
Polo	+	-	+
Protein kinase-like 17e	+	+	-
Protein tyrosine phosphatase 69d	-	-	+
Shaggy	+	-	-
Skittles	+	-	-
Strechin-mlck	+	-	-
Tao-1	+	-	+

+ significant deviation, - no difference

significantly strengthened our analysis and revealed that the regulatory network around FoxO is conserved in metazoans.

The most prominent hit in our screen was PKC53E, the *Drosophila* ortholog of the mammalian Protein kinase C alpha (PKCα). The knockout of the function of this gene in S2 cells had a very strong effect on dFoxO protein abundance as well as on localization, resulting in a reduction of dFoxO protein levels as well as a marked decrease of dFoxO in the nucleus. We found that results from PKC53E knockout were specific since simultaneous overexpression of PKC53E rescued the dFoxO sub-cellular localization phenotype. In addition, overexpression of PKC53E alone resulted in the opposite effect, an increase in dFoxO transcriptional activity and nuclear localization. The effect of PKC53E knockout was confirmed with endogenous dFoxO protein. We also explored the possibility that the PKC-dFoxO interaction is conserved in mammals. We used RNAi to knockout PKCα in HEK293 cells and found a similar effect as with the S2 cells: FoxO3 protein abundance was decreased in conjunction with its transcriptional activity as measured by a luciferase reporter (Article II, Figure 3 & 4). In summary, these results

demonstrate that PKC53E and its closest mammalian orthologue PKCα are regulators of FoxO activity.

FoxO is a regulator of cAMP signaling in Drosophila *(III)*

A microarray study of cells overexpressing constitutively nuclear dFoxOA3 mutant in S2 cells revealed *adenylate cyclase 76e* (*ac76e*) gene highly activated in this setting (Puig et al. 2003). We confirmed this transcriptional activation by using both qPCR and a luciferase based reporter assay. dFoxO was shown to increase the expression of *ac76e* several fold. We then mapped the putative FREs in the *ac76e* promoter and binding of dFoxO to these sequences was assessed by *in vitro* band shift assays and *in vivo* by chromatin immunoprecipitation (ChIP) (Article III, Figure 1). We cloned the *ac76e* ORF into an expression vector and showed that it encodes a functional protein with adenylate cyclase activity (Article III, Figure 2).

To further characterize the functional interaction between dFoxO and AC76E, we performed studies in flies. The expression pattern of the *ac76e* gene was analyzed by RNA *in situ* hybridization during embryonic development as well as in the third instar larva and adult flies. We found that *ac76e* has a very precise expression pattern throughout fly development. *ac76e* is strongly expressed in the third instar larva and adult corpus allatum (CA), and this expression is dependent on dFoxO since in the *dfoxo* null strain *ac76e* expression was notably reduced. Further evidence was provided from the observation that cellular cAMP levels were reduced in *dfoxo* null CA (Article III, Figure 3). Taken together our results present strong evidence that dFoxO has a role in modulating the cAMP signaling by activating the expression of *ac76e* gene *in vivo*.

dFoxO regulates Drosophila *development, size and starvation resistance through Adenylate cyclase 76E (III)*

To explore the role of the dFoxO-AC76E interaction *in vivo* we generated flies with a UAS-AC76E transgene, which is induced by the targeted spatiotemporal expression of Gal4 transcriptional activator. We employed a highly specific CA Gal4 line DI-11 to drive AC76E overexpression in those cells (Belgacem & Martin 2007). Overexpression of AC76E in CA caused retardation in larval development, manifested by delayed pupae formation, which was accompanied with reduced adult size (Article III, Figure 4). In addition, we sought the possibility that overexpression of AC76E in CA would modulate adult starvation resistance. Interestingly, we found that starvation resistance was enhanced, but only in females (Article III, Figure 5). Thus our results identify AC76E as a key regulator of stress resistance and development.

DISCUSSION

FoxO is a conditional, Insulin/TOR signaling dependent regulator of tissue growth (I)

The idea that FoxO regulates tissue growth conditionally in instances when Insulin signaling activity is low was introduced at 2003 by Junger et al. It was also known for some time that TOR signaling induces a negative feedback loop by S6K mediated phosphorylation of the Insulin receptor substrate (IRS), thereby shutting down the Insulin signaling pathway (Haruta et al. 2000, Manning 2004). Presumably, this mechanism would ensure that cells do not over-proliferate upon conditions of abundant nutrient/growth factors. We have shown that elevation of TOR signaling leads to activation of FoxO. This result is in favour of the idea that FoxO is part of the negative feedback regulation induced by attenuated TOR signaling. Whether this interaction is through a direct phosphorylation of FoxO by TOR/S6K or through PI3K/Akt inhibition is not known (Figure 1). Evidence supporting the latter can be found from the literature; Manning et al. (2005) have shown that *tsc2*$^{+/-}$ mice develop liver hemangiomas with nuclear FoxO1. However, the liver hemangiomas with double heterozygote *tsc2*$^{+/-}$ *pten*$^{+/-}$ mice were more severe and showed predominantly cytoplasmic FoxO1 localization, suggesting that regulation of FoxO1 in this setting goes through PI3K/Akt. This observation together with the finding that mouse embryonic fibroblasts (MEFs) lacking *tsc1/2* gene function exhibit strong attenuation of Akt and loss of FoxO regulation, strongly supports that elevated TOR signaling leads to down-regulation of Insulin signaling in these cells (Manning et al. 2005).

In this study, we tried to solve the question of how dFoxO activity is modulated by dTOR signaling but unfortunately the question remains unanswered. Our experiments failed to demonstrate the interplay between activated dTOR and Insulin signaling leading to regulation of dFoxO. For example, in S2 cells down-regulation of dTOR activity through RNAi mediated knockout of dTOR or dRaptor did not significantly change dFoxO localization (J. Mattila, unpublished observation). This result suggests that dTOR signaling affects dFoxO independently of the IRS mediated negative feedback loop. Evidence supporting this view has been presented by Luong et al. (2006) who showed that the Insulin insensitive dFoxOA3 mutant activity is dependent on dTOR signaling *in vivo*. It is therefore possible that dTOR modulates the activity of dFoxO independent of PI3K/Akt signaling. Interestingly, Patel & Tamanoi (2006) demonstrated that increased dTOR signaling sensitizes the flies to oxidative and nutritional stress. This is in complete contrast to the idea that dFoxO is activated through dTOR since in all the reported cases augmented FoxO activity leads to elevated stress resistance. Further research is needed to illustrate the regulatory mechanisms behind this complex network.

Regardless of the mechanism, our results clearly demonstrate that FoxO has a role in restricting overgrowth upon TOR hyperactivation. In addition, we showed that FoxO induces the expression of *scylla* and its mammalian homologue *redd1*. This gene is known for its negative impact on TOR signaling in mammals and in *Drosophila* (Reiling & Hafen 2004, Corradetti et al. 2005). It is therefore possible that FoxO plays an important role as a transducer of the negative feedback loop on TOR signaling ensuring ordered growth control upon abundant growth factors. The function of FoxO in this con-

text could also provide an explanation for the benign nature of the tumours in the human genetic disorder called tuberous sclerosis (Curatolo et al. 2008).

FoxO activity is modulated by an elaborate regulatory network (II)

The data shown in the original article II demonstrate that FoxO activity is under the control of several signaling pathways which balance its activity to meet the prevailing environmental conditions in terms of nutrient availability and stress. Several known dFoxO regulators were found in our screen and twenty one new dFoxO regulators were identified including nineteen kinases and two phosphatases. According to the *Drosophila* gene annotation (Flybase), among the hits were three inositol-lipid metabolism associated genes (*cg17026*, *fwd*, *sktl*), two cyclin dependent kinase genes (*cg7597*, *cdk9*), two PKC signaling associated genes (*dgkd*, *pkc53e*), two WNT-signaling associated genes (*gish*, *sgg*), three cell cycle associated genes (*gwl*, *mei-41*, *polo*) and four transmembrane receptor genes (*nrk*, *pvr*, *ptp69d*, *tao-1*). Based on our screening assays, the dFoxO regulators were classified upon their ability to modulate cytoplasmic localization, protein stability or transcriptional activity (Table 2). Several dFoxO regulators were identified with high probability. This statement is based on the scrutiny of the screen. In total, the hits were analysed with four different assays in the *Drosophila* S2 cells. In addition, an accompanying survey of the mammalian orthologues to regulate FoxO3 transcriptional activity was performed. Indeed, nine out of the ten tested orthologues were found to regulate FoxO3 activity in mammalian cell culture.

Among the hits, several interesting candidates exist. These include, for example, PKC, GSK-3β (*sgg*) and POLO. GSK-3β and POLO are implicated in glucose metabolism and cell cycle regulation, respectively, and PKC isoforms are well known for their role in the Insulin signaling pathway (Sampson & Cooper 2006). Additionally, a PKC upstream activating kinase, DGKδ was identified from the screen as a FoxO regulator. This finding further strengthens the idea that PKC is an important FoxO activity modulator. Interestingly, in our experimental conditions PKC did not modulate Akt phosphorylation, implying that the interaction between FoxO and PKC is independent of Insulin signaling. Co-immunoprecipitation experiments with overexpressed PKC and FoxO in *Drosophila* S2 cells revealed that FoxO was pulled down with a truncated PKC protein, lacking the kinase domain, but not with the full length protein (J. Mattila, unpublished observation). The significance of this observation was left unresolved and hence, future studies are needed to show the association between these proteins. Based on its role as a negative feedback regulator of the Insulin signaling (Leitges et al. 2002), it is not surprising that PKC was found to regulate FoxO as well. This could be seen as an additional level of regulation, ensuring rapid activation of FoxO and transcription. In order to discuss the nature of this regulation *in vivo*, some key questions are to be solved. Specifically, (1) what are the tissues and developmental processes that the regulation takes place and (2) how is the interaction induced? The utilization of the powerful genetic tools of *Drosophila melanogaster* would certainly be of great help in addressing these questions.

The strongest supporting evidence in the literature is provided for GSK-3β as being an important FoxO regulator. Initially, GSK-3β was identified as the kinase inhibiting the Glycogen synthase, the rate limiting enzyme of glycogen deposition. On the other

hand, GSK-3β is a known tumour suppressor whose inaccurate functioning is promoting many cancer types (Luo 2009). In addition, it is known that one of the key events in promoting cell survival is the inhibitory phosphorylation of GSK-3β by Akt (Cross et al. 1995, Grimes & Jope 2001). Finally, inhibition of GSK-3β has been shown to attenuate the expression of *pepck* and *g6pase* which are known FoxO targets, providing a direct link between GSK-3β and FoxO regulated gene expression (Lochhead et al. 2001). Taken together, these results strongly suggest that GSK-3β exerts at least some of its functions through FoxO.

In spite of the apparent ability of our screen to identify enzymes regulating FoxO activity, the results presented should be considered with some caution. The identified twenty one genes represent a list of putative dFoxO activity modulators and should not be considered as a comprehensive set of kinases and phosphatases regulating dFoxO activity. Moreover, it is possible that some of the hits represent false positives. This assumption is based on the limitations of the screening assay that was used. Primarily, FoxO activity assays utilized in this screen were based on transcription/translation of the reporter proteins luciferase and GFP. In any genome scale RNAi screen a number of targets are general regulators of these processes. Hence, these factors are picked up by the assay unless a suitable transcription/translation normalization procedure is used. In our primary and secondary screen the normalization was achieved by expressing an RFP plasmid under a constitutively active promoter and by measuring the sample overall protein concentration by the Bradford reagent, respectively. These measures were then used to normalize the assay output. However, the efficiency of the normalization procedures to completely exclude false positives, i.e. general regulators of protein biosynthesis, is uncertain and it is still possible that false positives were picked.

A second uncertainty of the screen comes from the fact that no inhibitors of FoxO activity were found. This observation could also reflect the utilization of the luciferase assay. Since overexpression of FoxO results into a regulatory loop finally shutting down the translational and/or transcriptional machinery, either directly through the induction of 4EBP or indirectly by sequestering general transcriptional/translational factors, it is possible that increase in its activity could not be recorded in a translation based assay (see figure S1A in the original article II; RFP intensity is greatly enhanced upon dFoxO RNAi). An attempt to overcome this problem was the utilization of the localization assay which is not hampered by the limitations described above. However, no new inhibitors (besides the already known Akt and PDK1) were found based on this assay either.

Finally, it is important to point out that the interaction per se does not yet imply a biological function *in vivo*. Additionally, these results could not distinguish between direct and indirect regulation. More experiments, such as co-immunoprecipitation and kinase assays are necessary to reveal these mechanisms. Further, to demonstrate a biologically meaningful interaction, *in vivo* studies are required. Hence, our list of FoxO regulators provides material for future studies, and hopefully aids in finding some still missing pieces in the complex puzzle of FoxO signal transduction.

Systemic regulation of development and size by dFoxO through AC76E (III)

The cAMP pathway components are conserved in *Drosophila*. In the fly genome there are five transmembrane AC genes related to the nine mammalian AC isoforms. In addition, a group of more distantly related AC isoforms, known as the DACX family, exists (Cann et al. 2000). At present, little is known about the AC isoforms in *Drosophila*. Few studies addressed their differential expression throughout embryonic development but their role *in vivo* or how their expression is regulated is not known. However, the activity of the pathway has been shown to be essential for the fly longevity and stress resistance. Tong et al. (2007) have reported that core components of the cAMP signaling pathway, adenylate cyclase *rutabaga*, cAMP phosphodiesterase *dunce* and *pka*, are transducing the Neurofibromatosis-1 dependent resistance to ROS and life span extension. It was further demonstrated that feeding flies with cAMP analogs dibutyryl-cAMP and 8-bromo-cAMP increased life span (Tong et al. 2007). Similarly, Wang et al. (2008) have shown that the *Drosophila* CREB coactivator TORC promotes resistance to ROS and starvation. These results raise the possibility that an analogous role of cAMP in regulating fasting metabolism is acting in *Drosophila*.

The data presented in our original article III outlines an important role for FoxO as a mediator of cAMP signaling. Our results indicate that dFoxO increases the cellular cAMP levels through its direct induction of *ac76e* expression. Flies with a *dfoxo* null mutation exhibited reduced *ac76e* expression as well as reduced cAMP levels. In addition, overexpression of the AC76E in a subset of its natural expression pattern, in the larval and adult CA, resulted in lengthening of the larval developmental time, dwarf flies and an increase in the female starvation resistance. Although we have not yet been able to show a mechanistic explanation for this, a strong downstream candidate for regulating the observed phenotype is juvenile hormone (JH). Several pieces of evidence are in favour of this hypothesis: (1) the observed phenotype, i.e. long larval developmental time and dwarf flies, are suggestive of defective endocrine signaling such as ecdysone or JH, which controls the larval moulting and growth (De Loof, 2008), (2) the regulation is taking place in CA which is the source of JH synthesis, (3) an increase of cAMP synthesis in the ring gland has been shown to decrease the JH synthesis/release (Richard et al. 1990), (4) flies with a null mutation in *dinr* are dwarf and defective in JH synthesis (Tatar et al. 2001), (5) targeted knockout of *dinr* in the CA results in dwarf flies suggesting that dFoxO is involved in emitting a signal from this tissue which systemically regulates growth (Belgacem & Martin 2007). Finally, it has to be mentioned that the observed phenotype, delayed development and small size, is analogous to the outcome of larval starvation. This implies that the *ac76e* expression through FoxO is regulated by nutrient availability.

In summary, our results demonstrate that AC76E is regulating systemically the timing of pupae formation. In addition, our results suggest that dFoxO is involved in this process by controlling the expression of *ac76e* in the CA. It was previously shown that dFoxO controls the synthesis of DILP2 (Hwangbo et al. 2004, Wang et al. 2005). These results are in favour of a mechanism where upon insufficient larval growing conditions dFoxO inhibits the Insulin signaling pathway by reducing DILP2 production in the IPCs, which is then followed by growth attenuation in the peripheral tissues. Simultaneously, reduced Insulin signaling results in dFoxO activation in the CA. This is then followed

by the activation of the cAMP pathway and its downstream systemic signal ensuring that moulting is not initiated prematurely (Figure 5). Taken together, in addition to its known role as a cell autonomous cell cycle brake, dFoxO seems to have a critical role in regulating the developmental growth non-cell autonomously.

Our study has three main limitations: First, the lack of an *ac76e* null mutant hampered our genetic studies where all results have been obtained by overexpression of the AC76E. We used strains with constructs driving RNAi against AC76E but the reduction in mRNA expression was not enough to fully eliminate its activity. Second, the lack of a reliable AC76E antibody prevented us from fully characterizing its function. We produced antibodies against AC76E but their specificity was not sufficient to be used in further studies. And finally, the lack of proper genetic interaction data *in vivo* prevented us to make a definitive conclusion about the role of dFoxO in this context. This was due to the early lethality of dFoxO overexpression in the CA. Therefore, at present the *in vivo* data relies solely on overexpression of the AC76E. These deficiencies need to be taken into account when interpreting the dFoxO-AC76E function.

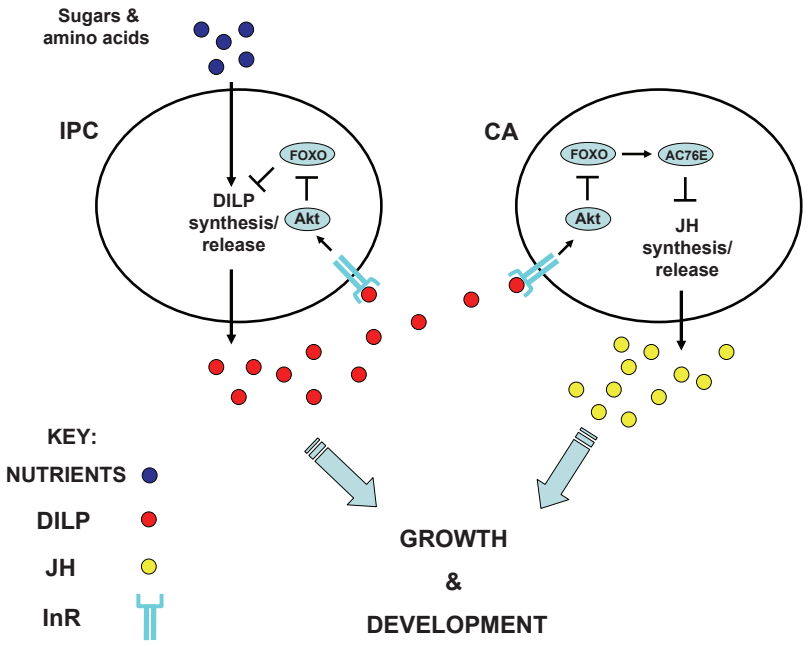

Figure 5. A model describing the role of dFoxO in systemic growth regulation in *Drosophila*.
Nutrients, i.e. sugars and amino acids, trigger *Drosophila* Insulin-like peptide (DILP) synthesis and release from Insulin producing cells (IPC). DILPs have auto- and endocrine functions in IPC and corpus allatum (CA) cells, respectively. Under stress or nutrient depletion, dFoxO inhibits the insulin signaling pathway by reducing DILP2 production in the IPCs, which is then followed by growth attenuation in the peripheral tissues (Hwangbo et al. 2004, Giannakou et al. 2004). Simultaneously, dFoxO activates cAMP production in CA which causes a reduction in JH synthesis, ensuring that moulting is not initiated prematurely (predicted from our results).

CONCLUSIONS

In summary, in this study we have provided valuable information that helps to understand better the role of FoxO in regulating critical processes like cell and organism growth and stress resistance. In the original articles I and III special emphasis was directed to the regulation of growth through dFoxO. We showed that dFoxO regulates the expression of *scylla* and *ac76e,* which presumably both have a role in growth control, although in different hierarchical levels. Whereas *scylla* has a role in the cell autonomous growth in the peripheral tissues by regulating the activity of the TOR signaling, *ac76e* is systemically orchestrating the developmental growth and timing of pupariation of the whole animal. These results provide supporting information in placing FoxO among the major metazoan growth regulators. In addition to downstream targets, several modulators of FoxO activity were identified in the original article II, highlighting the importance and range of biological functions in which this transcriptional regulator is participating. The obtained catalog of interactions will provide important information for researchers in their future attempts to reveal more insights into FoxO regulated processes.

FUTURE PROSPECTS

The results presented in this study raise several interesting questions and suggest fields for future studies. Specifically, the negative feedback regulation of Insulin and TOR signaling through FoxO is far from being completely understood. Given the important role this signaling network has in the pathogenesis of a variety of human carcinomas, the precise knowledge of how this regulatory loop behaves is of outmost importance. Advances in the understanding of this process could have important implications in the development and application of anti-tumour drugs. However, the unfolding of this complex network of interactions requires the combination of bioinformatics and experimental work. It is noteworthy that the mechanisms within this signaling vary between cell types and experimental conditions, making the task enormously challenging. Therefore, the integration of all available data to give a clear overall view requires the application of sophisticated systems biology tools. We assume the development of new prediction algorithms in the future years will revolutionize the experimental approaches used for signal transduction studies and will thus bring us closer to solving the nature of these interactions.

Currently the data presented in the original article II is mostly a database of interactions. To reveal the function of these regulatory interactions and their biological significances, individual characterizations are needed. In addition, given the number of parallel regulatory pathways, a remaining challenge is to understand how FoxO activity is balanced. We are confident that this set of data can provide valuable information to other researchers to open the way for a more detailed characterization of these interactions.

The accredited role of AC76E in the development and growth of the *Drosophila* larvae need further investigation to elucidate a more profound understanding of the biological role of the dFoxO-AC76E interaction. Furthermore, the observed phenotype requires a mechanistic explanation. The nature of the signal emanating from the CA has not yet been solved. As mentioned above, the obvious candidate for such a signal is the juvenile hormone. However, this hypothesis has not been directly demonstrated. In addition, the specific outcome of this systemic signal is yet to be described. For example, what are the downstream signaling events in the peripheral tissues and how do they contribute to the tissue growth?

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