Role of antimicrobial peptides in metabolism and innate immunity in *Drosophila melanogaster*

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1 INTRODUCTION

1.1 Layers of innate immunity

Throughout evolution, the ability of an organism to protect itself from microbial or other species invasion has been a key factor for survival. Living organisms are exposed daily to microbial infections and pathogens, and in order to defend themselves against the abrasive environment, they have developed potent defensive mechanisms called immunity (Hoffmann et al. 1999). Insects rely solely on innate immunity (Figure 1.1), which is manifested in three ways, first, activation of humoral response resulting in the production of antimicrobial peptides (AMP, Lemaitre et al. 1995), second, activation and phagocytosis of pathogens by blood cells, the so called plasmatocytes (Meister et al. 2004), and third, melanization by the activation of the phenoloxidase pathway (Bilda et al. 2009, Tang et al. 2009). This is its sole defense as it lacks an adaptive immunity system such as is found in mammals. The strong conservation of innate immunity systems in organisms from *Drosophila* to mammals, and the ease with which *Drosophila* can be manipulated genetically makes this fly a good model system for investigating the mechanisms of virulence of a number of medically important pathogens (Taeil et al. 2005). The first contact to microbes is always found at epithelial barrier tissues, like the gut, trachea or the epidermis, which are in contact with the external environment. These tissues represent a physical barrier, preventing microorganisms from entering the body cavity of the fly. AMPs are components of the innate immunity, forming the first line of defense used by many organisms against the invading pathogens (Jenssen et al. 2006). All species, from bacteria to humans, resist the invasion of microorganisms through a simple mechanism, but complex in function, involving AMPs. The induction process of AMP synthesis is prevalent in insects and has been particularly well studied in the fruit fly *Drosophila melanogaster* (Meister et al. 2000). AMPs are gene-encoded, short (<40 amino acids), amphipathic molecules with a broad-spectrum antimicrobial activity, displaying multiple modes of action, including bacteriostatic, microbial and cytosolic properties (Hancock et al. 2002). They represent a universal feature of defense systems existing in all living forms and their presence all along the evolutionary scale demonstrates their effectiveness and significance

in combating invading pathogens. AMPs are promptly synthesized and readily available shortly after an infection to rapidly neutralize a broad range of microbes. The ability to produce AMPs is well preserved in almost all living organisms and cell types (Boman et al. 2000). AMPs show broad-spectrum antimicrobial activities against various microorganisms, including Gram-positive and Gram-negative bacteria, fungi, and viruses (Zasloff et al. 2002). The cationic character of the AMPs, associated with their tendency to adopt an amphipathicity, facilitates their interaction and insertion into the anionic cell walls and phospholipid membranes of microorganisms (Oren et al. 1998). AMPs may directly kill microbial invaders (Papagianni et al. 2003). This defense mechanism is particularly important in protecting against infection. The systemic response is controlled by two conserved signaling cascades, which are called Toll and immune deficiency (Imd) in *Drosophila*. The function of these pathways is to detect pathogens in the haemolymph and to induce the production and release of specific effector molecules, to counteract the infection. The main organ of AMP production and release in the fly is the fatbody (Hoffmann et al. 2002) an equivalent of the mammalian liver (Figure 1.1).

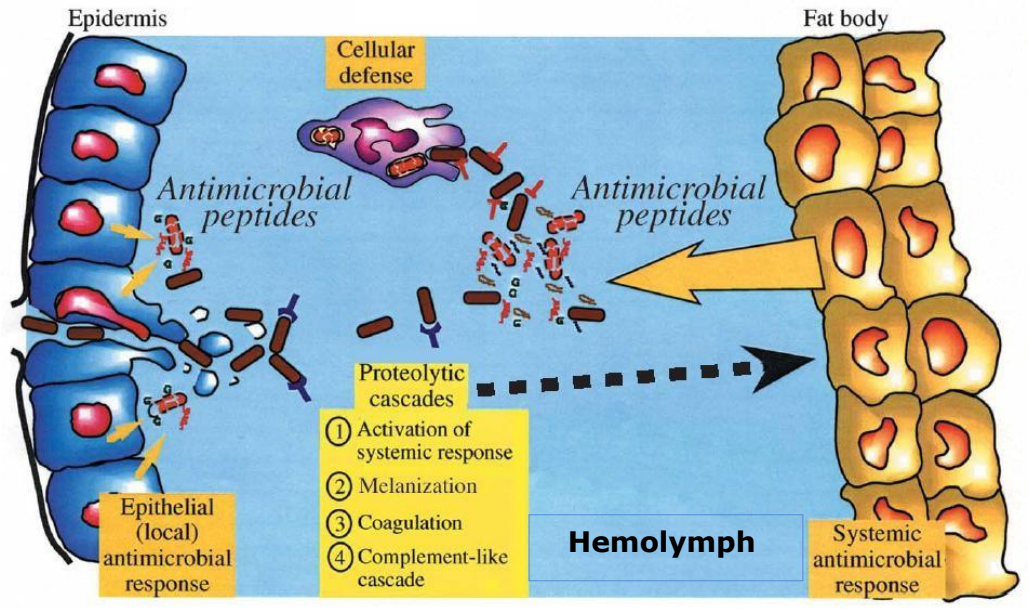


Figure 1.1. Epithelial, cellular and systemic innate immunity in *Drosophila*. The epithelial surfaces of the body serve as first-line defenses against microorganisms. The epidermis—the cells of the digestive and genital tracts—of the tracheae and of the Malpighian tubules all produce antimicrobial peptides (AMP), which inhibit microbial growth (Ferrandon et al. 1998, Tzou et al. 2000 and Onfelt et., 2001). Microorganisms that have succeeded in entering the general body cavity (called the hemocoele; *Drosophila* lacks an organized blood vessel system) are countered by both cellular and humoral defenses. The cellular defenses consist essentially of phagocytosis by

macrophage-like cells, called the plasmatocytes. Larger invading microorganisms are encapsulated by a specialized flattened cell type, called the lamellocytes. The hallmark of the humoral reactions is the systemic antimicrobial response. It corresponds to the challenge-induced synthesis by the fatbody - a functional equivalent of the mammalian liver - of AMPs that are secreted into the hemolymph. The humoral reactions also involve several proteolytic cascades. Bacteria are illustrated as brown rods; pattern recognition proteins as purple pincers; and putative opsonizing proteins as red T-shapes. Modified after Hoffmann et al. 2002.

1.2 AMPs, systemic and local expression

1.2.1 Systemic expression

AMPs are innate host defense molecules that are effective on bacteria (Gram-positive, Gram-negative), fungi (yeasts and filamentous) and parasites, and in some cases on enveloped viruses. They are found in evolutionarily diverse organisms ranging from prokaryotes to invertebrates, vertebrates, and to plants (Tossi et al. 2002, Pas et al. 2002, Bullet et al. 2004 and Ganz et al. 2003). AMPs are expressed in many types of cells and secretions. In addition to this systemic antimicrobial response, cells of most of the barrier epithelia of *Drosophila* produce AMPs that provide a local first line of defense against microorganisms (Ferrandon et al. 1998). Humans express several families of AMPs in myeloid cells. Over the past several years, we have come to realize that various epithelial surfaces from invertebrates and vertebrates can also express their own battery of defensive molecules. In humans, the three AMP families are (1) defensins, (2) cathelicidins (hCAp-18/LL-37 from human neutrophils), and (3) histatins (Ganz et al. 2005). In terms of structural diversity, the human arsenal in AMPs is rather limited (three main classes) compared to that of the fruit-fly *Drosophila* (Table 1.1). To date, eight distinct classes of AMPs have been identified in *Drosophila* (Hoffmann et al. 2003, Rabel et al. 2004), which can be classified in three groups depending on their main microbial targets. *Drosophila* Defensin (Def) is active against Gram-positive bacteria, while Drosocin (Dro), Cecropins (Cec), Attacins (Att), Diptericins (Dpt) and MPAC (truncated post-translationally modified pro-domain of AttC) are active against Gram-negative bacteria, and Drosomycin (Drs) and Metchnikowin (Mtk) efficient against fungi. Whereas, Andropin (Anp) is the only AMP that is not induced upon infection, but is expressed during mating in the male flies to protect the reproductive tract (Table 1.1).

Table 1.1. AMPs in *Drosophila* and their main expression in epithelial barrier tissues. Eight different AMP families with different spectra of activity have been identified in *Drosophila* (Hultmark etal., 2003, Bulet et al. 2004). The AMPs are expressed both locally at epithelial barrier and systemically in the fatbody. They are active against a broad range of microorganisms. Uvell et al. 2007.

Peptides, gene names	Number of genes	Antimicrobial activity	Note on *in vivo* expression and modes of regulation
Andropin; Anp	1	Gram-positive and Gram negative bacteria	Constitutive in male reproductive tract; no infection-induced expression
Attacin; Att	4	Gram negative bacteria	Local induction in the gastrointestinal tract; systemic induction in fatbody
Cecropin; Cec	4	Gram-positive and Gram negative bacteria and fungi	Constitutive in reproductive tract; and local induction in the gastrointestinal tract; systemic induction in fatbody
Defensin; Def	1	Gram-positive bacteria	Constitutive in female reproductive tract; weak induction in barrier epithilia; systemic induction in fatbody
Diptericin; Dpt	2	Gram negative bacteria	Local induction in the gastrointestinal tract; systemic induction in fatbody
Drosocin; Dro	1	Gram negative bacteria	Constitutive in female reproductive tract; local induction in respiratory and gastrointestinal tract; systemic induction in fatbody
Drosomycin; Drs	7	Fungi	Constitutive in salivary glands and female reproductive tract; local induction in respiratory organs (trachea); systemic induction in fatbody
Metchnikowin; Mtk	1	Gram-positive bacteria and fungi	Constitutive and local induction in the gastrointestinal tract; systemic induction in fatbody

1.2.2 Mode of action

Def is active on a large panel of Gram-positive bacteria strains and on a limited number of Gram-negative strains and filamentous fungi. Def disrupts the permeability barrier of the cytoplasmic membrane of bacteria, resulting in the loss of cytoplasmic potassium, a partial depolarization of the inner membrane, a decrease in cytoplasmic ATP and an inhibition of respiration. Thereby, increasing the salt at a relevant physiological concentration dramatically reduces the efficacy of the molecules (Cociancich et al. 1993). Drs and Mtk are potent antifungal peptides affecting the growth of filamentous fungi including human and plant pathogens at a micromolar level (Thevissen et al. 2004). Cecropins are highly effective against most of the Gram-negative strains. It is speculated that the helix-forming capability of Cec, in contact with the lipidic components of the

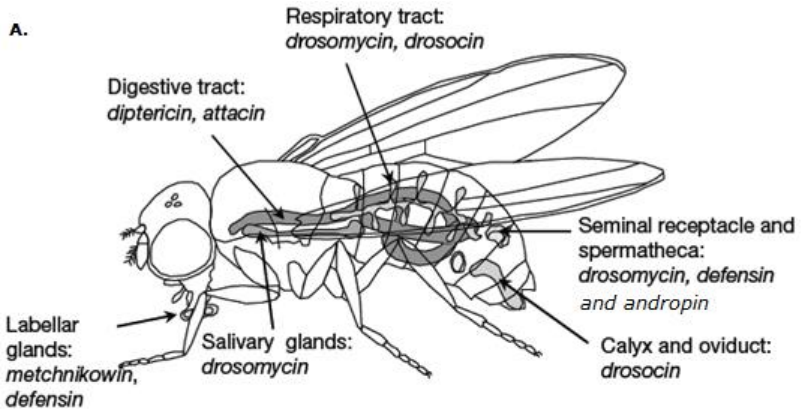
bacterial membrane, results in a general disintegration of the membrane structure and lysis of the bacteria (Bulet et al. 2004). Remarkably, Dro has a high selectivity toward Gram-negative bacteria especially the ones belonging to the *Enterobacteriaceae* family, while Gram-positive strains remain mostly non-susceptible. Dro binds to the multi-helical lid above the substrate-binding pocket of *Escherichia coli* (*E. coli)* heat shock protein (DnaK) resulting in the inhibition of chaperone-assisted protein folding (Kragol et al. 2001). Dpt was found to have an activity for only a limited number of Gram-negative bacteria and to kill these bacteria within an hour by increasing the permeability of the outer and inner membranes of the bacteria (Winans et al. 1999). Att interferes with transcription of the *omp* gene in the representative Gram-negative strain *E. coli*. The *omp* gene is involved in the synthesis of porines, which form protein channels in membranes. This results in a breakdown of the external membrane of *E. coli* (Imler et al. 2005).

1.2.3 Local expression

Important physiological functions such as nutrient absorption, reproduction, gas exchange, or excretion necessitate interaction between host cells and the environment. As a result, epithelial cells from the digestive, reproductive, respiratory tract or from the excretory system are frequently exposed to microorganisms. Local expression of AMPs in these tissues plays an important role as a first line of defense in mammals but also in insects, and particularly in *Drosophila*. Interestingly, epithelial expression of AMPs follows a complex pattern that is specific for each peptide (Figure 1.3A, Tzou et al. 2000). For example, Def and Mtk are expressed in two small glands that open at the beginning of the alimentary canal, the labellar glands; Drs is expressed in the salivary glands, Dpt, Att and to a lesser extent Dro and Mtk are expressed in the midgut, Dpt, Cec and Mtk are expressed in the Malpighian tubules (the excretory system) and Cec, Def, Drs, Dro, and to a lesser extent Mtk and Att are expressed in specific parts of the male and female reproductive tracts. In larvae, Cec expression can also be induced in the epidermis (Onfelt et al. 2001). In most cases, epithelial expression of AMPs is not constitutive, and is only observed in a restricted area of the tissue, in a fraction of the flies, suggesting a response to a local infection. Natural infection of the flies or larvae with the Gram-negative bacteria *Erwinia carotovora* triggers inducible expression of AMPs in several surface epithelia in a tissue-

specific manner. In all cases, this induction is dependent on the Imd pathway (Tzou et al. 2000, Onfelt et al. 2001). In particular, Dro expression, which is regulated by the Toll pathway during the systemic response, is regulated by Imd in the respiratory tract, thus demonstrating the existence of distinct regulatory mechanisms for local and systemic induction of AMPs in *Drosophila*. In some tissues, expression of the AMPs is constitutive. This includes the salivary glands for Drs, the female reproductive tract for Drs, Dro, Def and Cec, and the male reproductive tract for Cec. The constitutive expression of Drs in salivary glands and Cec in the ejaculatory duct was recently shown to be dependent on the homeobox gene product *Caudal* (Ryu et al. 2004). Curiously, the constitutive expression of Drs in the female reproductive tract is not dependent on *Caudal* (Figure 1.3B). Another interesting feature of the epithelial expression of AMPs is that it reveals an additional level of complexity in their genetic regulation. Indeed, the fact that AMP genes like Dpt and Dro, which are both controlled exclusively by the Imd pathway in the fatbody during the systemic response, are induced in different epithelial locations (e.g. digestive tract versus tracheae), points to the existence of tissue-specific transcription factors, which probably act in concert with Relish (Rel). The *Drosophila* Rel, is strongly induced in infected flies. Upon septic injury, Rel is rapidly processed and translocates to the nucleus, thereby, mediates induction of a subset of AMPs in a given epithelium (Tzou et al. 2000).

A.



B.

Tissue	Expression	Regulation
Fat body	Inducible	Toll
Trachea	Inducible	IMD
Salivary gland	Constitutive	Caudal
Spermatheca	Constitutive	?

Figure 1.3: Epithelial expression of AMPs in *Drosophila*. (A) The main sites of expression of AMP genes in a female fly are shown. **(B)** Tissue-specific expression and regulation of the Drosomycin gene.Modified after Imler et al. 2005.

1.3 AMPs regulation by immunity pathways

Since almost 20 years it is known that the *Drosophila* genome encodes for several classes of AMPs, which are active against Gram-positive as well as Gram-negative bacteria or fungi. All of the related promoter regions contain sequence motifs related to mammalian NF-κB response elements, which turned out to be crucial for AMP expression (Engstrom et al. 1993). Due to this reason, the later on discovered signaling pathways, which are responsible for AMP regulation, are called NF-κB-like signaling pathways. In the mid of the 1990's, it turned out that two distinct signaling pathways are controlling AMP expression, which are the Toll and the Imd pathways.

1.3.1 Toll Pathway

When the genes encoding several insect AMPs were sequenced, their upstream regulatory regions were found to contain sequence motifs similar to mammalian response elements of the inducible transactivator NF-kB (Hoffmann et al. 1997). Experiments with transgenic fly lines demonstrated that these nucleotide sequences conferred immune-inducibility to AMPs genes (Engstrom et al. 1993, Meister et al. 1994). The Toll pathway (Figure 1.4) is well known from dorso-ventral patterning during *Drosophila* embryogenesis (Belvin et al. 1996). In 1996, it was first described that the Toll receptor is crucial for proper organismal defense against fungi, which gave rise to a totally new function of this pathway in innate immunity (Lemaitre et al. 1996). It turned out that the Toll pathway is also responsible for recognition and defense against Gram-positive bacteria. The Toll receptor is a transmembrane protein, which shares sequence similarities with the vertebrate Interleukin-1 receptor (Hashimoto et al. 1988). Toll is activated via a cleaved form of the polypeptide Spaetzle, which is structurally similar to mammalian nerve growth factor (Charles et al. 2003). Processed Spaetzle interacts with the extracellular leucine-rich domain of Toll. The intracytoplasmic domain of this receptor has a TIR (Toll-IL receptor) homology domain, which is also present in DmMyD88 (Delamasure et al. 2002), in all *Drosophila* Tolls, in mammalian Toll-like–receptors (Imler et al. 2000), and in many plant proteins

involved in defense reactions (Thomma et al. 2001, Sessa et al. 2001). A receptor-adaptor complex is formed on the intracytoplasmic side of Toll, which comprises 3 death domain proteins: DmMyd88 (mentioned above), Tube, and the

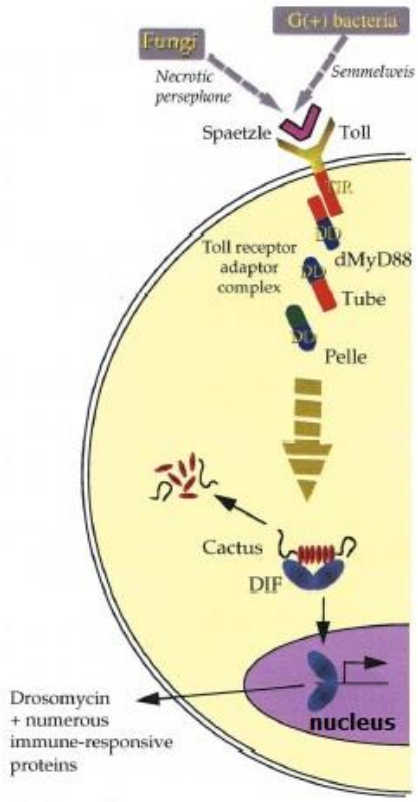


Figure 1.4. Toll Pathway of *Drosophila*. Toll-dependent induction of immune genes in fungal and Gram-positive bacterial infections in *Drosophila*. These microorganisms are sensed by circulating pattern recognition proteins, a process that is followed by proteolytic cleavage of the polypeptide Spaetzle; Spaetzle activates Toll, which leads to degradation of Cactus and nuclear translocation of the Rel protein DIF which activates the immune effector molecules called antimicrobial peptides (AMPs). β-GRP: β-glucan recognition protein, DIF: dorsal-related immunity factor, Drs: Drosomycin, PRRs: pattern recognition receptors, MyD88: death domain containing proteins. Modified after Hoffmann et al. 2002.

kinase Pelle. This complex signals to the ankyrin domain protein Cactus, which is phosphorylated by an undefined kinase (distinct from Pelle) and dissociates from the NF-kB/Rel protein DIF. Although Cactus becomes degraded, DIF translocates into the nucleus and directs the transcription of the Drs gene (Rutschmann et al. 2000) plus that of some 350 additional genes induced by natural fungal infection, many with unknown functions (Irving et al. 2001, De Gregorio et al. 2001, De Gregorio et al. 2002) for other genome-wide analyses of immune response in *Drosophila*. Notably, characterization of Toll signaling in *Drosophila* led to the

identification of Toll-like-receptor proteins in mammals. In addition to this, most other components of the *Drosophila* Toll pathway are conserved in vertebrates (Skaug et al. 2009).

1.3.2 Imd Pathway

The second NF-κB-like signaling pathway in *Drosophila* is the Imd pathway (Figure 1.5), named by the intracytoplasmic adapter protein Imd (Lemaitre et al. 1995) of a long time unknown transmembrane receptor. The Imd pathway is primarily activated by infection with Gram-negative bacteria and controls resistance to these microorganisms (Figure 1.5). This receptor belongs to the class of PGRP-LC proteins and interacts with Imd via a death domain. The Imd protein probably interacts with DmFADD (Leulier et al. 2002, Naitza et al. 2002) and the caspase-8 homologue DREDD (Leulier et al. 2000, Elrod-Erickson et al. 2000). Loss of function mutations in the genes encoding both DmFADD and DREDD silence the Imd pathway (Leulier et al. 2002, Naitza et al. 2002, Leulier et al. 2000, Elrod-Erickson2 et al. 2000). The mitogen-activated protein kinase kinase kinase (MAPKKK) dTAK1 acts downstream of Imd/DmFADD and activates an IkB kinase (IKK) signalosome equivalent (Vidal et al. 2001) consisting of *Drosophila* homologs of mammalian IKKb and IKKg/NEMO (NFkB essential modifier, Silverman et al. 2000, Lu et al. 2001, Rutschmann et al. 2000). Wild-type DmIKKb and DmIKKg are required for normal anti–Gram-negative responses (Lu et al. 2001, Rutschmann et al. 2000).

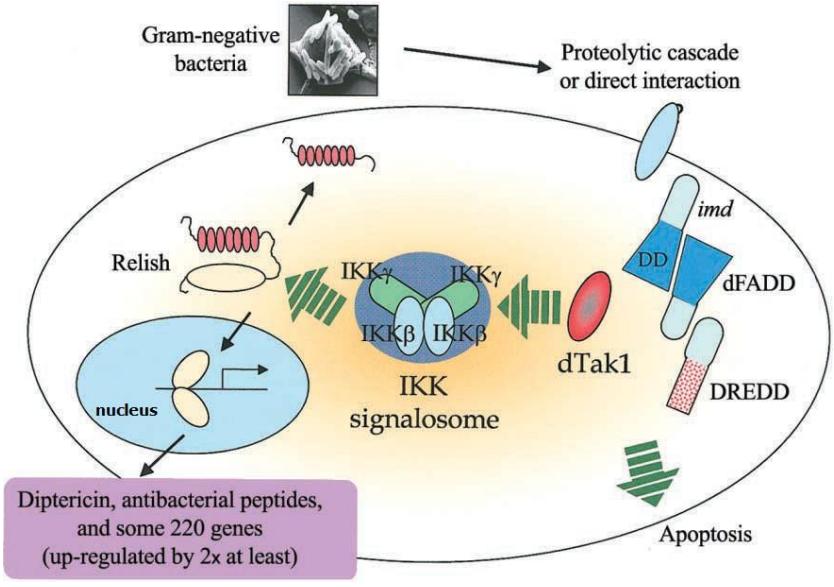


Figure 1.5. Imd pathway of *Drosophila*. Peptidoglycan from gram-negative bacteria activates the membrane receptor PGRP-LC. Imd acts downstream of PGRP-LC and activates Relish by IKK-mediated phosphorylation, and endoproteolytic cleavage, most likely by the caspase DREDD. The 68-kDa Rel domain from Relish then translocates to the nucleus where it can induce expression of antibacterial peptide genes. dFADD: *Drosophila* Fas-associated death domain protein, DREDD: caspase-8 homologue, dTak1: kinase, IKK: IκB kinase complex. Modified after Charles et al. 2003.

The NF- kB/Rel family member of the Imd pathway is the protein Relish, which is cleaved by an unknown caspase: the Rel homology domain translocates into the nucleus, whereas the ankyrin repeat domain remains in the cytoplasm (Stoven et al. 2000). Cleaved Relish activates the transcription of the genes encoding peptides, such as Dpt, but also those of many other, some with unknown function (Irving et al. 2001). Moreover, as seen for Toll signaling, also the Imd pathway is conserved in vertebrates since high homology to the TNF-α pathway can be found (Skaug et al. 2009).

1.4 AMPs and metabolism

The regulation of AMP genes, small cationic proteins that function by damaging microbial cell membranes, thereby causing stasis or lysis of the target microorganism (Yeaman et al. 2007), was further characterized. It turned out that transcriptional regulation of these genes is not restricted to NF-κB like immunity pathways, but also depending on IlS, which represents a novel link between metabolism and organismal defense.

1.4.1 Insulin/Insulin-like signaling

IlS is a conserved feature in all metazoans. It evolved with the appearance of multicellularity, allowing primordial metazoans to respond to a greater diversity of environmental signals. The IlS pathway is highly conserved in insects and particularly in *Drosophila*, where it has been extensively studied in recent years and shown to control metabolism, growth, reproduction, and longevity. As misregulation of the insulin/IGF pathway in humans plays a role in many medical disorders, such as diabetes and various types of cancer, unraveling the regulation of insulin/IGF signaling using the power of a genetically tractable organism like

Drosophila may contribute to the amelioration of these major human pathologies (Charles et al. 2006).

Insects have a single insulin/IGF system that may correspond to the ancestor of the dual insulin/IGF system. IlS is largely conserved in vertebrates and invertebrates. The architecture of this signaling cascade is simpler in the fly, since most components are present as single orthologs. While insects do not have a tissue, such as the pancreas, that is specialized in carbohydrate homeostasis, *Drosophila* do have a group of insulin-producing cells (IPCs) that are located in the brain and constitute an endocrine organ for the regulation of growth and sugar metabolism (Brogiolo et al. 2001, Rulifson et al. 2002). It is believed that all the *Drosophila* Insulin-like peptides (dILPs) activate the single insulin receptor, thereby specifying IlS activation in individual tissues or in context of different functions, but so far this model is not proven. A total of eight different *dILP*-encoding genes are found in the *Drosophila* genome (Brogiolo et al. 2001, Luo et al. 2013). The *Drosophila* insulin receptor (InR) is surprisingly similar in structure to the vertebrate insulin receptor, with a marked extension of the COOH-terminal-chain domain that is suspected to serve as a direct docking site for the downstream phosphatidylinositol (PI) 3-kinase. The intracellular adaptor of the InR is encoded by *chico* (Bohni et al. 1999), which mediates the signal of the autophosphorylated receptor to the phosphatidylinositol-3-kinase (PI3K). PI3K signaling is implicated in survival, regulation of the cell cycle, cell differentiation and intracellular traffic processes by activating the kinase Akt/PKB (Lars et al. 2004). Signaling by PI3K is counterbalanced by the tumour suppressor protein PTEN (phosphatase and tensin homologue deleted on chromosome 10). Downstream of PKB/Akt, IlS is divided into two branches with different tasks. One branch is responsible for transcriptional control, mediated by dFOXO. The fly genome encodes for a single dFOXO gene, which is conserved from worm to human and has extensively been described in context of cellular stress response and energy homeostasis (Arden etal., 2008, Gross et al. 2008). The dFOXO protein contains a forkhead box domain, which allows direct binding to the DNA via highly conserved recognition sequences. The PKB/Akt protein regulates dFOXO in an IlS dependent manner by phosphorylation. Increased IlS activity leads to enhanced dFOXO phosphorylation, retaining it in the cytoplasm. In contrast, dFOXO enters the nucleus when its phosphorylation status is low, subsequently followed by activation of dFOXO target gene expression (Calnan et al. 2008). The second branch, which is defined by the tuberous sclerosis (TSC) and target of

rapamycin (TOR) complexes, is mainly responsible for the regulation of translational control, autophagy and nutrient sensing (Hafen et al. 2004, Chang et al. 2009). The link between IIS and TOR signaling is established via the TSC2/TSC1 protein complex, which is directly regulated by PKB/Akt via phosphorylation of TSC2. This protein complex has been described in context of tumor formation downstream of the InR (Pan et al. 2004). Taken together, these two branches are responsible for all cellular processes in an IIS dependent manner (Figure 1.6).

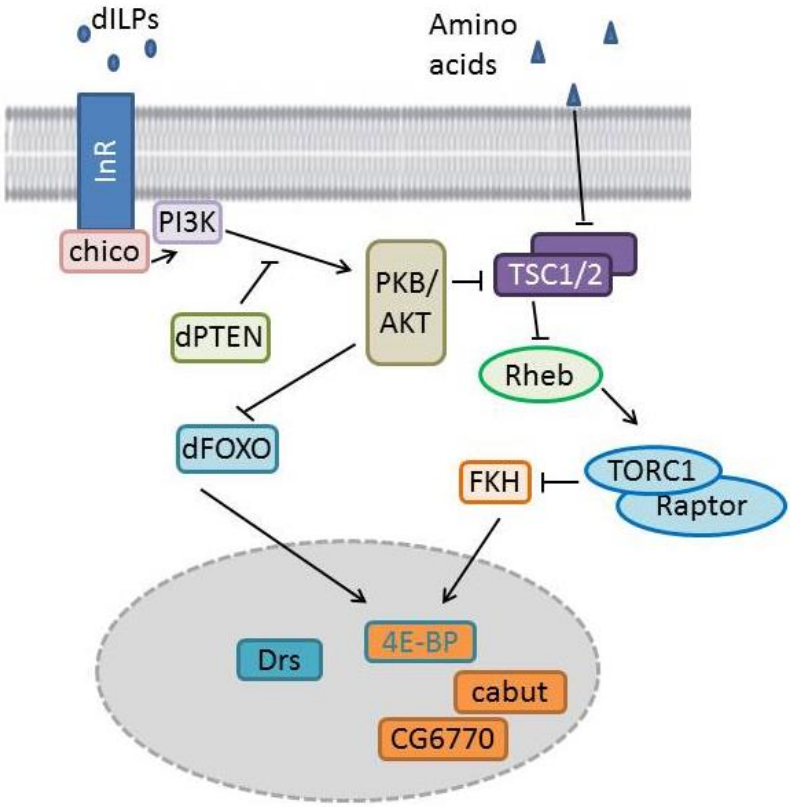


Figure 1.6: The IIS pathway of *Drosophila melanogaster*. The insulin/insulin-like growth factors signaling (IIS) gets activated under nutrient stress, thereby activating the transcriptional factor dFOXO. Under conditions of dietary protein abundance, the TOR signaling module is active and exerts a negative regulation on FKH, which is consequently sequestered in the cytoplasm and unable to modulate gene transcription. dILPs: insulin-like peptides, InR: insulin receptor, PI3K: phosphatidylinositol 3-kinase, dPTEN: lipid phosphatase, AKT/PKB: protein kinase B dFOXO: Forkhead box class O, 4E-BP: 4E binding protein, TORC1: target of rapamycin complex 1, Rheb: Ras homology enriched in brain, TSC1/2: Tuberous sclerosis 1/2.

1.4.2 Forkhead box class O transcription factor

FOXO proteins are a subgroup of the Forkhead family of transcription factors. This family is characterized by a conserved DNA-binding domain (the 'Forkhead box', or FOX) and comprises more than 100 members in humans, classified from FOXA to FOXR on the basis of sequence similarity. These proteins participate in very diverse functions: for example, FOXE3 is necessary for proper eye development, while FOXP2 plays a role in language acquisition. Members of class 'O' share the characteristics of being regulated by the insulin/PI3K/Akt signaling pathway (Lars et al. 2004). Forkhead (FKH), the founding member of the entire family (now classified as FOXA), was originally identified in *Drosophila* as a gene whose mutation resulted in ectopic head structures that looked like a fork. In invertebrates, there is only one FOXO gene, termed DAF-16 in the worm and dFOXO in the fly. In mammals, there are four FOXO genes, FOXO1, 3, 4, and 6 (Glauser et al. 2007). FOXO transcription factors are at the interface of crucial cellular processes, orchestrating programs of gene expression (production of proteins) that regulate apoptosis (cellular programmed death), cell-cycle progression, and oxidative stress resistance. Under severe starvation conditions, nuclear dFOXO presumably activates target genes that reduce cell proliferation. One of these target genes is 4E-binding protein (4E-BP), which encodes an inhibitor of translation initiation. Alternatively, FOXO factors can promote cell-cycle arrest. Additionally, FOXO factors facilitate the repair of damaged DNA. Other FOXO target genes have been shown to play a role in glucose metabolism, cellular differentiation, muscle atrophy, and even energy homeostasis (Edgar et al. 2006). FOXO factors have been shown to prolong lifespan in invertebrates. The worm ortholog, DAF-16, activates a program of genes that extend longevity by promoting resistance to oxidative stress, pathogens, and damage to protein structure (Partridge et al. 2010). In flies, overexpression of dFOXO is sufficient to increase longevity (Giannakou et al. 2008).

1.5 Crossregulation in *Drosophila* larvae

A completely new mechanism of crossregulation between IlS and innate immunity at the level of dFOXO was uncovered (Becker et al. 2010). In fact, this was the first description of a direct regulation of AMP genes by IlS, a signaling pathway known to regulate growth, energy homeostasis and lifespan. During the oscillatory

energy status, dFOXO has been shown to regulate AMPs independent of the classical innate immune pathways. The nutrient dependent regulation of dFOXO directly couples energy homeostasis to organismal defense against pathogens. Using the Drs promoter region, it was shown that dFOXO directly binds to conserved motifs found in the regulatory regions of nearly all AMP genes. Moreover, direct regulation of AMP expression by dFOXO turned out to be independent of Toll and Imd pathways and to function predominantly under non-infected conditions in fatbody and epithelial barrier tissues (Becker et al. 2010). These barrier epithelia are constantly exposed to omnipresent microorganisms and NF-κB like signaling is often reduced in these tissues to prevent necrosis, cancer formation and the induction of tolerance to pathogen-associated molecular patterns.

1.6 TOR pathway

TOR is an evolutionarily conserved nutrient sensing protein kinase that regulates growth and metabolism in all eukaryotic cells. As the name suggests, this kinase is a target for inhibition by rapamycin (Figure 1.6). Rapamycin was discovered as a byproduct of the soil bacterium *Streptomyces hygroscopicus* (Vezina et al. 1975). Rapamycin was originally studied and used for its potent antifungal properties and was later shown to inhibit growth of cells and also act as an immunosuppressant. TOR complex 1 (TORC1) together with Raptor is rapamycin sensitive and controls temporal aspects of cellular growth mediated mostly through S6 kinase 1 (S6K1) and initiation factor 4E-BP1 (Wullschleger et al. 2006). TOR is positively regulated by PKB/Akt. It is required for cell growth and proliferation and is linked to the insulin pathway. Growth stimulation by PI3K signaling requires TOR (Oldham et al. 2000, Zhang et al. 2000). TSC1 and TSC2 form a complex and was first shown to act parallel to the IIS pathway to inhibit TOR signaling (Gao et al. 2001, Niida et al. 2001). The small GTPase Rheb (Ras homologue enriched in brain) has been shown to be a direct target of TSC2 (Zhang et al. 2003). TOR is part of a complex network of signaling components. This is to be expected for a protein like TOR which is versatile and has to integrate various inputs to decide cellular fate. A key role for TOR is to match the growth rate to the availability of the resources in both intra- and extracellular environments. TOR achieves this by acting as a major hub for a complex network of signals. TOR plays a key role in aging, metabolism, stress response regulation,

coordination of growth regulation, regulation of protein synthesis, regulation of autophagy and in feeding behavior.

1.7 Forkhead box class A Transcription factor

Forkhead box A (FoxA) family proteins are characterized by the presence of a conserved Forkhead DNA binding domain (Weigel et al. 1990). The mammalian counterparts of FKH are the FOXA1, 2, and 3 proteins (Yagi et al. 2003, Lee et al. 2004), which are members of the larger family of FKH/HNF or Fox transcription factors (Weigel et al. 1990, Gajiwala et al. 2000, Sund et al. 2000). Similar to FKH, FOXAs play a role in specifying tissue-specific responses to steroid signaling, suggesting that aspects of FOXA function are evolutionarily conserved (Friedman et al. 2006). FoxA2 is the only one, which has a PKB/Akt phosphorylation site. Therefore, it can be regulated by IlS pathway.

The only *Drosophila* ortholog of FoxA proteins is FKH. It is an important factor in embryogenesis: it regulates terminal pattern formation, autophagic cell death in metamorphosis and salivary gland development (Lehmann et al. 2008). FKH is a key gene that is required for the development of all gut primordia (Weigel et al. 1988, Weigel et al. 1989). The gene is known to be expressed during the initial phase of gut formation in the foregut, the midgut and the hindgut anlagen. In FKH loss of function mutants the gut is not formed (Weigel et al. 1989). It is known that FKH is regulated by TOR signaling in *Drosophila* (Bülow et al. 2010).

1.8 Aims of the thesis

My first aim was to study the existence of the crossregulation (as shown in the larvae) between metabolism and innate immunity in the adult fly. In my thesis, I wanted examine the regulation of various AMPs during this oscillatory energy status independent of the classical innate immune pathways, which would help to unravel the connection not only to metabolism but also aging connected to IlS/FOXO.

The second aim was to analyze the different signal transduction pathways important for AMP expression. As FKH is a transcription factor related to FOXO, I focused on the possible role of the FKH/TOR pathway in immunity and their role in

activation of AMP genes.

2 MATERIALS

If not mentioned separately, all chemicals used were of *pro analysis* quality and ordered from one of the following companies: Faust, La Roche, Merck, Promega, Roth, Sigma, Invitrogen, Biorad, Macherey and Nagel or Stratagene. Consumable and plastic material was from Faust, Eppendorf, Roth, VWR or Greiner.

2.1 Consumable materials

Material	Company
1.5/2 ml reaction tubes	Eppendorf
Cell Strainer	BD Falcon
Cover slips	VWR
General laboratory equipment	Faust, Schütt
Microscope Slides	VWR
Paraffin	Medim Past
PCR reaction tubes	sarstedt
Plastic wares	Greiner
Syringe	Inject disposable 5ml Braun

2.2 Devices

Device	Composition
Autoclave	H+P Varioklav steam steriliser EP-2
Bacterial incubator	Innova 44 New Brunswick scientific
Balances	Sartorius BL 150 S; Satorius B211 D
Binocular	Zeiss stemi 2000
Centrifuges	5415R/5424 Eppendorf; Avanti J-26 XP Beckman Coulter; Biofuge primo R Heraeus; Rotina 420R
Confocal microscope	Zeiss LSM710
Electro pipette	Accu Jet
Fluorescence microscope	Zeiss AxioCam MRm; Olympus SZX12
Fly incubator	RuMed

Gel documentation	Biorad
Homogenizer	Precyllys Peqlab
Incubator/shaker	Biostep Dark Hood DH-40/50 (Benda) Heiz Thermo Mixer MHR13 HCL (Memmert), Innova 44 New Brunswick
Micro Hand Mixer	Roth
Microwave	Panasonic
Luminometer	Berthold Microlumat plus LB96V
PCR cycler	C1000 Thermal Cycler and S1000 Thermal Cycler-BIORAD
Photometer	Nano Drop 2000 Peqlab
Power supply	Bio-Rad Power Pac 3000
Real-time PCR cycler	I-Cycler with IQ5 optical unit (BioRad) Light Cycler 1.3 (Roche
Rotator	Snjiders test-tube-rotator
Speed Vac	Savant,SPD111V
Voltage source	Power Pac 3000 BioRad
Vortexer	Vortex Genie2
Thermomixer	Eppendorf Thermomixer comfort,HLC
Waterbath	Julabo SW22

2.3 Standards, kits, buffers and enzymes

Company	**Product**
Agilent Technologies	Strata Clone PCR Cloning Kit
BioRad	SYBRGreen 2x supermix
Biozol	DAPI-Flouromount G

Fermentas	DNA ladder mix DNA loading dye *Taq* polymerase Restriction Enzymes
Finnzymes	Phusion Hot Start II-High fidelity DNA Polymerase
Invitrogen	SybrSafe
Macherey Nagel	NucleoSpin Plasmid AX-100 kit NucleoSpin Extract II kit NucleoSpin RNA II kit NucleoSpin RNA XS kit
NEB	2-Log DNA ladder
Novagen-TOYOBO	KOD Hot Start DNA Polymerase
Promega	Luciferase assay system *Pfu* polymerase Go*Taq* polymerase Dual-Glo Luciferase Assay System
Qiagen	QuantiTect reverse transcription kit
Roche	PCR nucleotide mix rAPID Alkaline Phosphatase Restriction endonucleases and buffers T4 DNA ligase and ligation buffer
Roth	Lysozym Ampicillin

2.4 Buffers

If otherwise mentioned, all solutions and media were prepared with non-sterile, double deionised water (*aqua bidest*). All solutions were kept at room temperature unless a storage temperature indicated. All percent values are mass divided by volume. All solutions, which were made as concentrated stock solutions, the concentration factor is indicated.

Solution	**Composition**
Agarose	1 % agarose in TAE
Ampicillin (-20 °C) (1000x)	50 mg/ml
AP	100 mM NaCl; 50 mM MgCl2; 10 mM Tris,pH 9.5; 0.1 % Tween 20
Carbonate (-20 °C)	120 mM $Na2CO_3$, 80 mM $NaHCO_3$, pH solution to 10.2 with NaOH
Fixation solution	4% Paraformaldehyde (PFA) in PBS (Histofix, Roth)
Hybe (-20 °C)	100 mL formamide, 50 mL 20x SSC, 47 mL ddH_20, 20 mg tRNA, 20 µL Tween 20, DEPC ddH_20 to 200 mL.
Hybe B (-20 °C)	2 ml of 100 % formamide, 412 µL of H_2O, 480 µL of 5 M NaCl, 100 µL of 10% SDS, 80 µL of yeast tRNA (10 mg/ml), 80 µL of 50x Denhardt's solution, 40 µL of 1 M TrisHCL (pH 8.0), 800 µL of 50 % Dextran Sulfate, 8 µL of 0.5 M EDTA, DEPC ddH_20 to 4 mL.
Lysozyme (-20 °C)	10 mg/ml in TELT buffer
Nipagin solution	10 % 4-hydroxybenzoeacid-methyl-ester in 70% ethanol
PBS (20x)	2.6 M NaCl, 140mM Na_2HPO_4, 60 mM NaH_2PO_4 (pH 7.0)
PBT	0.1 % Tween 20 in PBS (1x)
Proteinase K stock solution (-20 °C)	20 mg/ml in DEPC
SSC (20x)	3 M sodium chloride and 300 mM trisodium citrate (adjusted to pH 7.0 with HCl).
Stop solution	0.2 M Sodium Acetate, pH to 6.0 with acetic acid
TAE	40 mM Tris acetate (pH 8.0); 1 mM EDTA
TELT	50 mM Tris (pH 7.5); 62.5 mM EDTA; 2.5 M LiCl; 0.4% Triton X-100
XGal	2 % 5-Brom-4-chlor-3-indoxyl-s D galactopyranosid stock solution

2.5 Solutions and chemicals

Chemicals	Company
Acetic Acid	Roth
Ethanol	Roth
Isopropanol	Roth
Methanol	Roth
Rapamycin	LC Laboratories
RU486	Sigma

2.6 Enzymes

Enzyme	Company
GoTaq polymerase	Promega
Levamisol	Sigma
Phusion Hot Start Polymerase	Thermo scientific
Proteinase K	Sigma Aldrich
Restriction endonucleases	NEB
RNase A	Sigma Aldrich
Shrimp Alkaline Phosphatase (SAP)	Roche
T4 DNA Ligase	Roche

2.7 Bacterial culture media

The bacteria are cultivated in the following media. All media are autoclaved for 20 min at 120 °C.

Materials	Composition
LB medium	10 g tryptone; 5 g yeast extract; 10 g NaCl; ad. 1 l aqua bidest. Adjust pH to 7.0.
LB agar	10 g NaCl; 10 g tryptophan; 5 g yeast extract; 20 g agar; ad 1 l aqua bidest, adjust pH to 7.0 and autoclave, plate when cooled to 55 °C. If necessary, add antibiotics before plating

LB-ampicillin medium	LB medium with 50 µg/ml ampicillin
LB-ampicillin agar	LB medium with 20g agar and 50 µg/ml ampicillin

2.8 Microorganisms

Name	**Genotype**	**Source**
E.coli DH5α	fhuA2 Δ(argF-lacZ)U169 phoA glnV44 Φ80 Δ(lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17	Stratagene,Heidelberg

2.9 Cell culture media and reagents

Medium/Reagent	**Source**
Cellfectin, DMEM, Schneiders, RPMI	Invitrogen
FCS	Biowest

2.10 Fly food

2.10.1 Stock amplification

Add 130 g agar to 15 l aqua bidest and boil until agar is dissolved. Add 248 g brewer's yeast, 1223g cornmeal and 1.5 l syrup to 5 l aqua bidest, solubilize and add to solubilized agar. Boil for 10 minutes and stir sporadically. Cool down to 65 °C, then add 300 ml 10% Nipagin solution and aliquot.

2.10.2 dFOXO heat shock experiments

7.5% Sugar and Yeast Food Amount/Volumes for 250 ml

Weigh 18.75g yeast and 5g Agar into a 500 ml bottle. Fill up with water to 200 ml. Autoclave the mixture and also additional of 200 ml of Water. Add 18.75g of sugar (glucose) and cool down. Add 7.5 ml of 10% Nipagin solution. Fill up with autoclaved water upto 250 ml. Mix well on a magnetic stirrer. Aliquot the food into the fly vials (4ml per fly vial).

2.11 Vectors

Name	Source
pAc	Hoch lab
pCa4B2G	Perrimon lab
pAHW	Hoch lab
PCRII Topo	Invitrogen
pGL3	Boutros lab
pMT	Hoch lab
pUAST	Hoch lab
pSC-A-amp/kan	Agilent Technologies

2.12 Oligonucleotides for SYBRgreen based real-time PCR

Name	Gene	Sequence
act Sy F1	actin	GTGCACCGCAAGTGCTTCTAA
act Sy R1	actin	TGCTGCACTCCAAACTTCCAC
Anp RT F1	andropin	GTCCTTCGGATGCAGTATT
Anp RT R1	andropin	TTAGCAAAGCAATTCCCAC
Att-a-Sy-F1	attacin-a	AGGAGGCCCATGCCAATTTA
Att-a-Sy-R1	attacin-a	CATTCCGCTGGAACTCGAAA
AttB RT F1	attacin-b	CTACAACAATGCTGGTCATGG
AttB RT R1	attacin-b	AAGACCTTGGCATCCAGATT
AttC RT F1	attacin-c	TCAGTCAACAGTCAGCCGCTT
AttC RT R1	attacin-c	ACGCCAACGATGACCACAA
Cec-a1-Sy-F1	cecropin-a1	TCTTCGTTTTCGTCGCTCTCA
Cec-a1-Sy-R1	cecropin-a1	ATTCCCAGTCCCTGGATTGTG
CecA2 RT F2	cecropin-a2	AAATCGAACGTGTTGGTCAG
CecA2 RT R2	cecropin-a2	AGATAGTCATCGTGGTTAACCT
Cec-c-Sy-F1	cecropin-c	TCATCCTGGCCATCAGCATT
Cec-c-Sy-R1	cecropin-c	CGCAATTCCCAGTCCTTGAAT
Def Real F1	defensin	ATTCCAGAGGATCATGTC
Def Real R1	defensin	GTTCCAGTTCCACTTGGA
Dpt-RT-F1	diptericin	ATTGGACTGAATGGAGGATATGG

Dpt-RT-R1	diptericin	CGGAAATCTGTAGGTGTAGGT
DptB RT F1	diptericin-b	GGCTTATCCCTATCCTGATCC
DptB RT R1	diptericin-b	CATTCAATTGGAACTGGCGA
Dro-Sy-F1	drosocin	TTTGTCCACCACTCCAAGCAC
Dro-Sy-R1	drosocin	ATGGCAGCTTGAGTCAGGTGA
Drs-Sy-F1	drosomycin	ACCAAGCTCCGTGAGAACCTT
Drs-Sy-R1	drosomycin	TTGTATCTTCCGGACAGGCAG
Drs_2 Real F1	drosomycin-2	ATGGTGCAGATCAAATTCCT
Drs_2 Real R1	drosomycin-2	CAAATACGTCGGCACATCTC
Drs_3 Real F1	drosomycin-3	TCCTGTTTGCTATCCTTGCT
Drs_3 Real R1	drosomycin-3	CCGAAAGTTCCAGATAGGCA
Drs_4 Real F1	drosomycin-4	TAAAGGATTGTTTGCTCTCCTC
Drs_4 Real R1	drosomycin-4	AAGGACCACTGAATCTTCCA
Drs_5 Real F2	drosomycin-5	GGAAGATACGGAGGACCCTG
Drs_5 Real R2	drosomycin-5	CAGCACTTCAGACTGGACT
foxo-sy-F1	foxo	AGCTTGCAGGACAATGCCTC
foxo-sy-R1	foxo	ATTGCCTC
InR-sy-F1	insulin receptor	AACAGTGGCGGATTCGGTT
InR-Sy-R1	insulin receptor	TACTCGGAGCATTGGAGGCAT
Mtk-Sy-F1	metchnikowin	CGATTTTTCTGGCCCTGCT
Mtk-Sy-R1	metchnikowin	CCGGTCTTGGTTGGTTAGGAT
Rp49-Real-F1	ribosomal protein 32L	GCTAAGCTGTCGCACAAATG
Rp49-Real-R1	ribosomal protein 32L	GTTCGATCCGTAACCGATGT
Thor-Sy-F2	4e-bp	CATGCAGCAACTGCCAAATC
Thor-Sy-R2	4e-bp	CCGAGAGAACAAACAAGGTGG

2.13 Oligonucleotides for analysis and cloning

Name	**Gene**	**Sequence**
CecA1_luci_900_F1	cecropin-a1	GGTCCTTCGGATGCAGTATT
CecA1 600 R1	cecropin-a1	ACTGCCATACAAAAGGCGAGAG
CecC_luci_2200_F1	cecropin-c	AGACTATCAGTCACTTAGTTCG

CecC_luci_R1	cecropin-c	TTTATAGCGAAGAATGAGATGC
Dro_luci_1500_F1	drosocin	GGATGTCAACTACTACCGTTT
Dro_luci_R1	drosocin	ATTTCAATCAGAGCACTTGG
LacZ INF Fwd	Beta-gal	ACCAACAACTCTAGAGGATCCACC GGTGGCCAAAAAGGCCGGCCGGA GCTGCTCAAGCGCG
LacZ INF Rev	Beta-gal	CCAACTAGTGGATCTGGATCCAAGC TTGGCTGCAGGTCG
CecA1InFusion fw	cecropin A1	ACCAACAACTCTAGAggatccGGTCC TTCGGATGCAGTATTTATTG
CecA1 InFusion rev	Cecropin A1	TTTTTGGCCACCGGTGGATCCACTG CGATACAAAAGGCGAGAG
CecC InFusion fw	cecropin C	ACCAACAACTCTAGAggatccAGACT ATCAGTCACTTAGTTCGCAATGG
CecC InFusion rev	cecropin C	TTTTTGGCCACCGGTGGATCC TTTATAGCGAAGAATGCGATGCC

2.14 Fly strains

2.14.1 Mutants

Name	**Genotype**	**Chromosome**	**Source**
foxo(21)	*w*;*foxo*21/TM6B	3	S. Cohen
Foxo(w24)	*w*;P{lacW}*foxo*W24/TM6B	3	M. Tatar
Foxo Δ94	w;+;FoxoΔ94.Tub^{GS}/TM6B	3	Hoch lab
dTOR$^{\Delta P}$	w;dTOR$^{\Delta P}$/cyo Kr.GFP;+	2	T.Neufeld

2.14.2 GAL4 strains

Name	**Genotype**	**Chromosome**	**Source**
hs-GAL4	*w*;P{GAL4Hsp70.PB}89-2-1	3	Bloomington stock center
Tubulin GeneSwtich	w;+; Tub^{GS}/ Tub^{GS}	3	M.Jünger & S.Pletcher
Caudal-GAL4	w;Cad-GAL4;+	2	B.Lemaitre

2.14.3 UAS strains

Name	Genotype	Chromosome	Source
UAS *dfoxo*™	*yw*; P{UAS-foxo™} /cyo;+	2	M. Tatar
UAS *dfoxo-gfp*	w;+;P{UAS-foxo-GFP}/TM3Ser	3	Hoch lab
UAS *dfoxoWT*	w;UAS *dfoxoWT/UAS dfoxoWT;*+	2	Hoch lab
UAS FKH	w;+;UAS FKH	3	Bloomington Centre
UAS TSC1/2	w;+;UAS TSC1/2	3	N. Tapon
UAS Rheb	w;+;UAS Rheb	3	H. Stocker

2.14.4 RNAi strain

Name	Genotype	Chromosome	Source
pMF-fkh RNAi	*w*;+;PMF-fkh RNAi/ PMF-fkh RNAi	3	Margret Buelow

2.15 RNA Samples for Real Time PCR Analysis

Heat Shock Experiments to read out all AMPs after FOXO™ overexpression

Time Point (hours)	Genotype	M/F
3	**+**;TM;+	F
3	**+**;+;hs	F
3	**+**;+;hs	M
3	+;TM;+	M
-hs	+;+;hs	M
6	+;+;hs	F

6	+;+;hs	M
-hs	+;+;hs	F
6	+;TM;+	M
3	+;TM;hs	F
-hs	+;TM;+	M
3	+;TM;hs	M
-hs	+;TM;+	F
6	+;TM;+	F
6	+;TM;hs	F
6	+;TM;hs	M

+:wild type genotype; TM: Foxo™; hs: Heat Shock; M: male; F: female
3 h and 6 h are time points where flies were collected after carrying out heat shock and stored at -80 °C.

2.16 Antibodies

2.16.1 Primary antibodies

Antibody	**Company/lab**	**Species**	**Concentration**
Forkhead	Margret Buelow	Rabbit	IF (1:250)
HA	Roche	Rat	IF (1:200)

Secondary Antibodies

Antibody	**Species**	**Source**	**Concentration**
α-rabbit-Cy3	donkey	Dianova	IF (1:100)

3 METHODS

3.1 Isolation and purification of DNA and RNA

3.1.1 Isolation of plasmid DNA (mini and midi)

For analytic purpose, 1.5 ml of an *Escherichia coli* culture was centrifuged for 5 minutes at 3500 rpm, resuspended in 200 µl TELT buffer with 1mg/ml lysozyme, incubated at room temperature for 5 minutes and boiled at 99 °C for 3 minutes in a thermomixer. After cooling on ice for 2 minutes, samples were centrifuged at 12000 rpm for 15 minutes at 4 °C, the supernatant was collected in a fresh tube and equal volume of isopropanol was added, incubated at room temperature for 5 minutes Plasmid DNA was pelleted by centrifugation at 13000 rpm for 15 minutes at 4 °C, washed with 500 ml of 70% ethanol, incubated at room temperature for 10 minutes and centrifuged at 13000 rpm for 15 minutes, air dried and resuspended in 30µl aqua bidest. For preparation of bigger amounts or highly pure plasmid DNA (e.g. for cell culture transfection), Nucleospin Plasmid AX-100 kit (Macherey Nagel) was used according to manufacturer's instructions.

3.1.2 Electrophoresis, DNA cleanup and determination of concentration

For separation of DNA fragments, gel electrophoresis with 1% agarose gels was used. Agarose was diluted in 1X TAE buffer and boiled until completely dissolved, then cooled to 60°C and plated. SyberSafe was mixed 1:10000 to fluid agarose before plating. Electrophoresis was done in gel chambers filled with 1X TAE, probes were diluted 1:6 with DNA loading dye. For cleanup of DNA fragments from enzymatic reactions or agarose gels: Nucleospin extractII kit (Macherey Nagel) was used according to manufacturer's instructions. The concentration of DNA or RNA in water was measured using SmartSpec plus photometer (BioRad) and Nanodrop (peQLab). Probes were diluted in a range of 1:5 to 1:100 with water and the optical density at 260 nm was measured. An optical density of 1.0 corresponded to 50µg/ml of DNA or 40µg/ml of RNA.

3.1.3 Isolation of genomic DNA from flies

For isolation of genomic DNA from flies, one to six animals were homogenized with a pestle in 400 µl buffer A and incubated at 65 °C for 30 minutes. 800 µl KAc / LiCl solution (1 part of 5M KAc to 2.5 parts of 6M LiCl) were added and incubated on ice for 10 minutes. Debris was removed by centrifugation at 13000 rpm/4°C for 10 minutes. 1 ml of the supernatant was added to 600 µl isopropanol and centrifuged at 13000 rpm/4°C for 20 minutes. The genomic DNA pellet was washed with 500 µl of 70% ethanol, air dried and resuspended in aqua bidest.

3.1.4 Isolation of total RNA from adult flies

For total RNA isolation from adults, RNA II kit (Macherey Nagel) was used. Adults were shock frozen in liquid nitrogen were transferred to 350 µl RA1 lysis buffer (supplied with RNA II kit, 3.5µl s-mercaptoethanol was added before) and homogenized with Ultra-Turrax T25 basic at full speed for 120 seconds. S2 cells and isolated tissues were directly transferred to filter column (supplied with RNA II kit). No filter column was necessary for S2 cell lysates. Total RNA was isolated according to manufacturer's instructions including DNase I treatment.

3.1.5 Reverse transcription of RNA into cDNA

cDNA of *Drosophila* total RNA probes was produced by reverse transcription using QuantiTect reverse transcription kit (Qiagen) including DNaseI treatment. 500 ng of total RNA were incubated with 1 µl of DNA wipeout buffer (supplied with the kit) and aqua bidest added upto 7 µl at 42°C for 5 minutes. Finally, 2 µl of reverse transcription buffer, 0.5 µl of primer mix and 0.5 µl of enzyme (all supplied with the kit) were added and reverse transcription was performed for 30 minutes at 42 °C, followed by an incubation at 95 °C for 3 minutes. Probes were filled up to 50 µl with aqua bidest before further use.

3.2 Cloning of DNA fragments

3.2.1 Preparation, ligation and transformation

The insert DNA was prepared using GoTaq DNA polymerase and then 3’ overhangs were added to the insert via TOPO cloning methods explained later. The ligation reaction mixture was prepared by combining (in order) the following components (half the volumes of standard procedure - Strata Clone PCR Cloning Kit,CA) 1.5 µl StrataClone Cloning Buffer was poured into an eppendorf tube to which 1 µl of PCR product (5–50 ng, typically a 1:10 dilution of a robust PCR reaction) or 2 µl of StrataClone Control Insert was added and then 0.5 µl StrataClone Vector Mix amp/kan was added and mixed well gently by repeated pipetting and then incubate the ligation reaction at room temperature for 5 minutes. When the incubation was completed, the reaction mixture was placed on ice. One tube of StrataClone SoloPack competent cells was thawed on ice for each ligation reaction. 3 µl of the cloning reaction mixture was added to the tube of thawed competent cells and mixed gently (do not mix by repeated pipetting). The transformation mixture was incubated on ice for 20 minutes. During the incubation period, the LB medium was pre-warmed to 42 °C. The transformation mixture was heat shocked at 42 °C for 45 seconds. Then the transformation mixture was incubated on ice for 2 minutes. 250 µl of pre-warmed LB medium was added to the transformation reaction mixture. Then the competent cells were allowed to recover for at least 1 hour at 37 °C with agitation (the tube of cells lay on the shaker horizontally for better aeration). During the outgrowth period, LB–Ampicillin plates were prepared for blue-white color screening by spreading 40 µl of 2% X-gal on each plate. 150 µl and 100 µl of the transformation mixture was spread plated on the color screening plates. Eventually the plates were incubated overnight at 37 °C. The white or light blue colonies were picked for plasmid DNA analysis. The mini-preps were prepared from the selected colonies using standard protocols. The restriction digestion analysis was performed of the miniprep DNA to identify colonies harboring the desired clone. The PCR product insertion site is flanked by *Eco*R I sites for convenient identification of insert-containing plasmids. To screen for clones with a specific insert orientation, the miniprep DNA was digested with a restriction enzyme with a single cleavage site in the insert DNA and one or a small number of sites in the vector DNA. Light blue colonies were picked.

3.2.2 Enzymatic digestion, vector preparation and ligation

Enzymatic digestion of DNA was done using Roche restriction endonucleases and buffers. 3-5µg of DNA was digested in a total volume of 30 µl, including 2 µl of the appropriate 10x buffer and 3-5 unit of enzyme per µg of DNA. After 2-3 h of incubation at 37 °C, DNA fragments were cleaned using Nucleospin extract II kit (Macherey Nagel) or separated by gel electrophoresis. Plasmid vectors were Plasmid vectors were digested as described above and dephosphorylated by adding 1 µl of rapid alkaline phosphatase (Roche) and 3 µl of the appropriate rAPid buffer to the sample. Dephosphorylation was done at 37 °C for 30 minutes, followed by inactivation of the enzyme at 75 °C for 2 minutes. Linearized plasmid vectors were separated by gel electrophoresis and cleaned using Nucleospin extract II kit (Macherey Nagel). Ligation of DNA fragments into plasmid vectors was carried out o/n at 16 °C in a total volume of 10 µl, including 1 µl 10 x ligation buffer and 1 µl T4 DNA ligase (Roche). The ratio of insert to vector was 3:1.

3.2.3 TOPO cloning

The required dATP overhang of the insert was added in a *Taq* polymerase reaction, which was performed at 72 °C for 15 minutes, followed by a cleanup using Nucleospin extract II kit (Macherey Nagel). The reaction included:

Go Taq polymerase Reaction-Addition of 3'overhangs to the insert DNA	
Water	Add upto 30µl
5X Green Go Taq Buffer	6µl
dATP	0.5µl
Template DNA	20µl
Go Taq Polymerase	0.25µl

3.2.4 Colony PCR

This technique is used to determine insert size and/or orientation in the vector. Alternately, the presence of an insert and its size can be determined by growing each colony in liquid, the plasmid purified by a boiling or alkaline preparation protocol, digestion of the plasmid with restriction enzyme(s) that excises the

insert, followed by separation by agarose gel electrophoresis.
Typical colony PCR reaction: Mix together the following on ice; always adding enzyme last. For multiple samples, make a large master mix and aliquot 50 µl in each PCR tube (also on ice).

Colony PCR	
Template DNA	1 µl plasmid DNA
Green GoTaq buffer	10 µl (PCR Buffe 5x)
Forward primer	0.5 µl
Reverse primer	0.5 µl
dNTPs	1 µl
25 mM MgCl2	3 µl
Polymerase	0.2 µl (Promega GoTaq)
Aqua bidest	ad. 50 µl

To each cold PCR tube containing the PCR reaction, a small amount of colony was added. To do this, a fine yellow pipette tip attached to a pipetter was used and pipette up and down to mix. Sufficient mixing results in complete cell lysis and high yields.
Program:

Cycle Step	**Temperature**	**Time**	**Number of Cycles**
Initial denaturation	95°C	1 min	1
Denaturation Annealing Extension	95°C 54-74°C 72°C	1 min 1 min 1 min/kB	30-40
Final extension	72°C 4°C	5 min hold	1

3.2.5 Sequencing DNA

Sequencing was performed by SeqLab. The DNA was prepared according to the requirement of the company.

3.3 PCR techniques

3.3.1 Primer design for PCR and real-time PCR

Primers were designed using Primer3 software (http://frodo.wi.mit.edu/). The following conditions were used:

Primers for cloning and analytical use		
Condition	Range	Optimum
Primer length	18-25 bp	20 bp
Melting temperature	50-65 °C	60 °C
% GC (of total)	35-60	50

Quantitative real-time primers		
Condition	Range	Optimum
Primer length	18-25 bp	20 bp
Product length	75-150 bp	120 bp
Melting temperature	60-64 °C	61 °C
% GC (of total)	40-60	50

Primers were synthesized by Invitrogen (Karlsruhe) without 5' and 3' modifications, desalted and shipped lyophilised. Before use, primers were resuspended in aqua bidest to a final concentration of 20 pmol/µl.

3.3.2 Semi-quantitative PCR for analytical purpose and cloning

For analytical purposes, *Taq* (Fermentas) or Go*Taq* (Promega) polymerases without proofreading capability were used, whereas for cloning *Pfu* polymerase (Promega) Phusion Hot Start Polymerase (Thermo scientific) and KOD Hot Start DNA polymerase (Novagen) with proofreading activity were taken. PCR reactions were set up as follows:

Go*Taq* PCR assays	
Template DNA	1 µl of genomic DNA from flies
Green GoTaq buffer	5 µl (Promega 5x)
Forward primer	0.5 µl
Reverse primer	0.5 µl
dNTPs	1 µl
Polymerase	0.125 µl (Promega GoTaq)
Aqua bidest	ad. 25 µl

***Pfu* PCR assays**	
Template DNA	2 µl
Pfu buffer	5 µl
Forward primer	0.5 µl
Reverse primer	0.5 µl
dNTPs	1µl
Polymerase	0.5 µl
Aqua bidest	ad. 50 µl

KOD Hot Start DNA Polymerase	
Template DNA	4 µl
10X Buffer for KOD Hot Start DNA Polymerase	5 µl(1X-final concentration)
25mM MgSo4	3 µl (1.5mM-final concentration)
dNTPs(10mM each)	1 µl (0.2mM each)
Forward Primer	1.5 µl (0.3µM-final concentration)
Reverse Primer	1.5 µl(0.3µM-final concentration)
KOD Hot Start polymerase(1U/µl)	1 µl(0.2U/ µl)
Aqua bidest	Ad.50 µl

Phusion Hot Start High Fidelity DNA Polymerase		
Component	Volume/20 µl reaction	Final Concentration
H_2O	13.4 µl	
5x Phusion HF buffer	4 µl	1x
10 mM dNTPs	0.4 µl	200 µM each
Forward Primer	1 µl	0.5 µM
Reverse Primer	1 µl	0.5 µM
Template DNA	1 µl	1pg-250ng (gDNA)
Phusion Hot Start DNA Polymerase (2U/ µl)	0.2 µl	0.02 U/ µl

Cycling and temperature profile for Phusion Hot Start High Fidelity DNA Polymerase:

Cycle Step	Temperature	Time	Number of Cycles
Initial denaturation	98°C	30s	1
Denaturation Annealing Extension	98°C 60-74°C 72°C	10s 30s 60s	25-35
Final extension	72°C 4°C	5-10 min hold	1

Cycling and temperature profile for KOD Hot Start DNA Polymerase

Cycle Step	**Temperature and time**
Polymerase activation	95 °C for 120 seconds
Denature	95°C for 20 seconds
Annealing	64°C for 10 seconds
Extension	69°C for 120 seconds
Repeat steps denature(2) to Extension - 40 cycles	

Annealing temperature was primer specific; elongation time was depending on the enzyme:

KOD Hot Start DNA polymerase: 25 seconds per 1000 bp

Pfu polymerase: 120 seconds per 1000 bp

Go*Taq* polymerase: 60 seconds per 2000 bp

3.4 Quantitative real-time PCR Cycling and temperature profiles

Temperature	**Time (s)**	**Action**	**Number of cycles**
95 °C	120	Denaturation	1
95 °C 52°C-61°C 72 °C	30 30 30-180	Denaturation Annealing Elongation	35 cycles
72 °C	300	Final elongation	1
12 °C	forever		

Quantitative real-time PCR experiments were done with the I-cycler and IQ5 optical system (BioRad) using SYBR-Green to detect amplification after each PCR cycle. cDNA probes of reverse transcribed total RNA were used as template.

Reactions were performed as duplicates in 96-well plates in a total volume of 25 µl. Gene expression studies were analyzed with IQ5 optical system software (BioRad). All expression data represent the statistical mean of at least two independent experiments, error bars show standard errors of the mean. Expression is always shown relative to a control condition and relative to an internal expression control, which was *rpl32* (*rp49*) in all experiments. Expression data were calculated according to the delta-delta-CT method.

Real-time PCR experiments for vertebrate genes were performed with a Light Cycler Taqman master kit and a universal probe library assay on a Light Cycler 1.3 instrument (Roche). For each gene, three replicate reactions were performed. Primers for real-time PCR assays were designed as described and tested for efficiency before use. Efficiency tests include dilution of template cDNA from 1:1 up to 1:125. Primers used for real-time PCR showed at least 80% efficiency up to a dilution of 1:25. All primers were optimized and used at an annealing temperature of 59 °C. The appearance of primer dimer was further ruled out by melt curve analysis.

SYBRgreen real-time PCR assay	
Template cDNA	1 µl
Forward primer	0.5 µl (5 pmol/µl)
Reverse primer	0.5 µl (5 pmol/µl)
2x SYBR-Green Supermix	12.5 µl
Aqua bidest	10.5 µl

Cycling and temperature profiles			
Temperature	**Time (s)**	**Action**	**Number of cycles**
95 °C	300	Denaturation, polymerase initiation	1
95 °C 59 °C 72 °C	30 30 30	Denaturation Annealing Elongation	40
55 °C to 95 °C (+0.5 °C per cycle)	30	Melt curve	81

3.5 Promoter studies

3.5.1 Identification of dFOXO binding motifs

A search for conserved dFOXO/Forkhead binding motifs was done within the 2 kB genomic region upstream of different antimicrobial peptides according to genomic sequences deposited in FlyBase (http://flybase.org/). Putative dFOXO/Forkhead binding motifs were identified by the following sequences:

Binding motif	**Description**	**Sequence**
dFOXO (for.d)	dFOXO binding motif on the plus strand	TTGTTTAC
dFOXO (rev.)	dFOXO binding motif on the minus strand	GTAAACAA
Forkhead (for.)	Forkhead binding motif on the plus strand	T(X)TTTA
Forkhead (rev.)	Forkhead binding motif on the minus strand	TAAA(X)A

X: Any nucleotide and **Y**: purine A or G

3.6 Work with Drosophila

3.6.1 Cultivation, crossing and recombination experiments

Drosophila stocks were kept on standard fly food at 18 °C and amplified at 25 °C. For amplification, adult flies were put to fresh food vials every three days. Stocks were kept homozygous or balanced, using chromosome specific balancers, to avoid changes in genotypes due to recombination. For crossing experiments, virgin female flies were crossed with male flies at 25 °C. A proportion of 2:1 (females to males) was used for crossing; genotypes were followed by genetic markers. Virginity was assured by isolating freshly hatched females in time, which were 5 h at 25 °C and 16 h at 18 °C. For recombination of chromosomes, virgin female progeny carrying two chromosomes in a transheterozygous combination were crossed with males carrying appropriate balancer chromosomes. Offspring being candidates for recombined chromosomes were selected, amplified and analyzed.

3.6.2 Fly work

If not further mentioned, fly stocks were obtained from the Bloomington stock centre. The FKH overexpression and RNAi lines were constructed by Margret Bü̈low. The TSC1/2 and Rheb overexpression lines were obtained from the Tapon and Stockers lab respectively. The loss-of function allele dTOR which is a deletion generated by imprecise P-element excision and removes the dTOR translation start site as well as the amino-terminal 902 codons, was a gift of Tom Neufeld. All the larvae including the mutants of TOR and Dif;Rel were staged by time after egg laying at 25°C, third instar (72**–**76 h). Larvae were kept on PBS-agar plates with yeast paste.

3.6.3 GAL4-UAS experiments and heatshock

Overexpression of specific proteins was done by using the GAL4/UAS system from yeast in *Drosophila*. To generate offspring that contain both, promoter dependent GAL4 expression and UAS dependent target gene sequences, flies carrying GAL4 elements were crossed with flies containing UAS sequences followed by a specific gene of interest. If not further mentioned, all GAL4/UAS experiments were done at 25 °C. GMR-GAL4 dependent overexpression in the eye was done at 28 °C. Overexpression in larvae or adult flies using the *heatshock* GAL4 system was achieved by incubation at 37 °C for 45 minutes, followed by incubation at 25 °C for 3h and 6h time point and adults were shock frozen in liquid nitrogen.

3.6.4 GeneSwitch system

This GAL4-UAS system is based on a chimeric gene (Gene-Switch) that encodes the GAL4 DNA-binding domain, the human progesterone receptor-ligand-binding domain, and the activation domain from the human protein, p65. In the presence of the antiprogestin, RU486, the chimeric molecule binds to a UAS and provides for ligand-inducible transactivation of downstream target genes. The Gene-Switch was used as an alternative *Drosophila* gene expression system. It provided the experimental control of transgene expression in both time and space. In the absence of an activator (uninduced), the GeneSwitch GAL4 protein is expressed in target tissues but remains transcriptionally silent; no expression of downstream UAS-linked genes therefore occurs. However, after systemic application of RU486

(induced), the binding of the RU486 ligand causes the GeneSwitch GAL4 protein to become transcriptionally active, resulting in ex-pression of UAS-linked genes (shown here in figure 3.1. as UAS-GeneX).

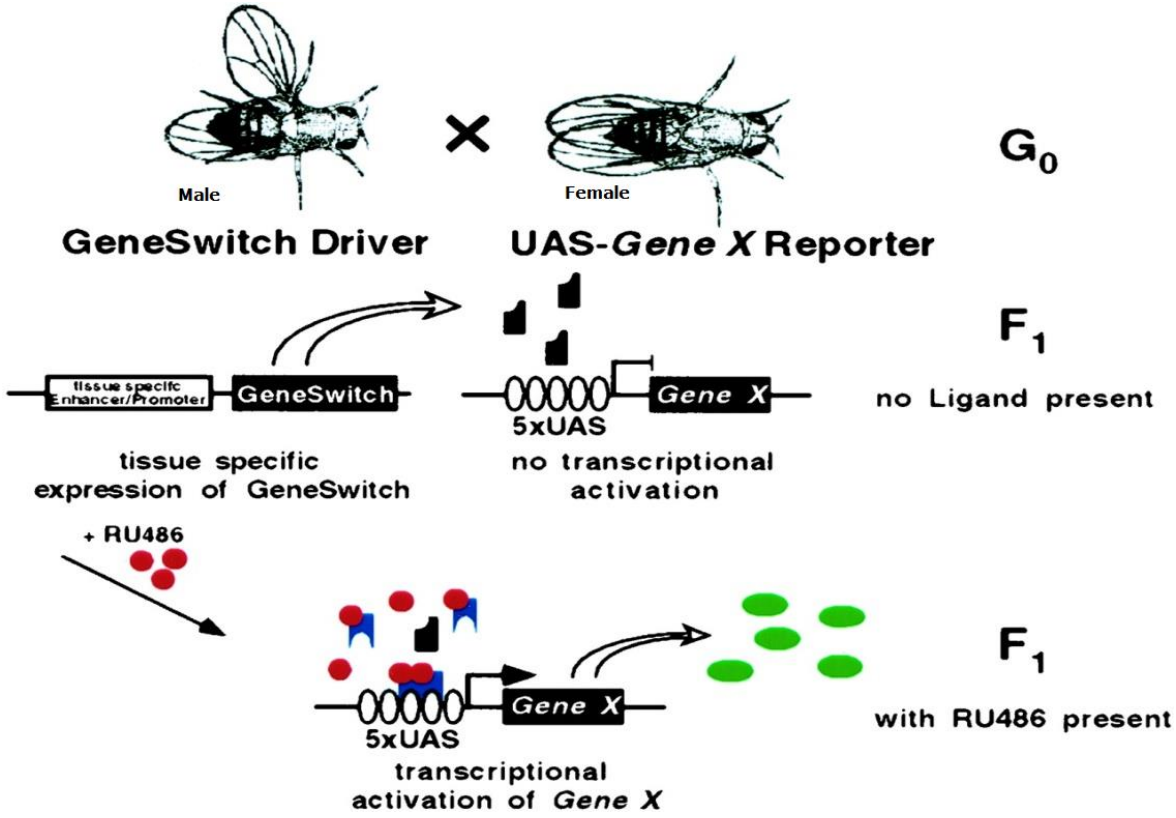


Figure 3.1. The GeneSwitch/UAS expression system in *Drosophila.* Driver lines expressing the transcriptional activator GeneSwitch in a tissue-specific fashion are crossed to UAS-reporter lines with genomic inserts of a target gene fused to five GAL4-binding sites arrayed in tandem (5× UAS). In the absence of an activator, the GeneSwitch protein is expressed in target tissues but remains transcriptionally silent (black); *Gene X* is therefore not expressed. However, after systemic application of RU486 (red), the GeneSwitch protein becomes transcriptionally active (blue), mediating expression of *gene X* (green) in only those tissues expressing GeneSwitch. Modified after Osterwalder et al. 2001.

3.6.5 Larval rapamycin feeding

If not stated otherwise, embryos were collected for 4 h on PBS- agar plates with yeast paste, which was prepared by suspending one cube of 42 g fresh yeast in 6.5 ml PBS. The larvae used as samples for various experiments depicted in the figures were treated as follows: for the ‘‘yeast’’ condition, larvae were kept on yeast paste until 72 h AED. For the ‘‘Rapamycin’’ condition, larvae were kept on yeast paste until 48 h AED, before 200 µl of a 50 µM rapamycin (LC Laboratories)

solution was added to the yeast paste and the plates were further incubated for 24 h at 25°C.

3.6.6 Larval mifepristone feeding

If not stated otherwise, embryos were collected for 4 h on PBS- agar plates with yeast paste, which was prepared by suspending one cube of 42 g fresh yeast in 6.5 ml PBS. The larvae used as samples for various experiments depicted in the figures were treated as follows: for the ‘‘yeast’’ condition, larvae were kept on yeast paste until 72 h AED. For the ‘‘mifepristone’’ (RU486) condition, larvae were raised on PBA agar plates to the desired age of 60 h AED. Up to 10 mg/ml of RU486 (Mifepristone, Sigma) was dissolved in ethanol or DMSO. For larval feeding, RU486 was diluted 25-fold from the working concentration in ethanol and directly mixed with the larval food to get a final concentration of 4% ethanol. The plates were further incubated for 24 h at 25°C.

3.6.7 Clonal Analysis

The Flp/Gal4 technique was used to overexpress 3xHA-FKH in cluster of cells in different organs of the larvae (Schematic 2). Females of the genotype hs-Flp;Sp/CyO; Act>CD2>Gal4, UAS-GFP/TM6B, Tb were crossed to homozygous UAS-3xHA-FKH males and allowed to lay eggs for 4 h. Early third instar Tb+ larvae were screened for clusters of cells expressing GFP and quickly dissected in PBS. In-situ hybridisation for Mtk was done as described, followed by alkaline phosphatase chemical staining and incubation with anti-GFP (Santa Cruz) antibody overnight at 4°C. The anti-GFP antibody was overlaid with Alexa488. Pictures were taken at a Zeiss LSM-710 confocal microscope for both transmission and laser light. For in-situ hybridisation, larvae were roughly dissected in PBS, fixed in fixation solution for 1 h at room temperature and washed in PBT (4x 15 minutes) on a rotator. Tissues were stepwise transferred to Hybe buffer by incubation in PBT + Hybe-B (1:1, room temperature; 50% formamid, 5x SSC), Hybe-B (65 °C) and Hybe-B + Hybe (1:1, 65 °C; 50% formamid, 5x SSC, 0,2 mg/ml sonicated salmon testis DNA, 0,1 mg/ml tRNA, 0,05 mg/ml heparin), each for 10 minutes. Pre-hybridisation was done in Hybe buffer at 65 °C (waterbath) for at least 1 h. RNA probes were diluted in Hybe buffer, boiled at 95 °C for 3 minutes and cooled on ice. Pre-hybridisation solution was removed from larval tissues, probes were

added and incubated overnight at 65 °C. Probes were then removed completely and tissues were stepwise transferred to PBT by incubation in Hybe-B + Hybe (1:1, 65 °C), Hybe-B (65 °C) and PBT + Hybe-B (1:1, room temperature), each for 10 minutes, followed by washing in PBT (4x 15 minutes). Before addition of the primary antibody, blocking in PBT + 5% donkey serum was performed for 1 hour. Anti-DIG antibody was added in blocking solution in a dilution of 1:200 at 4°C overnight, followed by washing in PBT (4x 15 minutes). Secondary antibodies Alexa 633 was diluted 1:200 in PBS+0.1% Tween-20+10% Donkey Serum and was applied for 1 h at RT. DAPI (1 mg/ml) was included in the last washing step before samples were mounted in Mowiol (Roth). Mounted tissue was analyzed using a Zeiss LSM 710 confocal microscope and images were further processed with the Zeiss LSM Image Software.

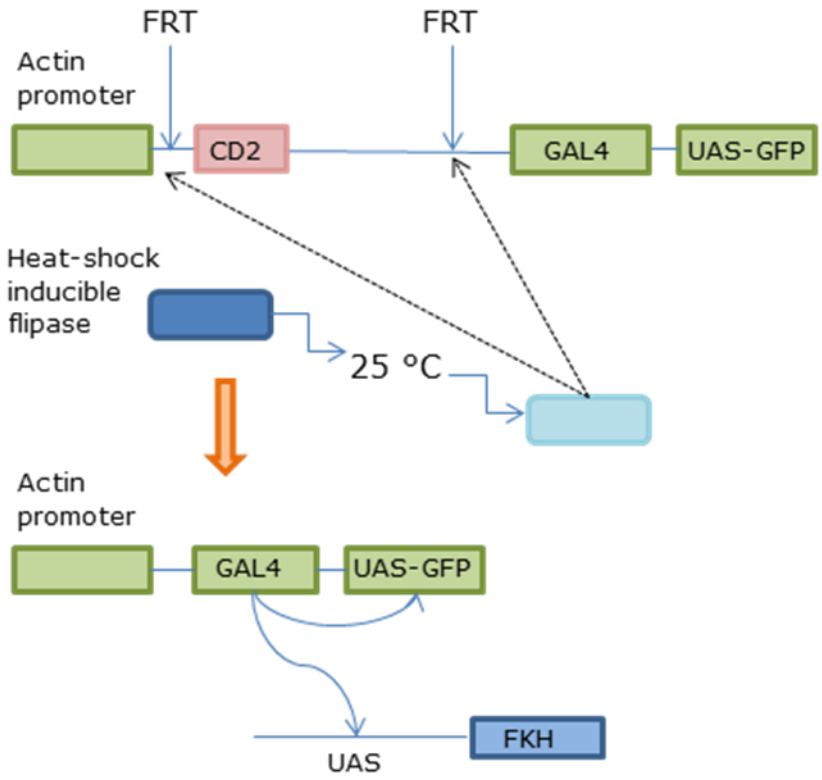


Figure 3.2. Heat-shock inducible Flip-FRT driver line to generate clones which express a responder construct and are labeled with GFP. Single cells can be manipulated and visualized using a driver line which carries a construct containing GFP under the control of an actin driver, interrupted by a FLP-out cassette and under the control of a heat-shock promoter. When crossed to the responder of interest and subjected to a temperature of 25°C, the FLP-out cassette is removed in random cells, allowing the expression of the responder construct and GFP at the same time, thereby labeling the affected cells. Cells which are not marked by GFP are unaffected by the responder construct and serve as wildtype control. The blue box is the switch in temperature. Modified after Pignoni & Zipursky, 1997.

3.7 Immunohistochemistry

3.7.1 Immunofluorescent staining

Antibody stainings were performed on white- (w^{1118}) larvae. The larval tissues were dissected in Drosophila Ringer's solution and fixed for 30 minutes in 4% formaldehyde in PBS. The tissue was then washed with PBS+0.3% Tween-20 for 30 minutes and further washed with PBS+0.5% Tween-20 for 10/10/10/10 minutes each. The tissue was then blocked with 10% Donkey Serum in PBS+0.5% Tween-20 for 30 minutes and washed before and after incubation with primary antibody in PBS+0,1% Tween-20 respectively, for 5/5/15/30 minutes. Incubation with primary antibodies diluted in blocking solution was performed overnight at 4°C. Fluorescence-coupled secondary antibodies were applied for 1 h at RT. DAPI (1 mg/ml) was included in the last washing step before samples were mounted in Mowiol (Roth). Primary antibodies used were rabbit α-FKH1 (dilution 1:250), mouse anti-GFP (Abcam, dilution 1:1000). Secondary antibodies donkey anti-rabbit Cy3 IgG (Dianova) and donkey anti-mouse Alexa 488 IgG (Dianova) were all diluted 1:200 in PBS+0.1% Tween-20+10% Donkey Serum. Mounted tissue was analyzed using a Zeiss LSM 710 confocal microscope and images were further processed with the Zeiss LSM Image Software.

3.7.2 JB-4 embedded tissue sections

Rapamycin-treated or normally yeast-fed third instar larvae were fixed in 4% paraformaldehyde at 4°C overnight. Larvae were dehydrated and embedded with the JB-4 Plus Embedding Kit (Polysciences) as described in the manufacturer's protocol. Polymerized blocks were cut into 7 µm thin sections using an ultramicrotome (Reichert-Jung). Immunohistochemical staining of the sections was performed as described below: Sections were rehydrated in a descending ethanol series. For antigen retrieval, sections were incubated for 5/5/5 min each with boiling citrate buffer (10 mM sodium citrate, pH 6.0) and were trypsinized for 1 h with 0.001% trypsin in 0.05 M Tris-HCl, pH 8.0. Sections were blocked with PBS+10% Donkey Serum for 30 min and were washed 5/5/5 minutes each with PBS. Incubation with primary α-FKH1 (dilution 1:250) antibody diluted in PBS+1% Donkey Serum was performed overnight at 4 °C. Sections were washed 5/5/5 minutes each with PBS. Fluorescence coupled secondary antibody donkey anti-rabbit Cy3 (diluted 1:150 in PBS+1% Donkey Serum) was applied for 2 h at RT.

After washing 5/5/5 minutes each with PBS, sections were mounted in Vectashield H-1500 with DAPI (Vector labs). Mounted sections were analyzed using a Zeiss LSM 710 confocal microscope and images were further processed with the Zeiss LSM Image Software.

3.8 Cell culture work

3.8.1 Cultivation and starvation

Drosophila Schneider cells were cultivated in plastic flasks with appropriate culture medium, which is Schneiders medium (Invitrogen) for *Drosophila* cells containing 10 % FCS. For starvation experiments, $1x10^5$ cells were seeded to 6-well plates. Starvation was done by incubating cells in appropriate medium without FCS for 24 h.

3.8.2 Transient transfection and induction

1x105 cells were seeded to 6-well plates with fresh medium containing 10 % FCS. For each well, 10 µl Cellfectin (Invitrogen), 100 µl cell culture medium and 1.5 µg of each plasmid were mixed in a 1.5 ml Eppendorf cap and incubated for 30 minutes at room temperature before addition to the cells. The transfection solution was incubated for 5 h and plates were gently shaken every hour. After this time, the medium was removed completely; cells were washed twice and incubated with fresh medium containing 10 % FCS.

3.8.3 Luciferase assays

The luciferase pGL3 constructs or empty pGL3 vector were cotransfected with pMT-GAL4 and UAS dFOXO-GFP into S2 cells. 16 h after transfection, dFOXO overexpression was induced by adding CuSO4 to a final concentration of 0.5 mM into the medium. Cells were incubated for 24h, followed by lysis in 350 µl RA1 buffer (Macherey Nagel) and RNA isolation. The *luciferase* expression was measured by real-time PCR (using primers Luc-Sy-F1 and Luc-Sy- R1) and normalized to GFP expression (with primers GFP-Sy-F1 and GFP-Sy-R1).

3.9 Statistics

Error bars represent the standard error of the mean (SEM) if not stated otherwise. Statistical significance was assessed using an unpaired two-tailed Student's t-test, comparing the experimental data with the respective controls. If not stated otherwise, all experiments were repeated by preparing RNA from independent biological samples at least three times. Asterisks indicate a p- value of less than 0.05 (*), 0.01 (**) or 0.001 (***).

4 RESULTS

4.1 Effect of dFOXO on the regulation of AMPs

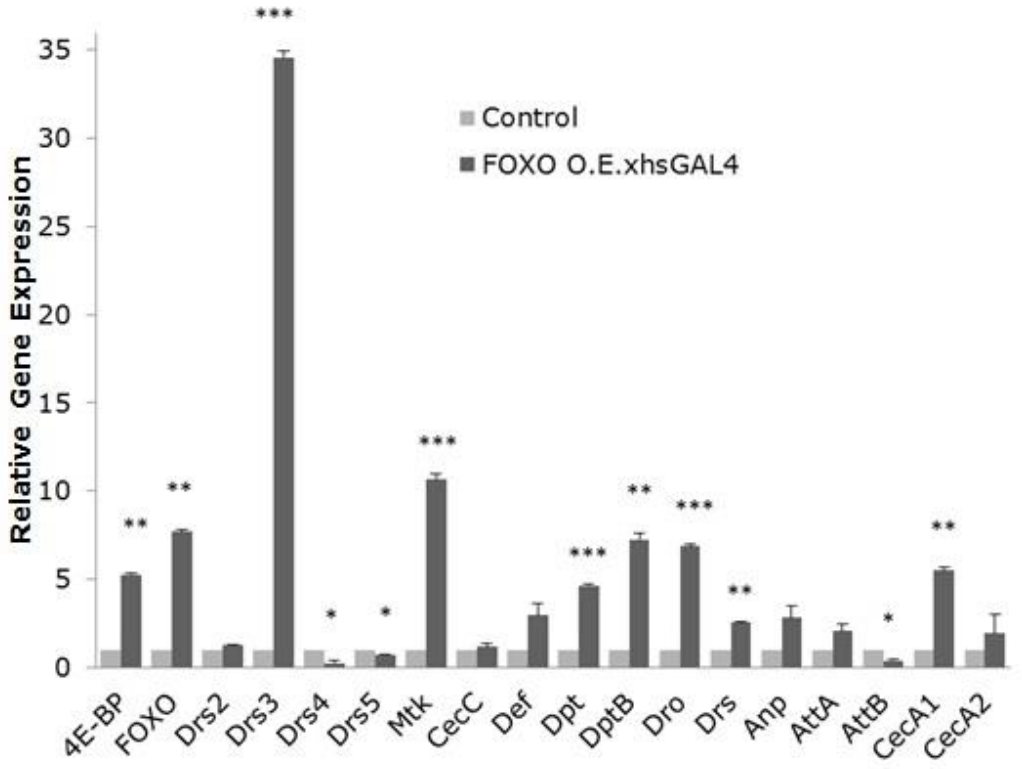
4.1.1 Analyses of AMP regulation upon expression of dFOXO

4.1.1.1 AMP expression upon dFOXO overexpression

In *Drosophila*, IlS is known to be tightly coupled to the abundance of food when larvae are growing (Zinke et al. 2002). As a direct result, IlS activity is quickly reduced when food is scarce and dFOXO is activated. To demonstrate whether dFOXO activity alone is sufficient to trigger AMP transcription, a series of overexpression experiments using the GAL4/UAS system were performed in adult flies. A modified dFOXO protein was used for overexpression, which is constitutively active in the nucleus due to the changed phosphorylation sites (dFOXO™, Junger et al. 2003). To control the time of expression under a particular condition the heat shock GAL4 driver line (hsGAL4) was chosen. As heat shock is an inducible system, the GAL4 driver was activated by shifting temperature from 25 °Celsius to 37 °Celsius, followed by re-incubation at 25 °Celsius. The reason to choose this driver line was 1) to prevent the target genes from always being active, as FOXO expression would be strong and thereby would activate many downstream signaling pathways, and 2) to see the immediate response of AMP gene regulation. The time point at which the flies (both males and females) were analyzed for AMPs' regulation after dFOXO overexpression was 3 hours and the study was carried out for 5 days as well as 30 days old flies (Figure 4.1). As control readout dFOXO expression was monitored. Additionally, 4E-BP as a target gene of dFOXO was analyzed. AMP expression was strongly increased in both 5 and 30 days old adult flies (Figure 4.1). In detail, the AMPs Andropin (Anp), Cecropin A1 (CecA1), Diptericin (Dpt), Diptericin-b (DptB), Drosocin (Dro), Drosomycin 3 (Drs3) and Metchnikowin (Mtk) were induced by dFOXO in 5 day old flies (Figure 4.1A). In addition to these AMPs, also Cecropin-c (CecC) was induced through dFOXO in 30 day old flies (Figure 4.1B). These data give strong indication for a dFOXO dependent regulation of AMP genes in *Drosophila* adult flies. Interestingly, Drosomycin 4 (Drs4), Drosomycin 5 (Drs5) and Attacin B (AttB) were slightly downregulated in 5 days old flies, which was not

the case in 30 days old flies (Figure 4.1). This could be due to the fact that with age there is an increase in expression of immune genes (Girardot et al. 2006).

A



B

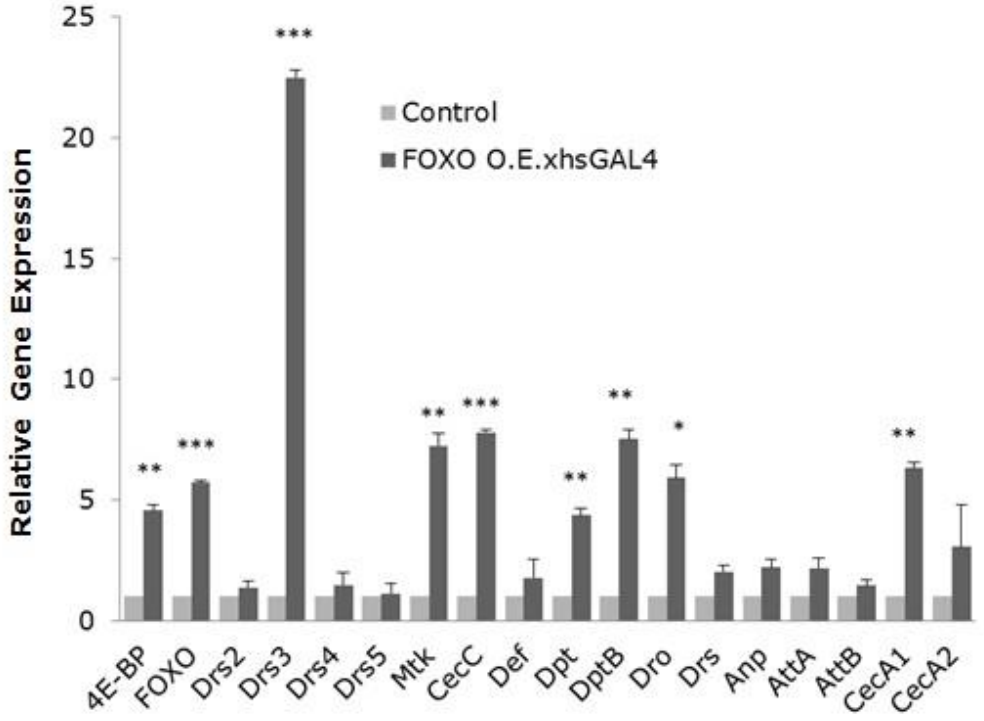


Figure 4.1. Upregulation of AMPs after overexpression of constitutively active dFOXO™. (A and B) Expression of AMPs in 5 **(A)** and 30 days old adults **(B)** observed at 3 hours after heat shock. The flies were reared on the 7.5 % Sugar and Yeast food. The experiment flies (FOXO O.E.xhs-GAL4) had the genotype +; FOXO^{TM}; hsGAL4 and the control used for this experiment was +; +; hsGAL4. 4E-BP: 4E binding protein, FOXO: Forkhead box class O, Anp: andropin, AttA: attacin A, AttB: attacin B, CecA1: cecropin A1, CecA2: cecropin A2, CecC: cecropin C, Def: defensin, Dpt: diptericin, DptB: diptericinB, Dro: drosocin , Drs: drosomycin, Drs2: drosomycin 2, Drs 3: drosomycin 3, Drs 4: drosomycin 4, Drs5: drosomycin 5 and Mtk: metchnikowin. A minimum of n = 3 has been analyzed for each set of experiment. Significance was tested using an unpaired 2-tailed Student's t-test. $P < 0.05$ (*); $p < 0.01$(**); $p < 0.001$ (***).

4.1.1.2 AMP expression in starved dFOXO mutants

To further analyze the physiological role of dFOXO concerning the AMP regulation, a series of starvation experiments in wild type adult flies were performed. Under starvation conditions, dFOXO translocates into the nucleus and activates the transcription of its target genes; among them is 4E-BP (Fuss et al. 2006). Therefore, it was of interest to examine whether physiologically upregulated dFOXO levels would also induce AMP expression. To show that this upregulation was really dFOXO dependent, dFOXO null mutant adult flies (*dfoxo*^{21}/*dfowo*W^{24}) were starved in parallel to the white- (hereafter referred to as wild type) flies. The expression of AMPs in wild type and dFOXO null mutant flies was compared after starvation (Figure 4.2). Some of the AMPs (CecA1, CecB, Dro, Drs2, Drs4 and Mtk) were similarly induced as in the wild type flies, although dFOXO was missing indicating that the expression of these AMPs is not solely dFOXO dependent. However, in the case of AttA, CecC, Def, and Drs3 the induction was abolished in dFOXO mutants hinting towards a dFOXO dependent activation (Figure 4.2). Taken together, these data give strong evidence for a dFOXO dependent regulation of AMP genes in *Drosophila* adult flies.

4.1.2 Analyses of tissue dependent AMP expression by dFOXO in adult flies

For NF-κB dependent regulation of AMPs it has been shown that innate immunity triggers expression of these peptides either locally in barrier epithelia or systemically in the fat body (Lemaitre et al. 2007). The tissue of choice is thereby only dependent on the type of infection. In contrast, dFOXO dependent AMP

regulation occurs in non-infected animals and is depending on the energy status of the cell, which uncouples AMP expression by dFOXO and by NF-κB.

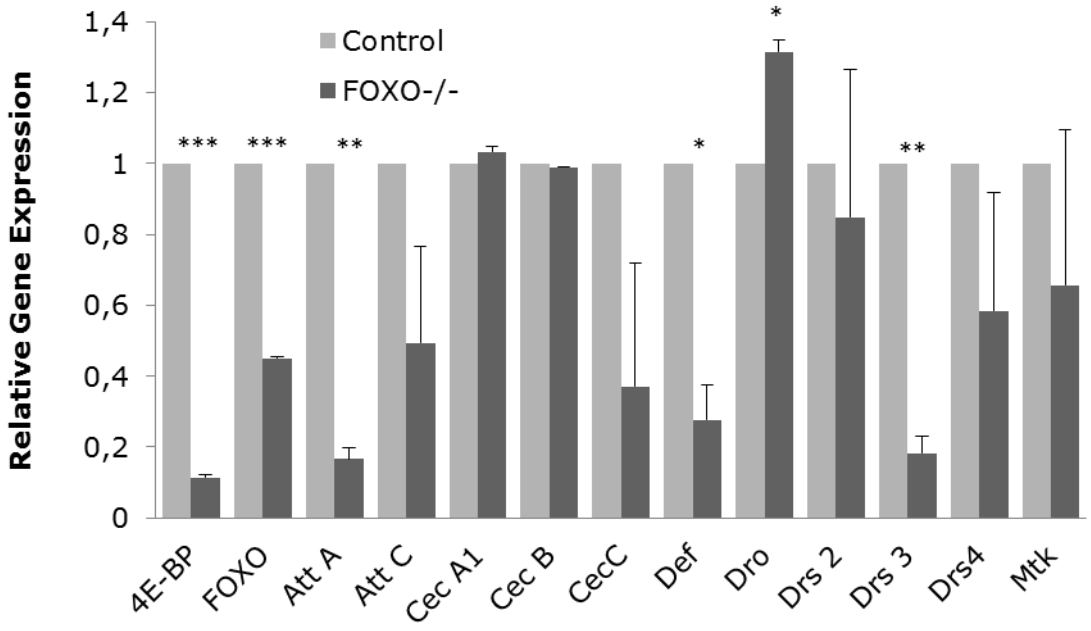


Figure 4.2. Expression of AMPs in dFOXO null mutant flies after starvation. Expression of AMPs in adult dFOXO mutants after starvation on PBS compared to starved wild type (Control) flies. FOXO-/-: w; +; $dFOXO^{21}/dFOXO^{24}$. 4eBP: 4E Binding Protein, FOXO: Forkhead box class O, AttA: attacin A, AttC: attacin C, CecA1: cecropin A1, CecB: cecropin B, CecC: cecropin C, Def: defensin, Dro: drosocin , Drs2: drosomycin 2, Drs 3: drosomycin 3, Drs 4: drosomycin 4 and Mtk: metchnikowin. A minimum of n = 3 has been analyzed for each set of experiment. Significance was tested using an unpaired 2-tailed Student's t-test. $p < 0.05$ (*); $p < 0.01$(**); $p < 0.001$ (***). Error bars represent SEM.

So far, it is unclear in which tissues a dFOXO dependent AMP regulation is taking place. *In-situ* hybridization experiments revealed that the fat body is targeted by this new mechanism, but the question remained whether also barrier epithelia show a nutrient dependent regulation of AMPs (Becker et al. 2010). To obtain further insight into AMP induction by dFOXO, various tissues were isolated after overexpressing dFOXO as well as modified protein (FOXO™). Expression analyses were done in isolated adult fly tissues, including the gut, which is the barrier epithelia in direct contact with the microorganisms, and the fatbody, the major site for the regulation of both energy homeostasis and systemic innate immunity in insects.

4.1.2.1 AMP expression upon dFOXO overexpression in the gut

The gut epithelium is frequently exposed to harmful pathogens and, therefore, it must be armed with an efficient and powerful immune system to protect itself. The fruit fly *Drosophila melanogaster* possesses a gut that is structurally and functionally similar to mammalian intestinal tract (Lemaitre and Miguel-Aliaga, 2013), which is constantly in contact with microbial pathogens as flies ingest large quantities of microorganisms through feeding on rotting fruits. *Drosophila* is a powerful model organism for deciphering innate immune responses (Lemaitre and Hoffmann, 2007). It is known that Caudal (Cad) acts as a gut-specific transcriptional repressor exerting its antagonistic role in commensal-induced NF-κB–dependent AMP induction. Furthermore, the overexpression of Cad in the gut could abolish the infection-induced AMP expression (Lemaitre and Hoffmann, 2007). Therefore, it was interesting to analyze AMP regulation in the gut of adult flies upon dFOXO overexpression. This was performed by overexpressing the dFOXO wild type protein in a Cad pattern. The expression of different AMPs was analyzed from either control (wild type crossed in Cad background (Cad-GAL4 x w-)) or experiment condition (dFOXO overexpressed in Cad pattern (Cad-GAL4 x UAS-FOXO^{WT}), and AMP upregulation in the latter tissues was calculated relative to the appropriate control tissue. The AMPs that were upregulated upon overexpression of dFOXO in the Cad pattern in the gut of adult flies were: AttA, CecC, Def, Dpt, DptB, Dro, Drs, Drs2, and Drs3 (Figure 4.3). In summary, the barrier epithelia gut expressed a broad spectrum of dFOXO dependent AMPs.

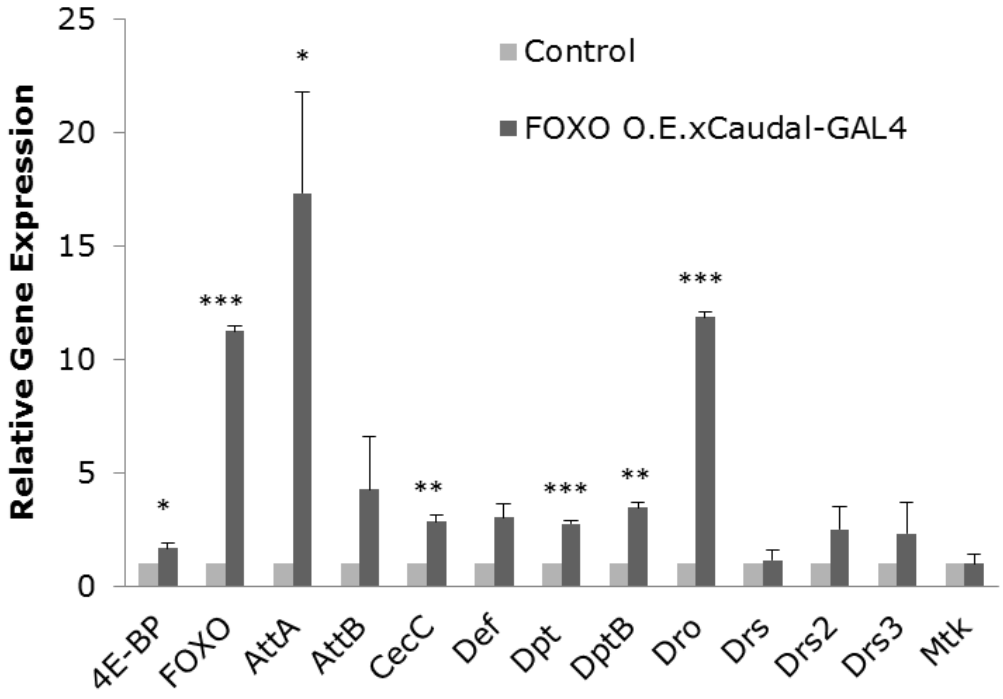


Figure 4.3. AMP expression upon overexpression of dFOXO^{WT} in the gut of adult flies. The experiment condition flies (Cad x FOXO^{WT}) were compared to the control flies (Cad x w-) and both were reared on the standard fly food. 4E-BP: 4E binding protein, FOXO: Forkhead box class O, AttA: Attacin A, AttB: Attacin B, CecA1: Cecropin A1, CecC: Cecropin C, Def: Defensin, Dpt: Diptericin, DptB: Diptericin B, Dro: Drosocin, Drs: Drosomycin, Drs2: Drosomycin2, Drs3: Drosomycin3 and Mtk: Metchnikowin. A minimum of n = 3 has been analyzed for each set of experiment. Significance was tested using an unpaired 2-tailed Student's t-test. $p < 0.05$ (*); $p < 0.01$ (**); $p < 0.001$ (***). Error bars represent SEM.

4.1.2.2 AMP expression upon dFOXO overexpression in the fatbody

The adult fatbody is the fly equivalent of the mammalian liver, and white adipose tissue is known to be the key organ for AMP production. The functions of the *Drosophila* fatbody include many of the metabolic activities of the mammalian liver, but also fat storage. Induced expression of dFOXO in the fatbody from the onset of adulthood increased the life-span, reduced the fecundity and increased resistance to paraquat in females (Partridge et al. 2004). It was thereby captivating to analyze the AMP regulation in a dFOXO dependent manner in the fatbody of the adult flies. The constitutive expression of UAS–*dFOXO* or UAS–*dFOXO*^{TM} was lethal for larvae when promoted from *actin*-GAL4, or when expressed in the fatbody with the fatbody specific driver line *adh*-GAL4. Therefore, conditional expression of *dFOXO* is required to bypass developmental lethality as well as to study its impact on AMP regulation exclusively in the adult stage.

The mifepristone (RU486) inducible-GAL4 system (annotated P{Switch} and GeneSwitch) was used to drive the expression of UAS-constructs in defined adult tissues. Ingested mifepristone strongly induced reporter expression at all ages (Hwangbo et al. 2003). The expression of UAS–FOXO^{TM} or UAS–dFOXO did not affect survival when induced with the P{Switch} strain $S_1$106, an efficient promoter in the fatbody (Roman et al. 2001). Therefore, this strain was used to induce the fatbody specific expression of dFOXO. Expression of different AMPs was analyzed from either control (P{Switch} strain $S_1$106 x w^-) or experiment condition (P{Switch} strain $S_1$106 x UAS-FOXO^{TM}) and AMP upregulation in the latter condition was calculated relative to the appropriate control condition. The AMPs that were upregulated upon overexpression of FOXO™ in the fatbody were AttA, AttB, AttC, CecA1, CecA2, CecC, Dpt, DptB, Dro, Drs, Drs3 and Mtk. The fatbody being the main site for production of AMPs, it corresponds with the broader range of AMPs expressed upon dFOXO overexpression.

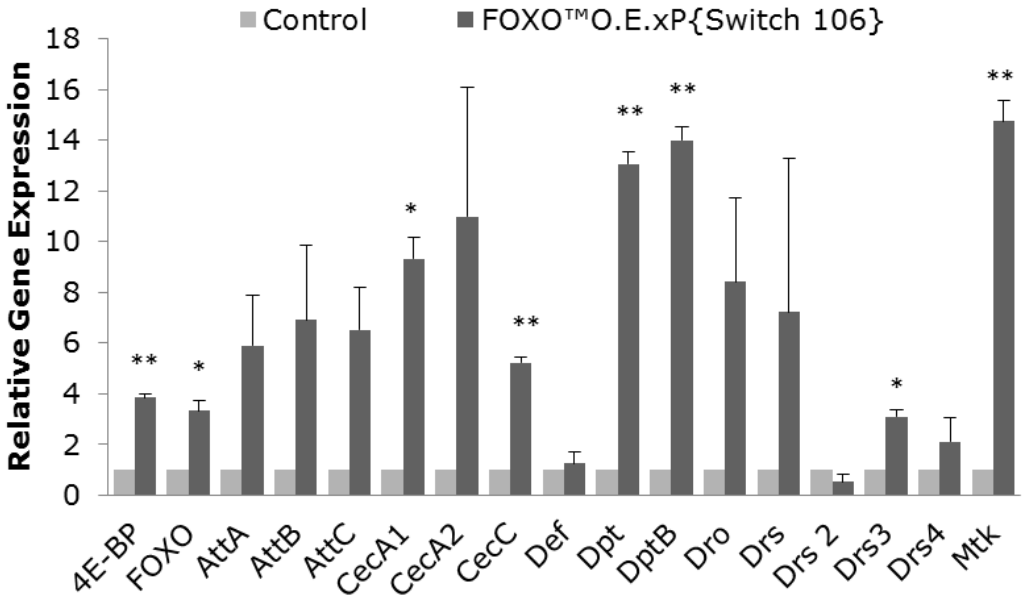


Figure 4.4. AMP expression upon overexpression of dFOXO^{TM} in the fatbody. The experiment condition flies (UAS-FOXO™/cyo X P{Switch 1}106 on RU486 food) were compared to the control flies (UAS-FOXO™/cyo X P{Switch 1}106 on food without RU486). 4eBP: 4E binding protein, FOXO: Forkhead box class O, AttA: Attacin A, AttB: Attacin B, AttC: Attacin C, CecA1: Cecropin A1, CecA2: Cecropin A2, CecC: Cecropin C, Def: Defensin, Dpt: Diptericin, DptB: Diptericin B, Dro: Drosocin, Drs: Drosomycin, Drs2: Drosomycin2, Drs3: Drosomycin3, Drs4: Drosomycin 4 and Mtk: Metchnikowin. A minimum of n = 3 has been analyzed for each set of experiment. Significance was tested using an unpaired 2-tailed Student's t-test. $p < 0.05$ (*); $p < 0.01$(**); $p < 0.001$ (***). Error bars represent SEM.

4.1.3 Impact of dFOXO on a subset of AMPs

4.1.3.1 Sequence analysis of AMP gene promoters

Since almost 20 years it is known that the *Drosophila* genome encodes for several classes of AMPs, which are active against Gram-positive as well as Gram-negative bacteria or fungi. The AMPs are neighbors in the genome. They occur in clusters based on their similarity in the scaffold folding, identity in signal peptide as well as mature peptides. For example Andropin is placed right next to the Cecropin cluster as it has identical signal peptide sequence (Hultmark et al. 1991). All of the related AMP promoter regions contain sequence motifs related to mammalian NF-κB response elements, which turned out to be crucial for AMP expression (Engstrom et al. 1993). Due to this reason, the later on discovered signalling pathways, which are responsible for AMP regulation, are called NF-κB-like signaling pathways.

As mentioned before, dFOXO is the main signal transducer of IlS on the level of transcriptional regulation in *Drosophila* (Puig et al. 2003). Binding of FOXO to DNA molecules is in general restricted to specific binding motifs, which are conserved in metazoans (Furuyama et al. 2000) and located in the regulatory region of a gene. The three types of FOXO binding motifs that I concentrated on were the FOXO binding motif (TTGTTTAC-perfect match), the FOXO binding motif (TTGTTTA**C**- last base changed) and the Forkhead (FKH) binding motif (TXTTTAY), where Y is any pyrimidine (with C preferred) and X is A or G. The strongest binding occurs with FOXO binding motif and then is the FKH binding motif. First, I studied the FOXO binding sites in the AMPs found to be dFOXO dependent, by searching for the presence of these FOXO binding sites. The putative as well as main FOXO binding sites were observed in the studied regions (Figure 4.5). Several putative binding motifs of all three types were identified in the Cecropin cluster and *Dro* promoter sequence strengthening the possibility that dFOXO has a direct role in controlling immunoresponsive genes.

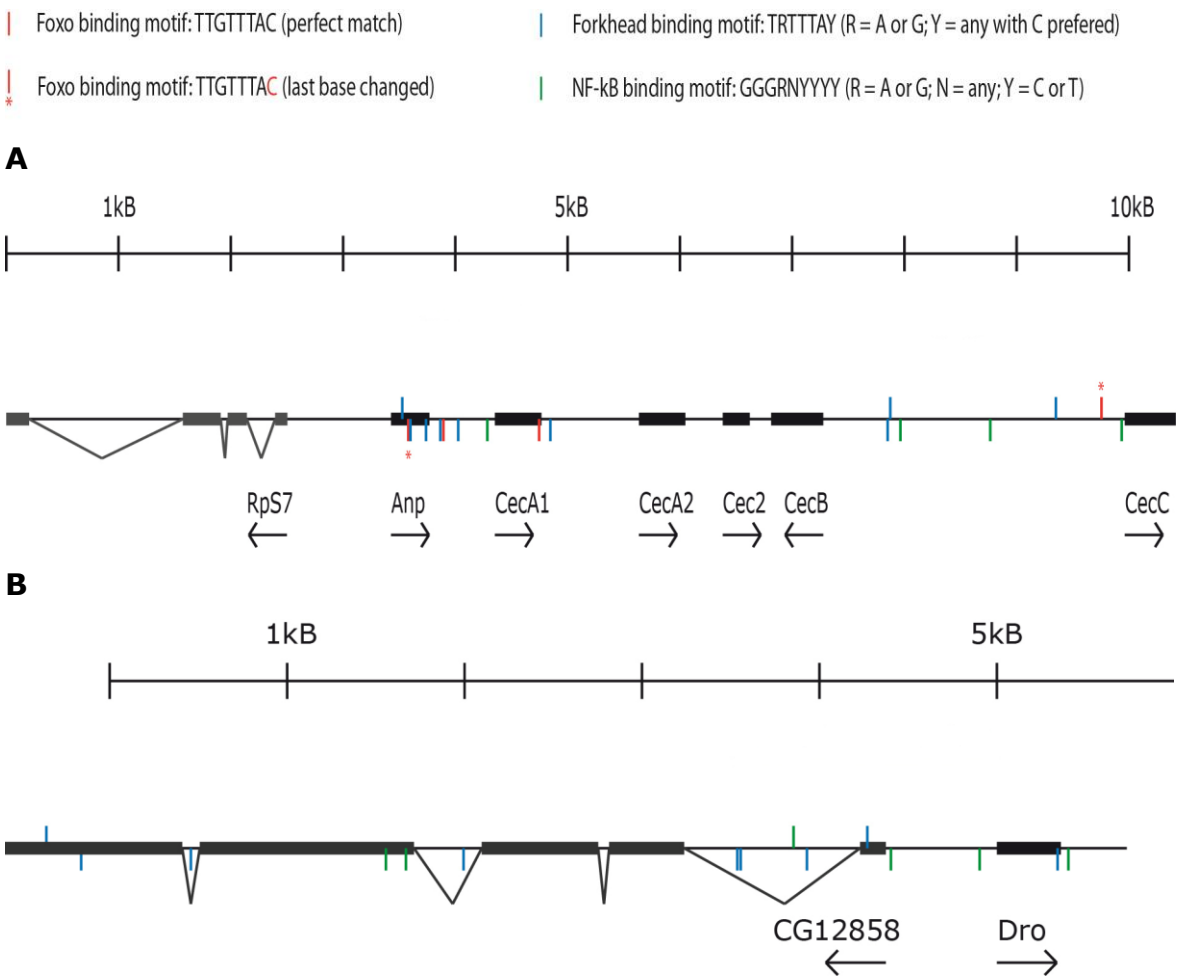


Figure 4.5. Schematic overview of the 5 kb upstream region of different AMPs. (A and B) Binding motifs for FOXO, FKH and NF-κB are shown. An upward bar indicates orientation of the binding motif in forward direction; a downward bar indicated reverse orientation. Anp: andropin AttA: *attacin A*, AttB: *attacin B*, CecA1: *cecropin A1*, CecA2: *cecropin A2,* CecB*: cecropin B,* CecC*: cecropin C* and Dro: *drosocin*.

4.1.3.2 Cloning of a Dro and CecC promoter reporter constructs

To study the usage of the clustered dFOXO binding sites, a cell culture and an *in vivo* approach was chosen. Different promoter constructs covering the Dro *and* CecC promoters were cloned (Figure 4.6). For all cell culture constructs the pGL3 *luciferase* vector was used, where the promoter region was cloned upstream of the *luciferase* gene. The *in vivo* constructs for usage in transgenic flies were based on the approach of β-galactosidase (β-gal) as a marker. The Dro construct contained the characteristic dFOXO binding cluster 1.5 kb upstream upstream of

the start of the open reading frame (ORF). The CecC construct contained the characteristic dFOXO binding cluster 2.2 kb upstream of the ORF (Figure 4.6).

A major obstacle to creating precisely expressed transgenes lies in the epigenetic effects of the host chromatin that surrounds them and the regulatory element of the neighboring genes. To overcome this problem, a GAL4-inducible luciferase assay is employed to systematically quantify position effects of host chromatin and the ability of insulators to counteract these effects at phiC31 integration loci randomly distributed throughout the *Drosophila* genome. The loci that could be exploited to deliver precise doses of transgene expression to specific tissues were identified. The property of the gypsy retrovirus insulator was discovered to induce gene expression to levels several-fold greater than at most or possibly all un-insulated loci, in every tissue tested. The gypsy insulators flanked on either sites of the loci of interest blocked the effect of neighboring genes onto the gene of interest inserted at the chosen loci. These findings provided the first opportunity to create a battery of transgenes that can be reliably expressed at high levels in virtually any tissue by integration at a single locus, and conversely, to engineer a controlled phenotypic allelic series by exploiting several loci (Markstein et al. 2008). This enabled the identification and modification of loci for optimal transgene expression.

The phiC31 integrase mediates recombination between the bacterial and phage attachment sites, attB and attP, and has been shown to efficiently integrate attB-containing plasmids into attP 'landing sites' that have been previously inserted in the genome. The attP40 site (II Chromosome) was chosen for insertion of CecA1 and CecC reporter constructs.

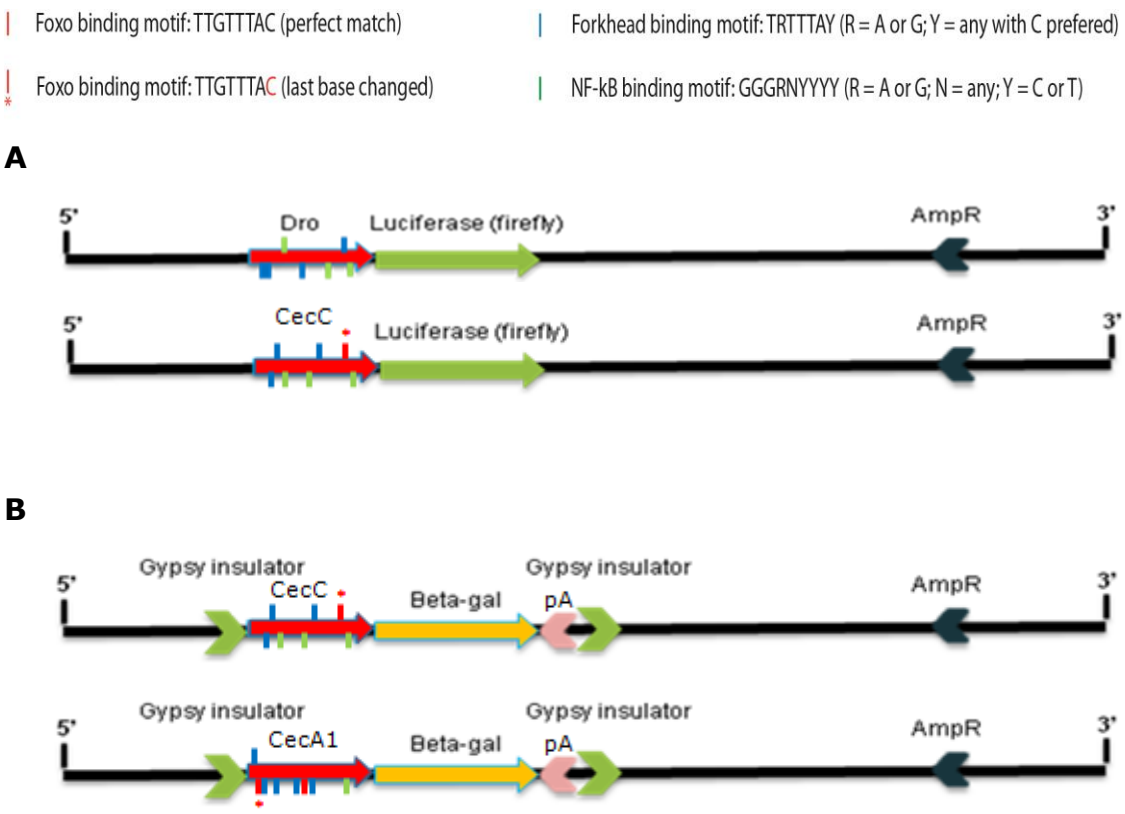


Figure 4.6. Overview of the reporter constructs. (A) Graphical map of the luciferase constructs for the AMPs Dro and CecC. The promoter was cloned behind the *luciferase* gene which contained its own basal promoter in the pGL3 vector. **(B)** For transgenic flies the promoter region of CecA1 and CecC was cloned behind the *β-galactosidase* gene in the pCa4B2G vector. CecA1: CeCC: Dro: AmpR; pA: SV40 polyA.

4.1.3.3 In vitro analysis of dFOXO binding motifs

For *in vitro* analyses S2 cells were used to verify the cluster of dFOXO binding motifs and its dependence on it for regulation. The Dro construct was co-transfected with plasmids pMT-GAL4 and UAS-dFOXO-GFP. The two latter plasmids allowed GAL4/UAS dependent overexpression of dFOXO-GFP in transfected cells under control of a metallothionein promoter, which is inducible by addition of CuSO4 (Fuss et al. 2006). Induction of dFOXO-GFP overexpression was done 16 h after transfection, followed by 24 h incubation before cells were harvested and subjected to RNA preparation. The assay was analyzed by quantitative Realtime qPCR, which allowed the normalization of *luciferase* transcription according to dFOXO-GFP activity only in transfected cells. Comparing Dro construct to the empty pGL3 vector showed that dFOXO-GFP was indeed able

to induce *luciferase* expression indicating that dFOXO is able to bind to the promoter region and induce the expression of Dro.

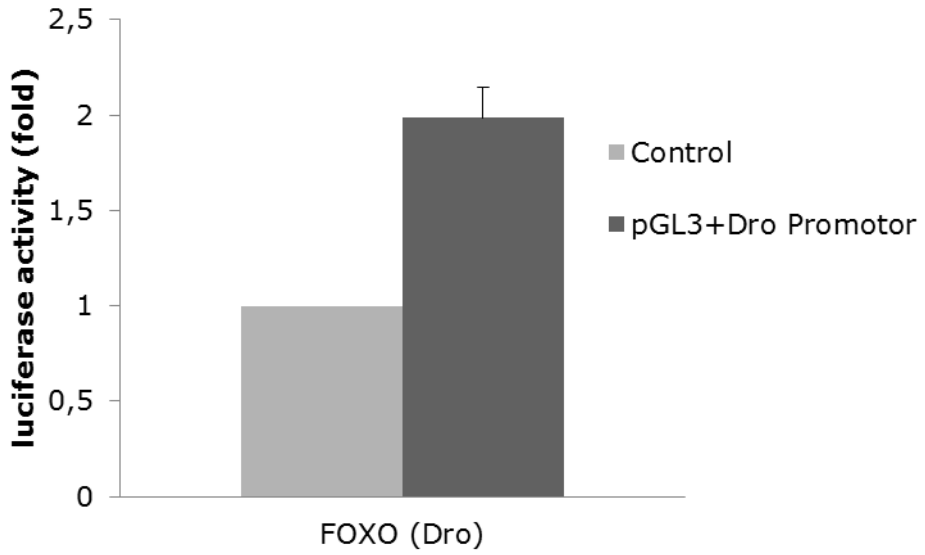


Figure 4.7. Luciferase assay in S2 cells. Lusiferase expression in S2 cells, transfected with Dro-luciferase constructs. The conditions chosen for the experiment were as follows:Control: pGL3(Dro); transfection with pGL3+Drosocin, pUAST-Foxo-GFP & pMTGAL4. GAL4 expression was performed by usage of Cu^{2+} ions. A minimum of n = 3 has been analyzed for each set of experiment. Error bars represent SEM.

The effect of dFOXO on CecC was analyzed with a Dual-Luciferase Reporter Assay using the activities of firefly (Photinus pyralis) and Renilla (Renilla reniformis, also known as sea pansy) luciferases, which are measured sequentially from a single sample. The CecC promoter (2.2 kB upstream region) incorporated into the pGL3 vector was co-transfected into S2 cells along with pAHWB-FOXO (with ubiquitous expression of dFOXO under the control of an actin promoter). In the control condition the pGL3 vector alone was co-transfected into the cells with the pAHWB-FOXO. In both cases, also the psiCHECK vector comprising the Renilla luciferase was transfected in order to normalize the lusiferase activity to the transfection efficiency. The luciferase assay was then carried out according the user manual instructions and the activity levels of Renilla luciferase were recorded first followed by firefly luciferase levels. CecC expression was indeed upregulated when dFOXO was present, indicating that CecC regulation is dFOXO dependent (Figure 4.8).

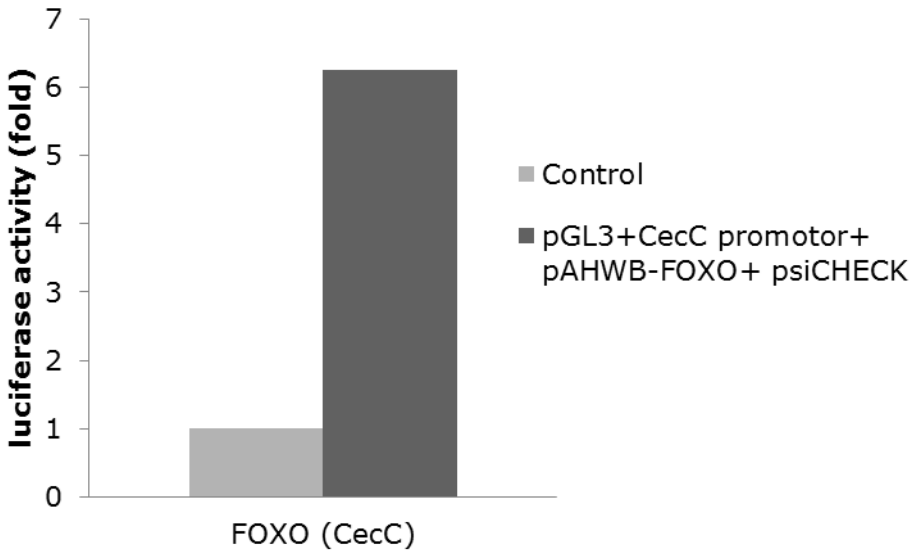


Figure 4.8. Effect of dFOXO on CecC expression in S2 cells. The luciferase assay was performed using the S2 cell lysates. In the control condition, S2 cells were co-transfected with empty pGL3 vector, pAHWB-FOXO vector and psiCHECK. In the experiment condition (dFOXO) pGL3 vector cloned with CecC promoter region, pAHWB-FOXO vector and psiCHECK vector were transfected. n = 1 has been analyzed.

4.1.3.4 In vivo analysis of AMP promoter activation through dFOXO

The CecC enhancer region contains a main FOXO binding site. Supporting the hypothesis that dFOXO could be a transcription factor that drives CecC expression, came from cell culture studies. Therefore, it was the next step to study the effect of dFOXO on the regulation of CecC *in vivo.* Transgenic flies were made carrying the CecC-β-gal construct (2.2 kB) to show that the dFOXO dependent regulation, which was observed in S2 cells, is also essential *in vivo*. The CecC construct was shuttled from pGL3 vector to pCa4B2G vector, which was used for germline mediated transformation into attP 40 embryos. Beside the *CecC* promoter region, the *β-gal* ORF contained in the pCaSpeR-AUG-β gal vector was also transferred to allow β-gal expression in adult flies. The β-gal activity in transgenic adult flies was quantified using the Realtime-qPCR and normalized to total protein of the animals. Using the *tubulin* GeneSwitch system dFOXO protein was overexpressed in adults carrying the CecC construct. To test the *in vivo* significance of dFOXO binding motifs in a more physiological way, endogenous dFOXO activity was triggered by starvation instead of overexpressing a constitutively active protein. To analyze this, a series of starvation experiments in transgenic adult flies were performed. Male adult flies were starved for 24 hours on PBS and the control transgenic flies were fed on yeast mixed with PBS for the same amount of time. Under these

conditions, dFOXO translocates into the nucleus and activates transcription of its target genes, among them are 4E-BP, lipase3 (Puig et al. 2003, Fuss et al. 2006). The genes also induced upon starvation are brummer and glutaminase (Groenke et al. 2007 and Desvergne et al. 2006). If the regulation of CecC is dependent on dFOXO, the expression of β-gal, being downstream of the CecC promoter, should be upregulated as well. As seen in S2 cells, the CecC construct responded strongly to enhanced dFOXO activity also in adults (Figure 4.9). This demonstrates that dFOXO is essential for upregulation of CecC when IlS is reduced. To this end, experiments in cell culture and *in vivo* revealed an essential role for the cluster of dFOXO/FKH binding motifs in IlS dependent *CecC* regulation.

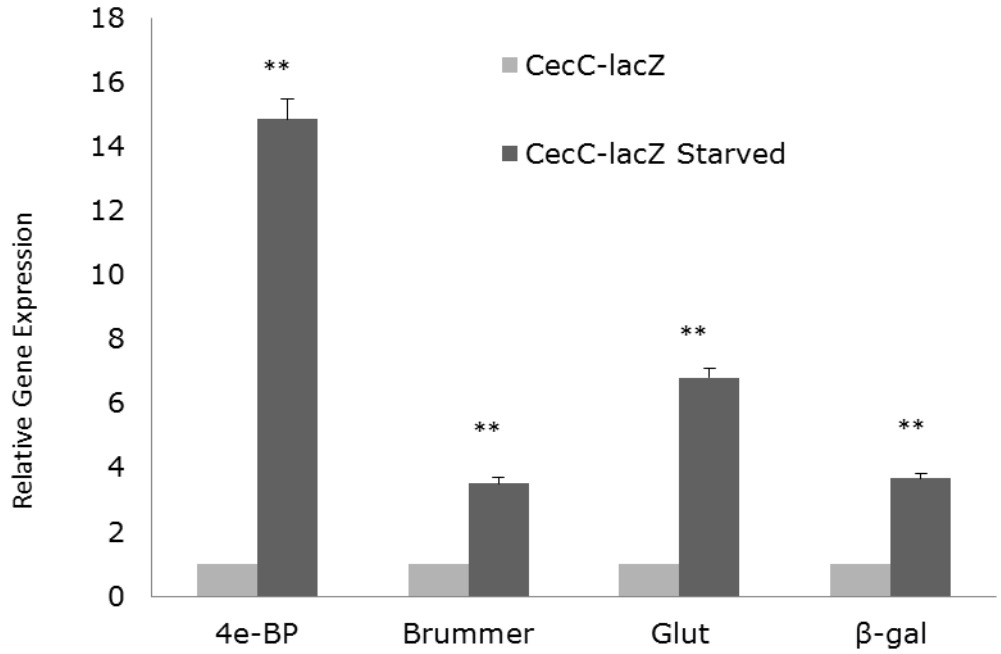


Figure 4.9. β-galactosidase levels in transgenic adults carrying CecC construct. β-galactosidase (β-gal) levels in 10 days old transgenic adults *(w;CecC-lacZ/CecC-lacZ;MKRS,Sb/TM6B,Hu)* after starvation on PBS (Cec-lacZ Starved) compared to full nutrition fed adult flies (Control: *w;CecC-lacZ/CecC-lacZ;MKRS,Sb/TM6B,Hu)*. Realtime-qPCR was performed to quantify mRNA levels in adult flies extracts. 4e-BP: 4E-binding protein, Glut: Glutaminase and β-gal: β-galactosidase A minimum of n = 3 has been analyzed for each set of experiment. Significance was tested using an unpaired 2-tailed Student's t-test. $p < 0.05$ (*); $p < 0.01$(**); $p < 0.001$ (***).

Furthermore, the *tubulin*-GAL4 GeneSwitch (Tub^{GS}) driver line was chosen for analysis of β-gal level regulation in a dFOXO dependent manner. The Tub^{GS} enabled the ubiquitous overexpression of endogenous dFOXO in the adults. The experiment condition was activated by adding RU486 whereas the control condition was reared on the same food but without RU486. Upon induction of dFOXO levels, also the transcriptional levels of β-gal were induced up to 5-fold (Figure 4.10). This further supports the hypothesis that dFOXO binds to the promoter and can specifically activate the transcription of *CecC*.

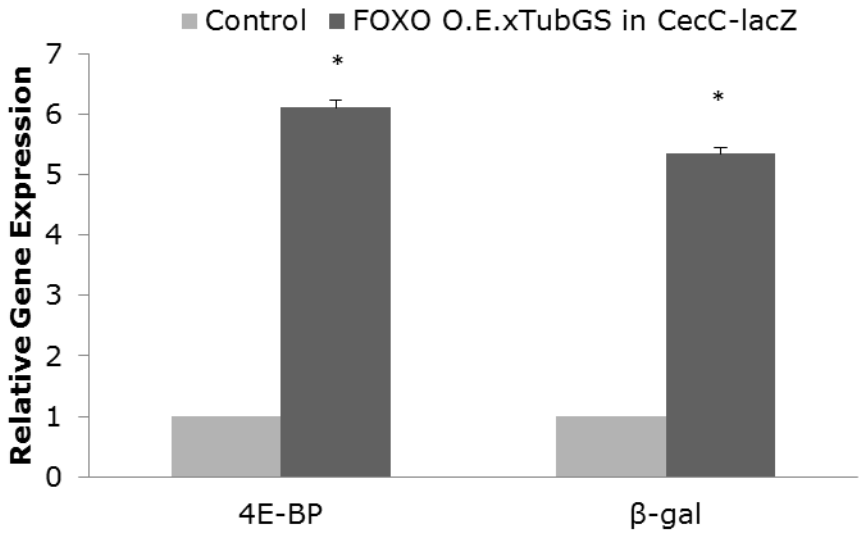


Figure 4.10. β-galactosidase induction upon dFOXO overexpression.
The adult flies with the desired genotype (w; UAS-$FOXO^{WT}$/CecC-lacZ; Tub^{GS}/TM6B) after being fed with RU486 (FOXO O.E.xTubGS in CecC-lacZ) compared to the control flies fed with food without RU486 (w; UAS-$FOXO^{WT}$/CecC-lacZ; Tub^{GS}/TM6B). Realtime qPCR was performed to quantify mRNAs in adult flies extracts. 4E-BP: 4E-binding protein, Beta-gal: beta galactosidase. A minimum of n=3 has been analyzed for each set of experiment. Significance was tested using an unpaired 2-tailed Student's t-test. $p < 0.05$ (*); $p < 0.01$(**); $p < 0.001$ (***). Error bars represent SEM.

4.2 Action of FKH on the regulation of AMPs

Drosophila responds to immune challenges by upregulating AMP expression in the fatbody and gut via the Toll and the Imd signaling pathways (Figure 4.11A) (Lemaitre & Hoffmann 2007). Previous work from our laboratory has shown that AMPs are not only regulated by immune pathways, but also by the insulin pathway (Figure 4.11B) and that the expression of Drosomycin and other AMPs is increased upon nutrient stress (starvation) by dFOXO (Becker et al. 2010). Since dFOXO shares target genes with a transcription factor from the same family, Forkhead (FOXA2, FKH), which acts downstream of TOR signaling (Bülow et al. 2010), I tested the hypothesis whether AMPs are also regulated by the TOR pathway. I quantified the gene expression of AMPs from the eight known classes in TOR mutant larvae and used CG6770, a target gene downstream of TOR signaling (Bülow et al. 2010), as a positive control. CecC, Dpt and Mtk were significantly upregulated, while Def and Dro were downregulated (Figure 4.11C). Since Dpt and Mtk were the two genes which were the most prominently upregulated AMPs, I decided to focus on these two in further studies. Mtk is a proline-rich peptide, which was found to inhibit the growth of filamentous fungi and Dpt is O-Glycopeptide directed against Gram-negative bacteria (Levashina et al. 1998, Imler and Bulet 2005). *Drs* was used as a negative control as it is differentially regulated from *Dpt* and *Mtk, namely* as a target of IlS rather than TOR signaling (Becker et al. 2010).

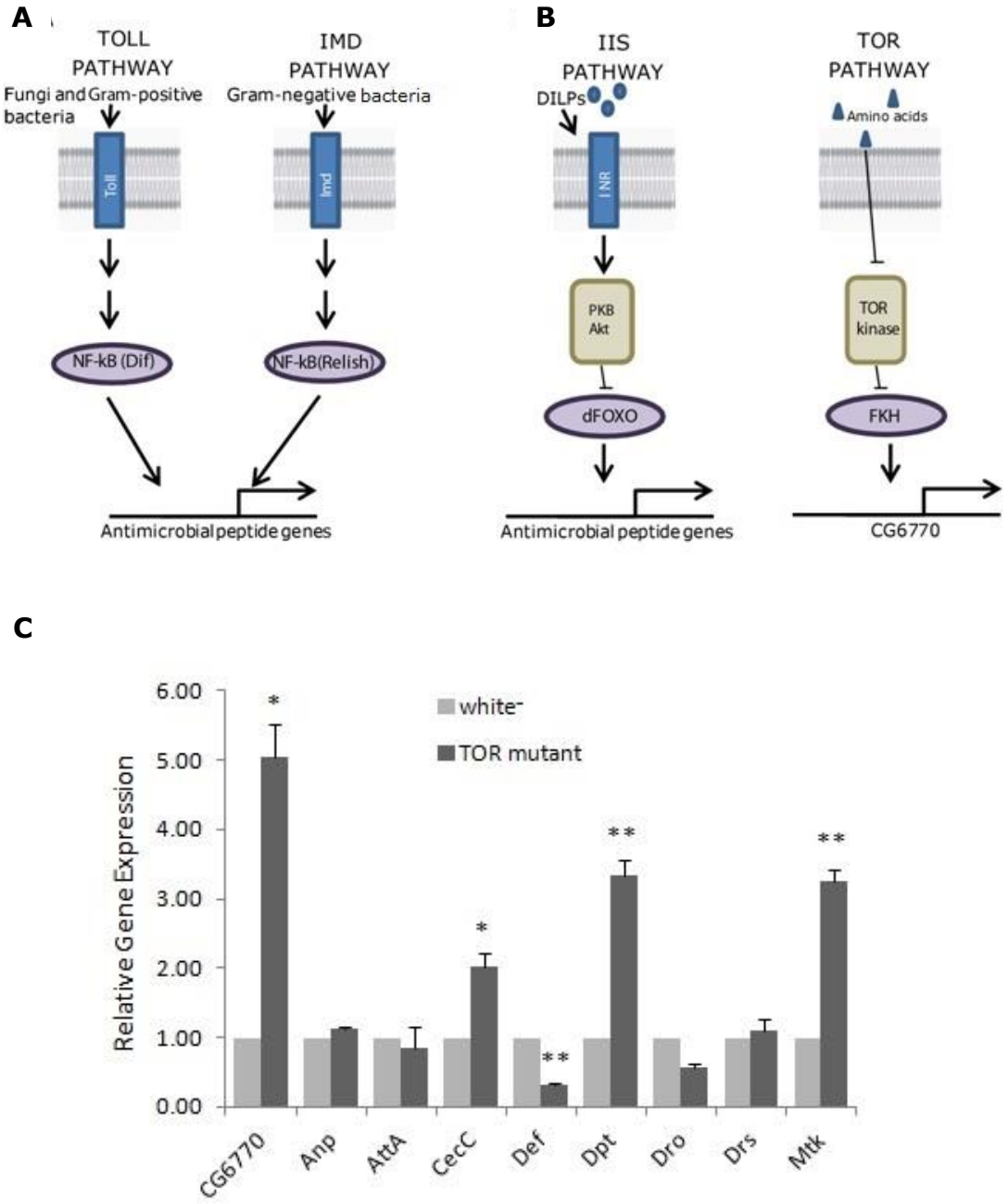


Figure 4.11. Key players of Immune, Metabolic Pathways of *Drosophila* and nutrient-dependent geneexpression by TOR. (A) Presents a view of Toll-dependent induction of immune genes in fungal and Gram-positive bacterial infections in *Drosophila*. These microorganisms are sensed by circulating pattern recognition proteins, a process that is followed by proteolytic cleavage of the polypeptide Spaetzle; Spaetzle activates Toll, which leads to degradation of Cactus and nuclear translocation of the Rel protein DIF which activates the immune effector molecules called antimicrobial peptides (AMPs). The Imd pathway regulates the *Drosophila* defense against Gram negative infection. The infection leads to translocation of the Rel protein Relish into the nucleus to activate the AMPs. **(B)** The *D. melanogaster* insulin/insulin-like growth factors signaling (IIS) gets activated under nutrient stress, thereby activating the transcriptional factor dFOXO. Under conditions of dietary protein abundance, the TOR signaling module is active and exerts a negative regulation on FKH, which is consequently sequestered in the cytoplasm and unable to

modulate gene transcription. (**B**) When TOR complex 1 activity is inhibited by rapamycin or protein deprivation, the repression of FKH activity is diminished. A significant fraction of the cellular FKH pool accumulates in the nucleus and activates expression of the growth-inhibiting genes *CG6770*, *cabut* and *4E-BP*. **(C)** Overview of the AMPs that are transcriptionally upregulated in heterozygous TOR mutants (y w; dTORΔP/+) fed on protein rich yeast food. The control is white$^-$ larvae reared on protein rich yeast food. A minimum of n = 3 has been analyzed for each set of experiment. Significance was tested using an unpaired 2-tailed Student's t-test. $p < 0.05$ (*); $p < 0.01$(**); $p < 0.001$ (***). Error bars represent SEM. Anp: Andropin, AttA: Attacin-A, CecA1: Cecropin A1, CecC: Cecropin C, Def: Defensin, Dif: dorsal-related immunity facor, DILPs: insulin-like peptides in *Drosophila*, Dpt: Diptericin, Dro: Drosocin, Drs: Drosomycin, Imd: Immune Deficiency, INR: Insulin Receptor, Mtk: Metchnikowin, NF-kB: nuclear factor kappa-light-chain-enhancer of activated B cells and PKB/Akt: Protein Kinase B.

4.2.1 Genetic and pharmaceutical manipulation of the TOR pathway leads to changes in AMP expression

The TOR pathway contains two important factors, which act upstream of the TOR kinase: the tuberous sclerosis (TSC1/TSC2) complex (Tapon et al. 2001), and Ras homology enriched in brain (Rheb) (Stocker et al. 2003, Saucedo et al. 2003). The TSC1/TSC2 complex is a negative regulator of TOR signaling which inhibits growth. It acts inhibitory on Rheb, a positive regulator of TOR and of growth (Figure 4.12). TSC1/TSC2 was overexpressed using the ubiquitous, mifepristone (RU486)-inducible driver Tubulin-GeneSwitch-Gal4 (Osterwalder et al. 2001). This led to the upregulation of Dpt and Mtk, while the dFOXO target Drs was not regulated (Figure 4.13A). Since TSC1/TSC2 suppresses TOR signaling, these results indicate in line with the result from the TOR mutant, that inhibition of TOR signaling leads to an upregulation of the expression of AMPs. To further narrow down the impact of the TOR pathway on AMP expression, Rheb, which hyperactivates the TOR pathway, was overexpressed. This resulted in the reduction of Dpt and Mtk mRNA levels, while Drs was upregulated (Figure 4.13B). The data further demonstrate that active TOR signaling, as it occurs under conditions of high amino acid availability, suppresses the expression of AMPs.

TOR kinase activity can be manipulated pharmaceutically using the anti-cancer drug rapamycin (Oldham et al. 2000). I fed white$^-$ larvae for 24 hours with 50 µM rapamycin, which inhibits TOR signaling. In line with my previous results, I found that Dpt and Mtk are upregulated. Drs was not regulated under this condition (Figure 4.13C). Taken together, these findings demonstrate that regulation of AMP expression occurs downstream of TOR signaling.

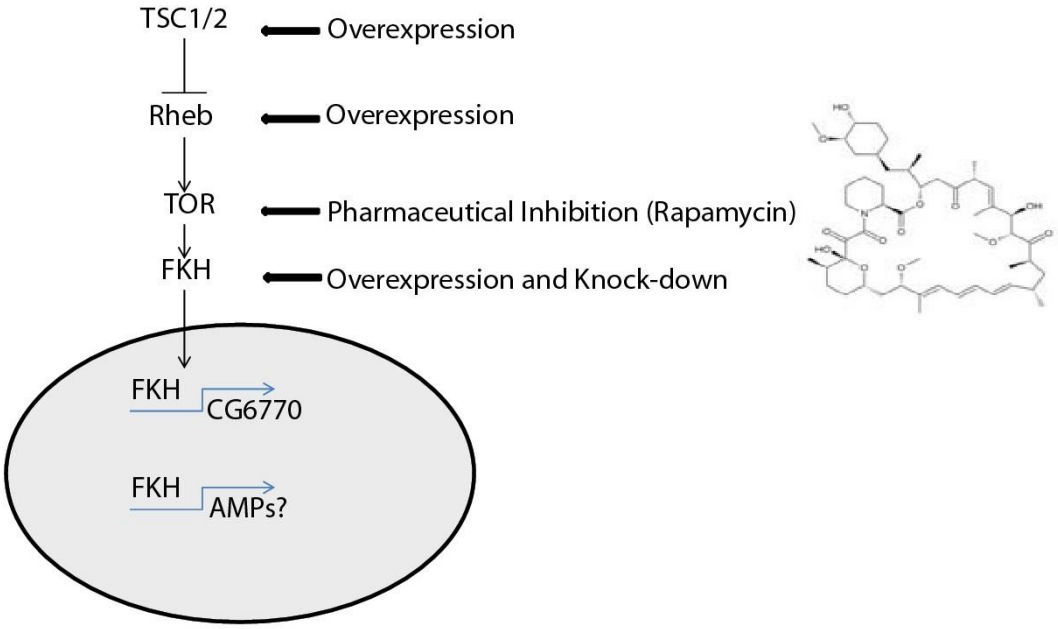
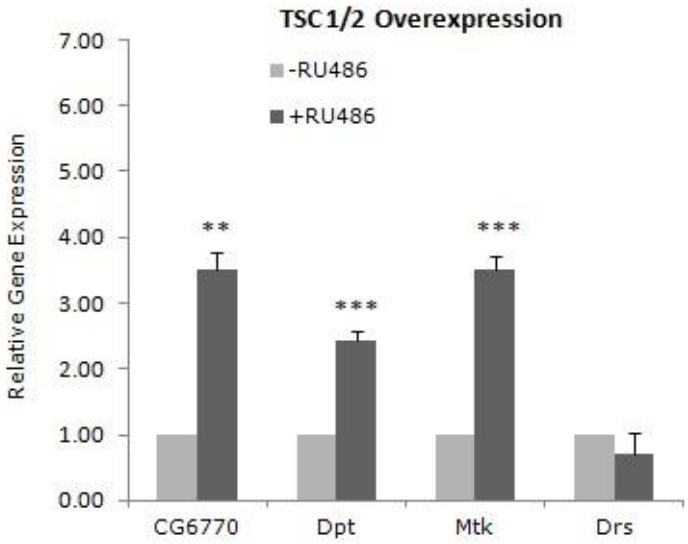


Figure 4.12. A simplified schematics of TOR pathway. It depicts the various key components that were used to analyze the AMP regulation by TOR. This included the use of pharmaceutical inhibitor of TOR called rapamycin. Realtime qPCR was performed to quantify mRNAs in larval extracts.

A


TSC1/2 Overexpression
-RU486
+RU486
Relative Gene Expression
7.00
6.00
5.00
4.00
3.00
2.00
1.00
0.00
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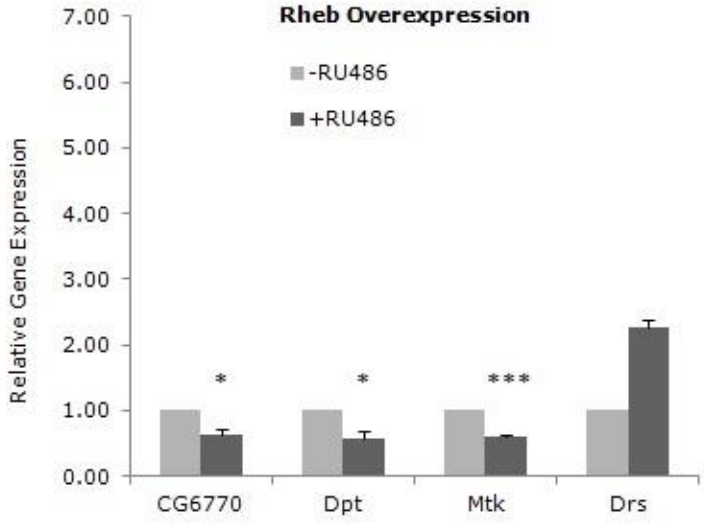
***
***
CG6770
Dpt
Mtk
Drs

B


Rheb Overexpression
-RU486
+RU486
Relative Gene Expression
7.00
6.00
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4.00
3.00
2.00
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0.00
*
*
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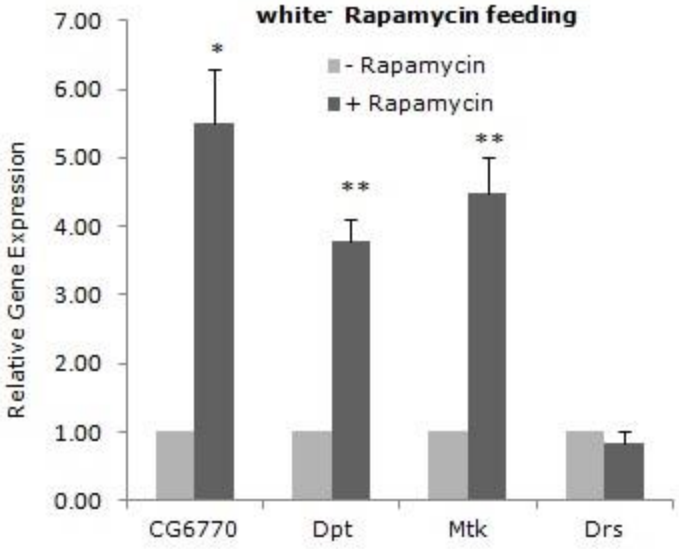
CG6770
Dpt
Mtk
Drs

C


white- Rapamycin feeding
- Rapamycin
+ Rapamycin
Relative Gene Expression
7.00
6.00
5.00
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3.00
2.00
1.00
0.00
*
**
**
CG6770
Dpt
Mtk
Drs

Figure 4.13. Transcription of Diptericin and Metchnikowin is regulated by TOR. **(A)** Diptericin (Dpt) and Metchnikowin (Mtk) mRNA levels are significantly elevated upon TSC1/TSC2 overexpression (w;;UAS-TSC1,UASTSC2/ TubulinGene Switch). The control (-RU486) larvae are fed with RU486 devoid food. In the experiment condition (+RU486) larvae are fed with RU486 containing food. **(B)** Dpt and Mtk mRNA levels are significantly low upon Rheb overexpression (w;;UAS-Rheb/TubulinGene Switch). The control (-RU486) larvae are fed with RU486 devoid food. The experiment condition (+RU486) larvae are fed with RU486 containing food. **(C)** *CG6770,* Dpt and Mtk transcription is induced in white$^-$ larvae (72 h old) fed with 50 µM rapamycin. The control (-Rapamycin) is white$^-$ larvae reared on protein rich yeast food. The experiment condition (+Rapamycin) is fed with 50 µM rapamycin. A minimum of n = 3 has been analyzed for each set of experiment. Significance was tested using an unpaired 2-tailed Student's t-test. $p < 0.05$ (*); $p < 0.01$(**); $p < 0.001$ (***). Error bars represent SEM. Dpt: Diptericin and Mtk: Metchnikowin.

4.2.2 The transcription factor FKH regulates Dpt and Mtk

The TOR pathway controls growth by phosphorylation of 4E-BP (Gingras et al. 1999), but also by controlling gene expression via the transcription factor Forkhead (FKH). The IlS and TOR pathway act in parallel through dFOXO and FKH, which are excluded from the nucleus under conditions of high nutrient availability. To test whether Dpt and Mtk are regulated by FKH, I analyzed their expression under conditions of FKH overexpression and RNAi knock-down. Upon FKH overexpression, reflecting low TOR signaling, Dpt and Mtk are upregulated and Drs is downregulated (Figure 4.15A), while upon FKH knock-down, reflecting high TOR signaling, Dpt and Mtk are downregulated and Drs expression is unchanged (Figure 4.15B). The promoter regions of Dpt and Mtk (done in cooperation with Margret Bülow) were analyzed and numerous FKH binding sequences were found in both (Figure 4.14A,B).

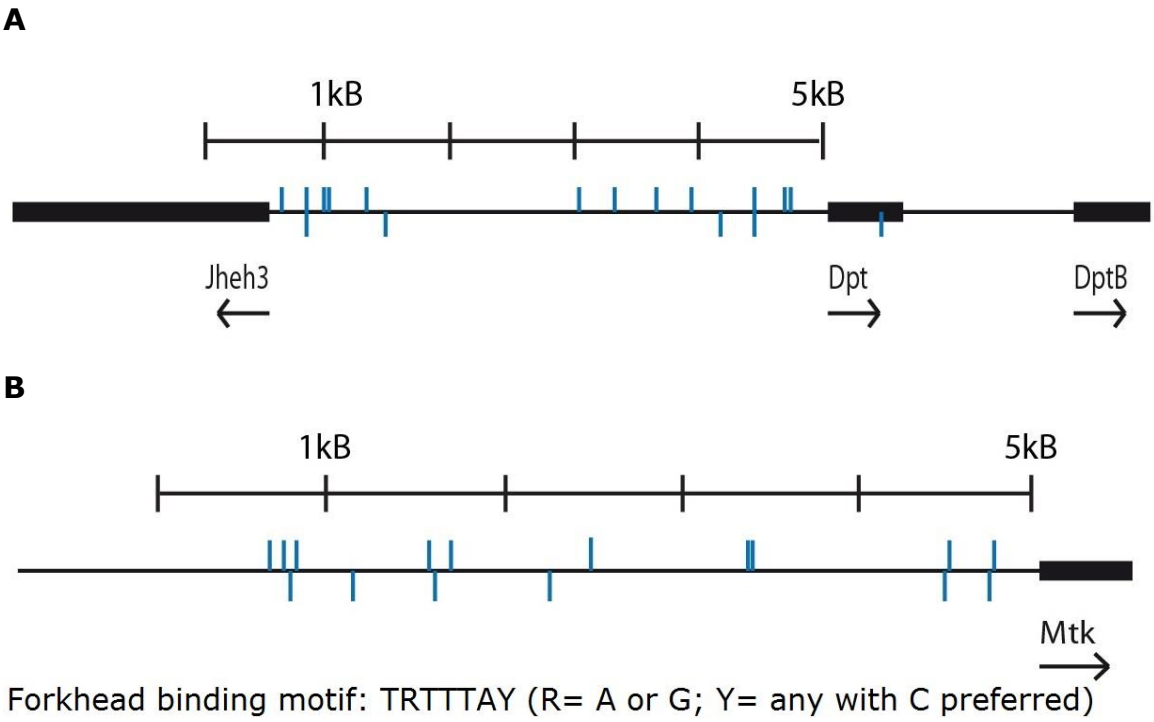
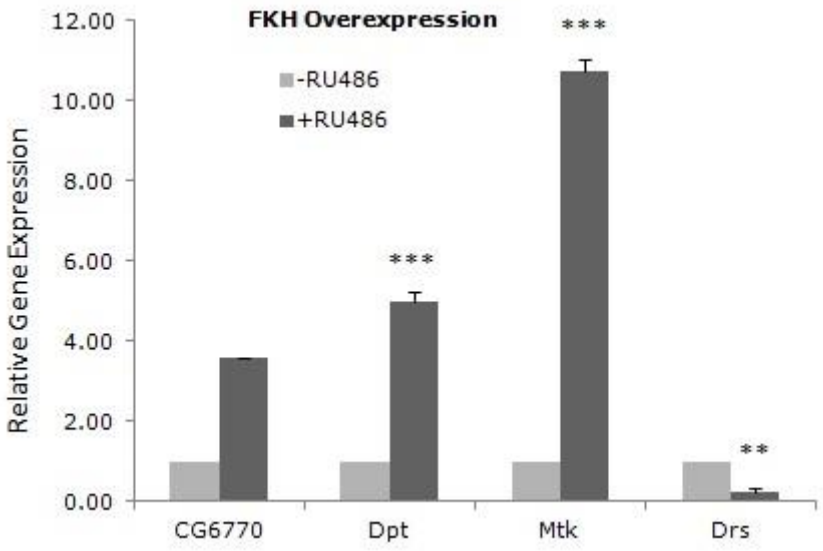


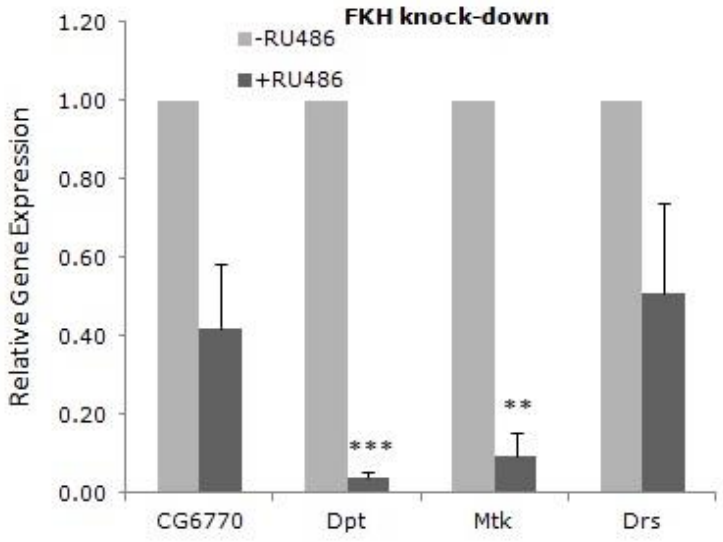
Figure 4.14. (A-B) Schematic overview of the 5 kb upstream region of *Dpt* and *Mtk* respectively. Binding motifs for FKH are shown in blue bars. An upward bar indicates orientation of the binding motif in forward direction; a downward bar indicated reverse orientation. *Dpt*: *Diptericin and Mtk*: *Metchnikowin.*

To further demonstrate the regulation of AMPs by FKH, fluorescent *in situ* hybridization was performed in clones overexpressing FKH. FKH construct was expressed in a heat-shock inducible GAL4 line, which allows random flip-out events in all tissues while labeling the positive, FKH-overexpressing clones with GFP. There was an increase in the signal from the Mtk probe in the clones, further supporting the hypothesis that Mtk is a target of FKH (Figure 4.15C).

A


FKH Overexpression
-RU486
+RU486
Relative Gene Expression
12.00
10.00
8.00
6.00
4.00
2.00
0.00
***
***
**
CG6770
Dpt
Mtk
Drs

B


FKH knock-down
-RU486
+RU486
Relative Gene Expression
1.20
1.00
0.80
0.60
0.40
0.20
0.00
***
**
CG6770
Dpt
Mtk
Drs

C

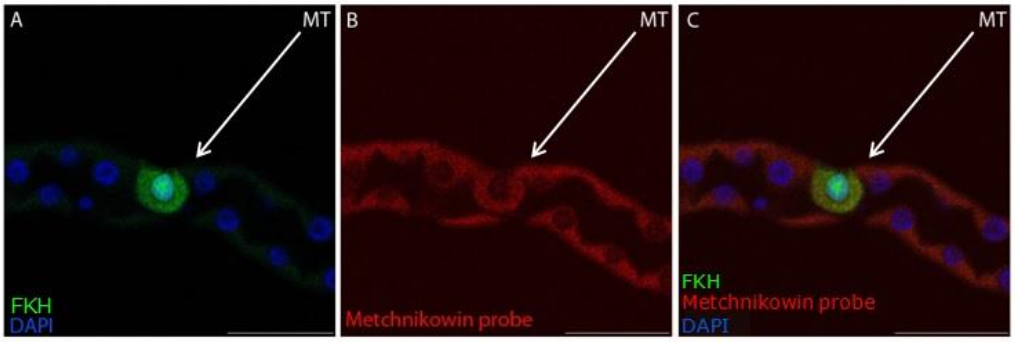

A
MT
FKH
DAPI
B
MT
Metchnikowin probe
C
MT
FKH
Metchnikowin probe
DAPI

Figure 4.15. Transcription of Diptericin and Metchnikowin is regulated by FKH. **(A)** Diptericin and Metchnikowin mRNA levels are significantly elevated upon FKH overexpression (w;;UAS-FKH/TubulinGene Switch). The control (-RU486) larvae are fed with RU486 devoid food. The experiment condition (+RU486) larvae are fed with RU486 containing food. **(B)** Diptericin and Metchnikowin mRNA levels are significantly low upon FKH-RNAi overexpression (w;;UAS-FKH-RNAi/TubulinGene Switch). The control (-RU486) larvae are fed with RU486 devoid food. The experiment condition (+RU486) larvae are fed with RU486 containing food. **(C)** Pictures show confocal sections of larval Malpighian tubules (MT), using the fly line yw hs-flp;; Act.CD2.Gal4 UAS-GFP to drive UAS-FKH, overexpression and control responder lines. Cells expressing the transgene are marked by the co-expression of GFP, whereas the non-fluorescent serve as wild type controls within the same tissue sample. Larvae were reared on yeast for 72 h AED, tissue was stained with α-GFP (green), α-DIG (red) and DAPI (blue). (Figure E: A–C) show the clone in the MT along with DAPI and a co-expression of *Metchnikowin* in the same clone upon overexpression of FKH. A minimum of n = 3 has been analyzed for each set of experiment. Significance was tested using an unpaired 2-tailed Student's t-test. $p < 0.05$ (*); $p < 0.01$(**); $p < 0.001$ (***). Error bars represent SEM. *Dpt*: *Diptericin, Mtk*: *Metchnikowin* and AED: after egg deposition.

4.2.3 Regulation of AMPs by TOR signaling is independent from immune pathways

The phosphoinositide-3 kinase/protein kinase-B/mammalian target of rapamycin (PI3K/Akt/mTOR) pathway is known to be important in regulating the innate and adaptive immune responses (Mao et al. 2013). In mice mTOR has been shown to regulate the anti-inflammatory cytokine IL-10 (Hazlett et al. 2013). I therefore first asked whether an alteration of TOR levels *in vivo* would have an effect on innate immunity in *Drosophila.*

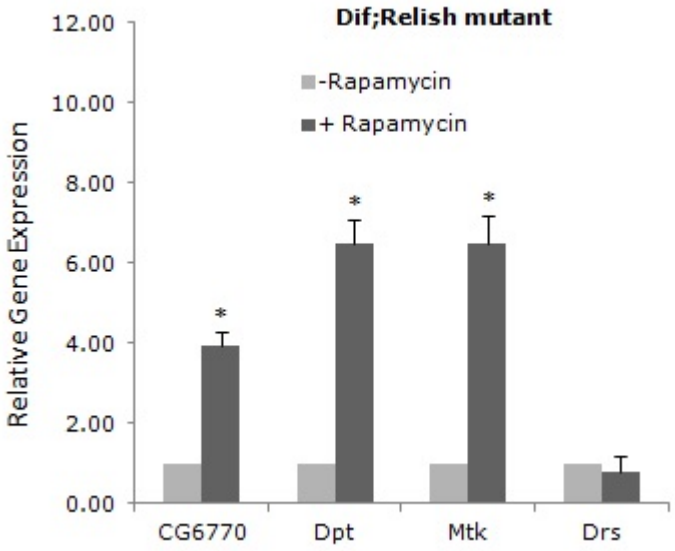
To test whether inhibition of TOR and the subsequent upregulation of AMPs occur due to an induction of the immune pathways, I treated a mutant for Dif and Relish, the transcription factors downstream of the Toll and Imd pathway, with 50 µM rapamycin for 24 hours (Figure 4.16A). In this double mutant, both the Imd and the Toll pathway are defective, resulting in immune deficient animals that have been shown to be unable to respond to bacterial, fungal challenge and AMP upregulation (Taylor et al. 2004). Thereby, the treatment with rapamycin in the mutant flies leads to the inhibition of TOR pathway, in the absence of Toll and Imd pathways. The expression of Dpt and Mtk and was upregulated as already shown by feeding rapamycin to white^{-} larvae (Figure 4.13C), indicating that the regulation of AMPs by the TOR pathway is independent from the immune

pathways. The expression of Drs was unaffected by the Dif;Relish mutation as well as the rapamycin treatment.

Furthermore, the dFOXO mutant *dFOXO*$^{\Delta 94}$ (Slack et al. 2011) was treated with rapamycin to see whether the TOR related effects observed on the regulation of AMPs are dFOXO-dependent. The *dFOXO*$^{\Delta 94}$ mutant is a new deletion mutant of *dFOXO* by imprecise excision of a P-element positioned upstream of the first noncoding exon of the *dFOXO* gene. This deletion spans over 20 kb of the *dFOXO* locus, removing part of the predicted promoter region as well as several coding exons. The homozygous mutants are completely devoid of dFOXO transcript expression. Consequently, this deletion appears to represent a true null allele of *dFOXO* (Slack et al. 2011). I found that Dpt and Mtk expression is induced by the rapamycin treatment regardless of the dFOXO mutation, indicating that TOR regulates Dpt and Mtk independent from dFOXO (Figure 4.16B).

To further demonstrate that the TOR-dependent regulation of Dpt and Mtk is FKH-dependent, FKH knock-down animals were treated with rapamycin (done in cooperation with Margret Bülow). Rapamycin strongly induces Dpt and Mtk in wildtype larvae. However, this response is blocked in larvae, which express FKH RNAi underlining the FKH-dependent expression of Dpt and Mtk (Figure 4.17A).

A



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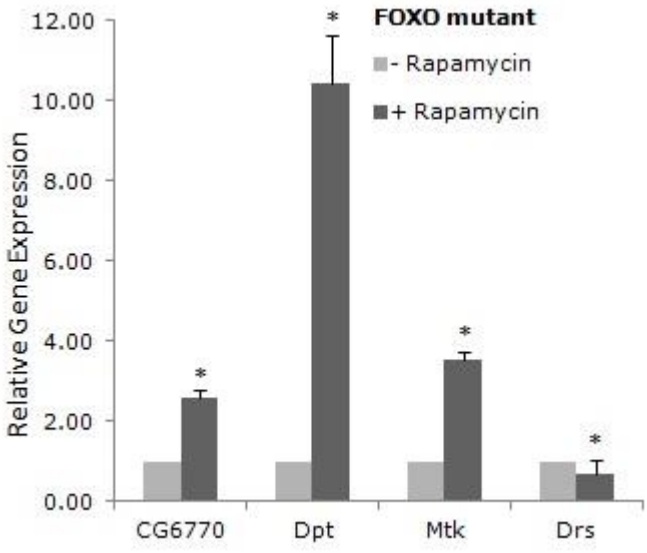
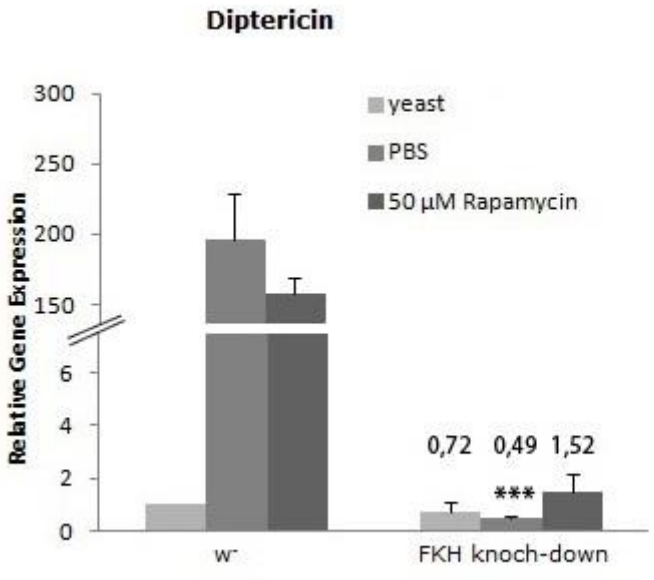


Figure 4.16. Regulation of AMPs by TOR signaling is independent of dFOXO and immune pathways. Realtime qPCR was performed to quantify mRNAs in larval extracts. **(A)** *CG6770*, *Diptericin* and *Metchnikowin* transcription is induced in the larvae (72 h old w;;Dif,Rel) fed with rapamycin. The control condition larvae (-Rapamycin) were fed with ethanol containing food. The experiment condition (+Rapamycin) larvae were fed with 50 µM rapamycin. **(B)** *CG6770*, *Diptericin* and *Metchnikowin* transcription is induced in the larvae (72 h old w;;FOXO$^{\Delta 94}$. TubulinGene Switch) fed with rapamycin. The control condition (-Rapamycin) larvae were fed with ethanol containing food. The experiment condition (+Rapamycin) larvae were fed with 50 µM rapamycin. A minimum of n = 3 has been analyzed for each set of experiment. Significance was tested using an unpaired 2-tailed Student's t-test. $p < 0.05$ (*); $p < 0.01$(**); $p < 0.001$ (***). Error bars represent SEM. *Dpt*: *Diptericin* and *Mtk*: *Metchnikowin*.

A



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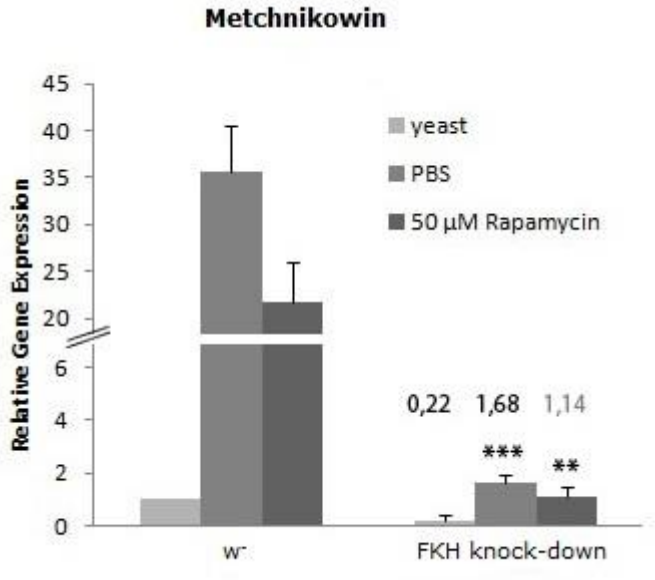


Figure 4.17. Regulation of AMPs by TOR signaling via FKH. Realtime qPCR was performed to quantify mRNAs in larval extracts. **(A-B)** Both *Diptericin* and *Metchnikowin* are upregulated upon starvation (PBS) and rapamycin treatment. *Diptericin* and *Metchnikowin* upregulation upon PBS or rapamycin feeding is blocked in Forkhead knock-down animals (w;;FKHRNAi). A minimum of n = 3 has been analyzed for each set of experiment. Significance was tested using an unpaired 2-tailed Student's t-test. $p < 0.05$ (*); $p < 0.01$(**); $p < 0.001$ (***). Error bars represent SEM. *Dpt*: *Diptericin* and *Mtk*: *Metchnikowin*.

4.2.4 Forkhead protein shuttles between cytoplasm and nucleus/nucleolus in the midgut

dFOXO protein is regulated by its subcellular localization: the insulin pathway leads to phosphorylation of dFOXO and retains it in the cytoplasm, while low insulin signaling, like upon starvation leads, to nuclear shuttling and the subsequent expression of dFOXO target genes. FKH acts under a similar mechanism downstream of TOR signaling. Although FKH was long thought to be constitutively nuclear, it has been shown for fatbody tissue that it changes its subcellular localization in response to TOR signaling (Bülow et al. 2010). FKH is nuclear under both fed and rapamycin treated conditions in tissues of an ectodermal origin like the Malpighian tubules (Figure 4.19A, 4.18B), the hindgut (Figure 4.19B, 4.18A) and the salivary glands (Lehmann et al. 2007), sections (done in cooperation with Yanina-Yasmin Pesch) show that FKH is cytoplasmic in midgut and fatbody cells (Figure 4.19C, E).

In midgut and fatbody cells of rapamycin-treated larvae, a fraction of the FKH protein is still cytoplasmic but in addition, also shows a clear nuclear and nucleolar localization, indicating a nutrient-dependent shuttling also in this metabolically and immunologically relevant tissue (Figure 4.19D, F). I analyzed the expression of FKH and its target genes CG6770, Dpt and Mtk in isolated midgut and fatbody tissue, and found that CG6770, Mtk and FKH are significantly upregulated in a rapamycin-dependent manner in the midgut (Figure 4.19G). Expression of all three target genes is strongly induced upon rapamycin feeding in the fatbody. This could be due to an overall higher gene expression rate in the systemic immune response.

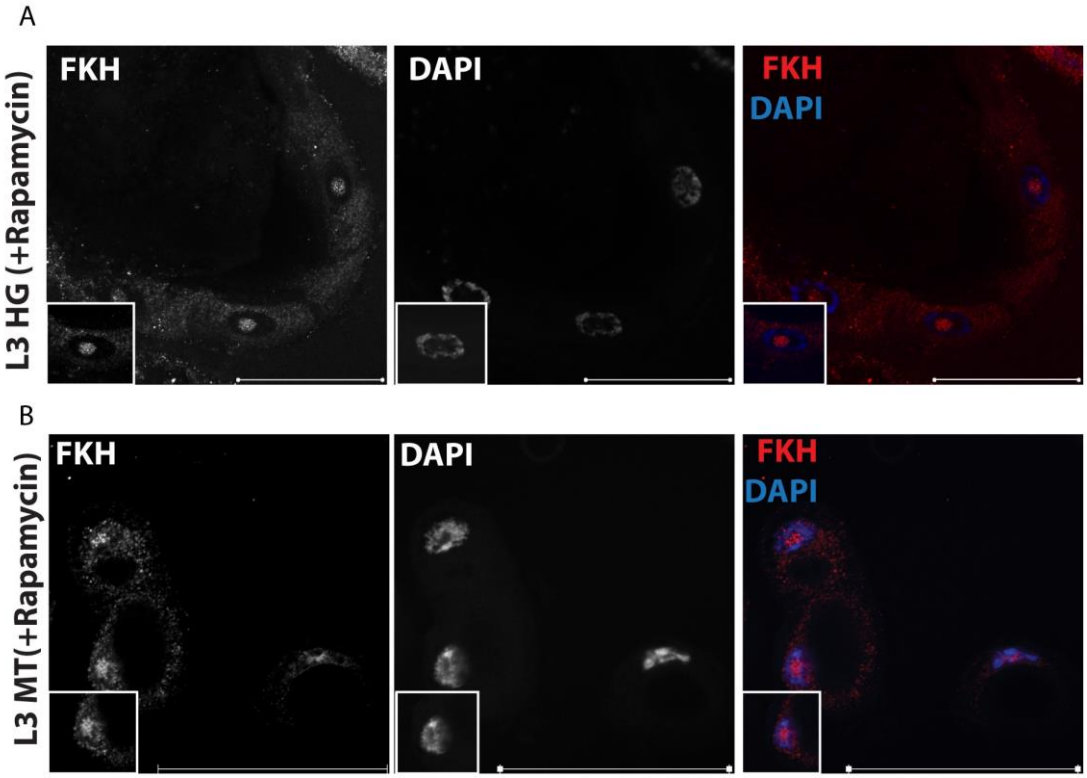


Figure 4.18. Forkhead protein is constitutively nucleolar in the ectodermal tissue. (A and B) Pictures show confocal JB-4 embedded tissue sections of white-larval hindgut (HG) and Malpighian tubules (MT) respectively. FKH transcription is induced in white- larvae (72 h old) fed with rapamycin. The experiment condition (+Rapamycin) larvae were fed with 50 μM rapamycin. FKH is constitutively nuclear under the rapamycin fed conditions in the Malpighian tubules and in the hindgut, respectively.

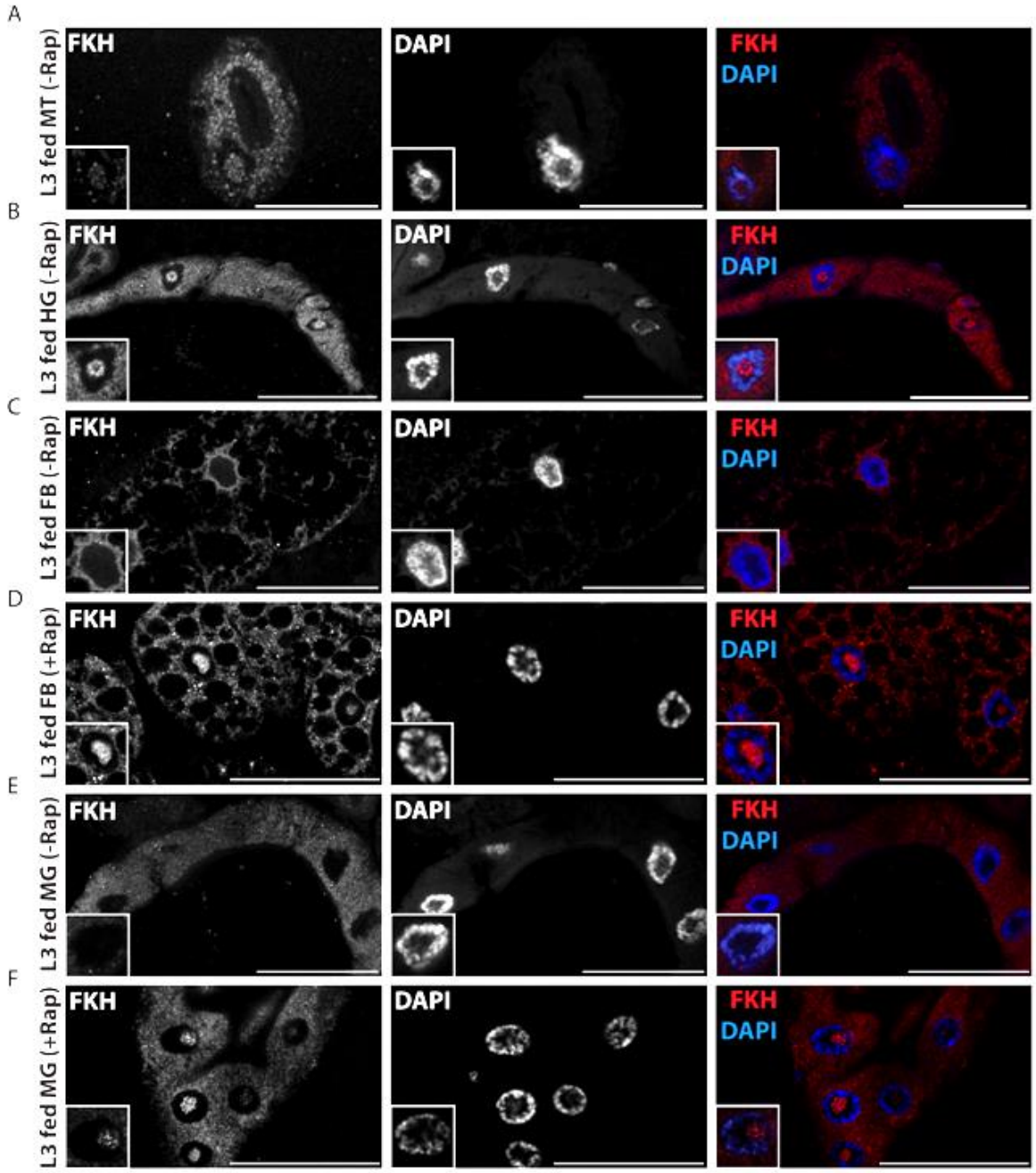
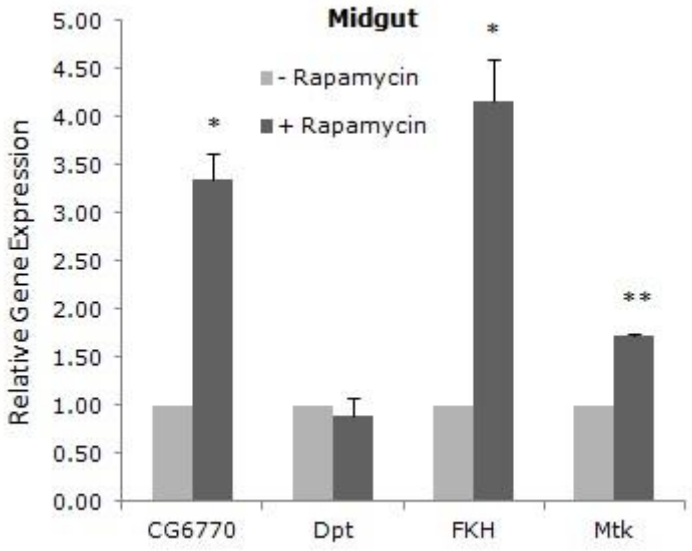


Figure 4.19. Forkhead protein shuttles between cytoplasm and nucleus in the midgut. (A-F) Pictures show confocal JB-4 embedded tissue sections of white$^-$ tissues stained with α-FKH (red) and DAPI (blue).**(A-B)** larval Malpighian tubules (MT) and hindgut (HG). FKH transcription is induced in white$^-$ larvae (72 h old) fed with protein rich yeast food. FKH is constitutively nuclear under fed conditions in the MT and in the HG, respectively. **(C-D)** larval fatbody(FB), FKH is cytoplasmic in FB cells. The larval fatbody, treated with rapamycin. In the fatbody cells of rapamycin treated larvae FKH is still cytoplasmic but also shows a clear nuclear and nucleolar localization. **(E-F)** midgut cells (MG). The MG treated with rapamycin. In the midgut cells of rapamycin treated larvae, FKH is still cytoplasmic but shows a clear nuclear and nucleolar localization. Tissue was stained with α-FKH (red) and DAPI (blue).

A



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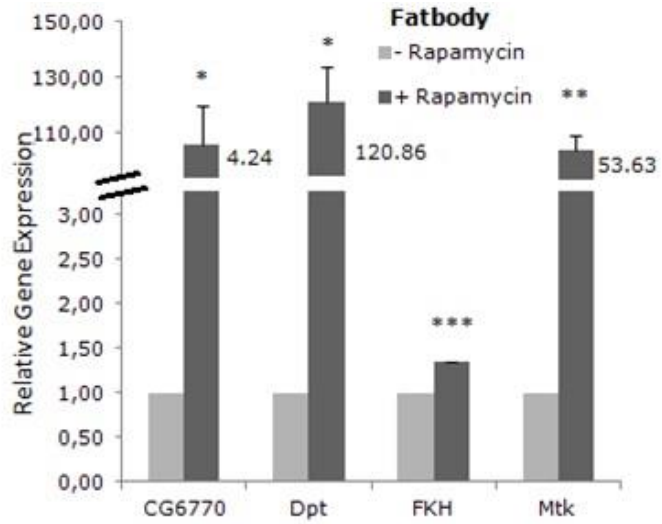
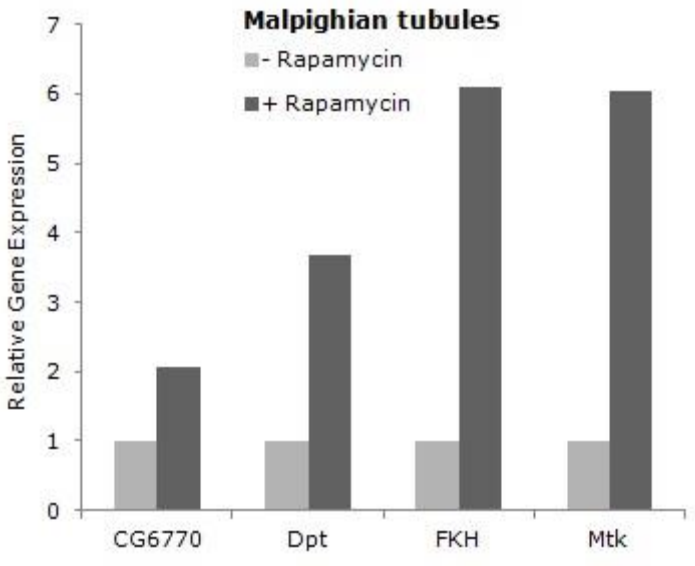


Figure 4.20. (A, B) Spatial expression of *CG6770*, *Dpt*, *Mtk* and *FKH* in the midgut and fatbody respectively, in white^{-} larvae (72 h) treated with rapamycin. The control condition larvae (-Rapamycin) were fed with protein rich yeast food containing ethanol. The experiment condition (+Rapamycin) larvae were fed with 50 µM rapamycin. A minimum of n = 3 has been analyzed for each set of experiment. Significance was tested using an unpaired 2-tailed Student's t-test. $p < 0.05$ (*); $p < 0.01$(**); $p < 0.001$ (***). Error bars represent SEM. Scale bars represent 50 µM. *Dpt: Diptericin*, *FKH*: *Forkhead* and *Mtk*: *Metchnikowin*.

Additionally, I analyzed the expression of FKH and its target genes CG6770, Dpt and Mtk in isolated Malpighian tubules and hindgut tissue, and found that CG6770, Mtk and FKH were downregulated upon rapamycin feeding in the hindgut (Figure 4.21B). Expression of Dpt and Mtk was strongly upregulated in a rapamycin-dependent manner in the Malpighian tubules (Figure 4.21A). The expression of Mtk correlates well with Mtk probe detection in the *in situ* hybridization (Figure 4.15C). This could be due to the fact that, MT are free floating in the hemolymph and are one of the first epithelial tissues to sense systemic invasion of microbe. Since epithelial cells from *Drosophila* and human share substantial similarities (Wagner et al. 2008), MT appear to be highly suitable for modeling human renal diseases related to dysfunction of innate immune system (Dow et al. 2010). These findings suggest that FKH exerts its function by a more complex mechanism than dFOXO with tissue-specific, nutrient-dependent shuttling.

A



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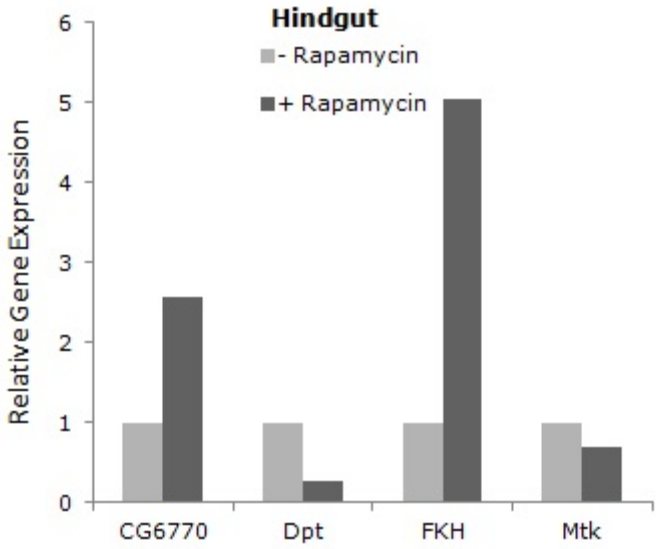

Figure 4.21. The spatial expression of AMPs in different parts of the gut. (A-B) Spatial expression of CG6770, Dpt, Mtk and Fkh in the Malpighian tubules and hidgut respectively, in white^{-} larvae (72h) treated with rapamycin. The control condition (-Rapamycin) larvae were fed with ethanol containing food. The experiment condition (+Rapamycin) larvae were fed with 50 μM rapamycin. n=1 has been analyzed. Dpt: Diptericin, FKH: Forkhead and Mtk: Metchnikowin.

5 DISCUSSION

5.1 Regulation of AMPs via dFOXO in adults

Insulin/Insulin-like signaling (IlS) is one of the major signaling pathways in *Drosophila*, which has been found to be involved in such diverse processes like regulation of organismal growth, cell size, cell proliferation, energy homeostasis, apoptosis, protein synthesis, autophagy and lifespan determination (Hafen, 2004). It thereby plays a direct role in determining the size of an animal or functions as a global modulator of other genetic programs controlling organismal size. The main transcription factor present downstream of IlS pathway is dFOXO. It gets activated upon reduced IlS (Jünger et al. 2003). The fly genome encodes for a single dFOXO gene, which is conserved from worm to human and has extensively been described in context of cellular stress response and energy homeostasis (Arden, 2008, Gross et al. 2008). The dFOXO protein contains a forkhead box domain, which allows direct binding to the DNA via highly conserved recognition sequences. Forkhead box O (FoxO) transcription factors (TFs) are involved in a plethora of cellular processes to regulate whole-organism physiology and are major determinants of animal lifespan (Partridge and Bruning, 2008). It has been shown by Becker et al. 2010 that there is a connection of metabolism and innate immunity through dFOXO. A dFOXO dependent mode of AMP expression probably allows modular adaptation of organismal defense against microorganisms to environmental conditions without the severe side effects induced by activation of NF-κB like signalling pathways. The larvae feed continously at all times, whereas the adult flies fly to different places in search of food. They come in contact with various kinds of microorganisms and have a much more complex metabolism. The adult stage is the final stage of *Drosophila,* thereby, this stage is void of all development related fluctuation in gene expression. Therefore, one of the main goals of this thesis was to study dFOXO regulation of AMP genes in the adult flies.

5.1.1 dFOXO directly regulates AMP expression

FOXO proteins bind to the DNA by their Forkhead box domain, using conserved binding motifs of approximately eight bases as recognition sites (Furuyama et al.

2000). dFOXO/FKH binding motifs were identified in the promoter region of the different AMPs, like *CecA1*, *CecC* and *Dro* genes, hinting at a direct dFOXO dependent regulation. Based on this observation, some more approaches to reduce IlS and thereby triggering dFOXO activity were performed and analyzed for AMP expression. A physiological way to reduce IlS is nutrient depletion, which is quite effective in adult male flies in a span of 24 hours, whereas for females IlS is reduced dramatically in a span of 72 hours and the signaling activity is strongly coupled to the abundance of nutrients. It was shown that starvation had an impact on CecC transcription, strengthening a theory of direct dFOXO dependent regulation of this gene.

The coupling of AMP induction to the abundance of food could represent an ancient protection system, supporting organismal defense when the energy status is low. Nevertheless, starvation is not strictly IlS specific, but has an influence on different signaling pathways and cellular processes independent of dFOXO (Zinke et al. 2002, Pletcher et al. 2002). In contrast to larvae, adult flies are discontinuous feeders and organismal growth is already finished. Whereas in larvae IlS activity is predominantly used to control cellular and organismal growth, in adult flies metabolic balance, reproduction (Flatt et al. 2008) and determination of the life-span are more tightly associated with IlS. Concerning this latter point, IlS and FOXO activity have been correlated with longevity in invertebrate (Hwangbo et al. 2004, Giannakou et al. 2007, Partridge 2008) and vertebrate model organisms (Bartke, 2008, Papaconstantinou, 2009). Starvation signifies a normal physiological situation for an animal while searching for food. As a consequence, the energy status of cells is oscillating all the time. Under starvation situation, dFOXO translocates to the nucleus and activates the target genes. The regulation of AMPs via dFOXO reveals that dFOXO is indispensible for IlS dependent AMP expression. Constant activation of the classic innate immune pathways lead to a reduced lifespan (Libert et al. 2006). This hints that activation of the NF-κB like pathways also play a role in processes apart from AMP induction, which have a negative effect on lifespan determination.

5.1.2 Tissue dependent AMP expression by dFOXO

An important aspect was to know in which tissues the dFOXO dependent regulation of AMP expression is used. Eight different classes of AMPs were

described in Drosophila (Uvell et.al. 2007), being expressed in a highly variable pattern. In case of an infection, AMPs are produced either systemically by the fat body (Tzou et al. 2002, Ferrandon et. al., 2007) or locally by barrier epithelia (Ferrandon et al. 1998, Tzou et al. 2000). Analysis of isolated adult tissues after overexpression of dFOXO revealed that AMPs were expressed in a dFOXO dependent manner in both, the fat body as well as epithelial tissue including the gut. Notably, an induced expression of AttA, CecC, Def, Dpt, DptB and Dro was found in the gut, whereas a clear fat body derived expression showed a regulation of these genes as well as AttC, CecA1, CecA2, Drs, Drs3 and Mtk. This could be due to the fact that fatbody is the key organ for AMP production. This allows speculation about the expression profile of AMPs being expressed differently in epithelial barrier tissues in a dFOXO dependent manner. The expression of AMPs varies in different tissues. This can be explained, as every AMP may have a different set of regulatory elements that bind to its promoter region. It could be that dFOXO alone is responsible for the expression of one AMP, whereas, Cad and NF-κB might be pivotal for the expression of another AMP or FKH and dFOXO can only act in a synergistic manner to upregulate AMPs in a specific tissue (Figure 5.1).

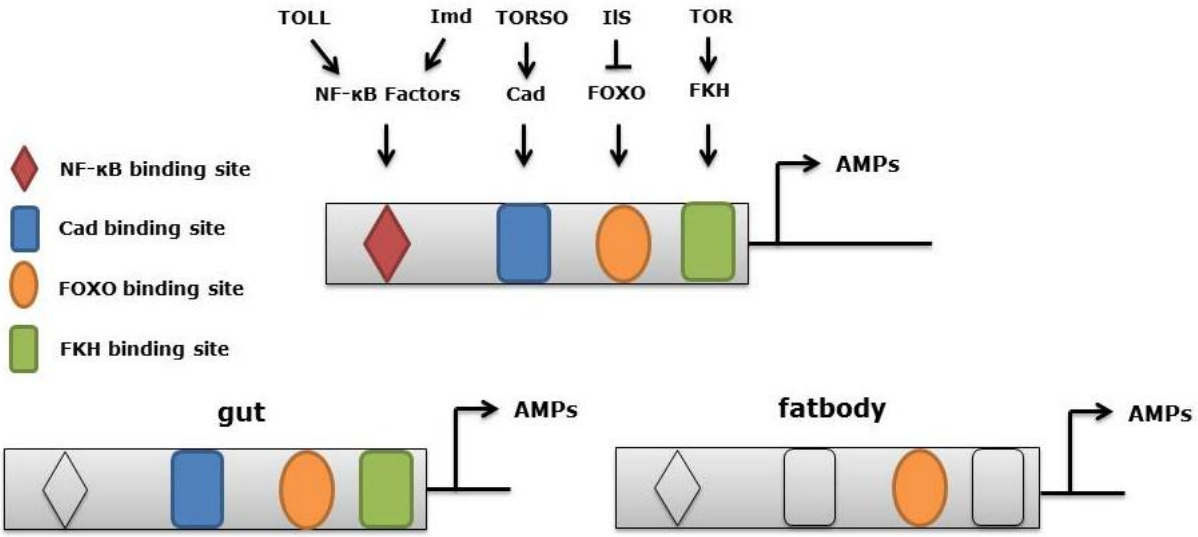

Figure 5.1. Hypthetical tissue specific regulation of AMPs. Different AMPs possess various binding sites for the transcription factors NF-κB, Caudal (Cad), FOXO and FKH. The expression of AMPs varies in different tissues dependent on the interplay between the regulatory elements.

Formerly, it has been shown that dFOXO regulates AMPs independent of NF-κB signaling pathways. Especially, expression levels of AMPs derived by dFOXO signaling are comparatively low as compared to those found by NF-κB dependent activation after infection. This raises the question about the importance of such a mild mechanism of regulation, as AMP expression levels derived by NF-κB like signaling were up to 1000 fold stronger. In contrast, quick reduction of transcriptional level after an infection is essential since high AMP expression levels are detrimental to the host (Becker et al. 2010). Furthermore, it is known from studies in barrier tissues in *Drosophila* and mammals that TLR signaling and activation of immune effector genes are downregulated to avoid chronic inflammation, which is associated with necrosis and cancer formation (Libert et. al. 2006, Abreu et. al. 2005). These tissues are permanently in contact with microorganisms and prolonged exposure to lipopolysaccharides or lipoteichoic acid is known to result in tolerance and cross-tolerance to other pathogen-associated molecular patterns. Similarly, it has been shown in *Drosophila* that Imd signaling and the induction of NF-κB dependent immune effector gene expression in the gut epithelium is repressed by the intestinal homeobox gene *caudal*, thereby allowing and regulating symbiotic interactions of commensal bacteria with the intestinal epithelium (Ryu et. al. 2008). In this context, a dFOXO dependent regulation could ensure the sparse production of AMPs in barrier epithelia in healthy individuals, thereby maintaining and strengthening the defense barrier of these tissues, in particular when animals are suffering from energy shortage or stress.

It has recently been shown, that activation of dFOXO and DAF-16 in the gut/fat body does not require dFOXO/DAF-16 elsewhere to extend lifespan (Rera et al. 2013). Rather, in *Drosophila*, activation of dFOXO in the gut/fat body or in neuroendocrine cells acts on other organs to promote healthy aging by signaling to other, as-yet unidentified factors. FOXO TFs may promote longevity cell non-autonomously by a feedback signaling mechanism (FOXO-to-FOXO) or towards other factors (FOXO-to-other) in distal tissues. Whereas, FOXO-to-FOXO signaling appears to be required for metabolic homeostasis, it has been pinpointed that FOXO-to-other signaling is an important mechanism through which localized FOXO activity ameliorates aging (Alice et al. 2014). This in-turn opens an interesting and intriguing field whereas the AMPs being directly regulated by dFOXO could play a vital role in longevity.

5.2 Role of other metabolic pathways in AMP regulation

Nutrition is a key regulator of tissue growth. In animals, nutritional status is monitored and signaled at both the cellular as well as systemic levels. The main mediator of cellular nutrient sensing is the protein kinase target of rapamycin (TOR). TOR receives information from levels of cellular amino acids and energy, and it regulates the activity of processes involved in cell growth, such as protein synthesis and autophagy (Teleman et al. 2008). IlS is the main mechanism of systemic nutrient sensing and mediates its growth-regulatory functions largely through the phosphatidylinositol 3-kinase (PI3K)/AKT protein kinase pathway. Other nutrition-regulated hormonal mechanisms contribute to growth control by modulating the activity of IlS. The pathways mediating signals from systemic and cellular levels converge, allowing cells to combine information from both sources (Hietakangas et al. 2009).

Forkhead transcription factors of the FOXO subfamily regulate gene expression programs downstream of the IlS network. It is unclear which proteins mediate transcriptional control exerted by TOR signaling, but recent studies in nematodes suggest a role for FoxA transcription factors downstream of TOR. It has been shown that the FoxA protein Fork head (FKH) regulates cellular and organismal size downstream of TOR. FKH overexpression has a negative effect on growth under fed conditions, and this phenotype is not further exacerbated by inhibition of TOR via rapamycin feeding (Bülow et al. 2010). It is known that the alteration of FKH levels has an effect on cellular and organismal size, and that FKH function is required for the growth inhibition and target gene induction caused by low TOR signaling levels (Bülow et al. 2010). Additionally it is depicted that under conditions of starvation or low TOR signaling levels, knockdown of FKH diminishes the size reduction associated with these conditions. Subcellular localization of endogenous FKH protein is shifted from primarily cytoplasmic on a high-protein diet to a distinct nuclear amassing in animals with reduced levels of TOR or pharmaceutical inhibition with rapamycin (Bülow et al. 2010).

5.2.1 Metabolic pathways regulate antimicrobial peptides

The transcription factor dFOXO is an important regulator of various stress responses It does not only inhibit growth upon nutrient scarcity in response to low IlS but also regulates genes for the oxidative stress response (Junger et al. 2003).

This study provides the first evidence that also the pioneer of the forkhead box transcription factor family, FoxA or FKH (Weigel et al. 1989; Weigel and Jäckle, 1990) regulates the expression of a subset of AMPs in the fat body and the gut. FKH acts, however, downstream of TOR and induces AMPs such as Mtk and Dpt when TOR activity is downregulated in response to rapamycin feeding or genetic manipulation of TOR pathway components. I further demonstrate that the FKH-dependent activation of AMPs occurs independently from the infection-triggered classical Toll and Imd immune pathways, similarly as has previously been found for the FOXO-dependent activation of AMPs under fasting or starvation conditions (Becker et al. 2010). Together, my results further demonstrate the existence of a dFOXO and FKH-dependent crossregulation of metabolism and innate immunity that seems to act under normal physiological conditions of oscillating energy levels and in parallel to the infection-triggered classical immunity pathways on AMP regulation.

It has been shown previously that FKH, in contrast to dFOXO, is constitutively nuclear in ectodermal tissues such as the salivary glands, the fore- or the hindgut (Weigel et al. 1989; Hoch and Pankratz, 1996). Previous work, however, has demonstrated in mammals (Wolfrum et al. 2003) and flies (Bülow et al. 2010) that in hepatocytes and fat body tissue, FKH exerts its function by shuttling from the cytoplasm to the nucleus. Here, I show that also in midgut FKH shuttles from cytoplasm to the nucleus when TOR is downregulated by rapamycin treatment. This shows an important regulatory function for FKH in the two major organs for metabolic and immune system processes, the fatbody and the gut. It thus seems that FKH is constitutively nuclear in tissues of ectodermal origin, while it shuttles in a TOR-dependent manner in the fat body and the midgut, which are derived from mesoderm and endoderm, respectively. This indicates a more complex regulation of FKH than of dFOXO which has to be explored in more detail in future studies.

5.2.2 AMP expression by FKH and NF-κB like signaling

Earlier it was shown that dFOXO regulates AMPs independent of the classical innate immune pathways (NF-kB signaling pathways). Thereby, dFOXO and the NF-kB signaling pathways work in parallel to protect the organism from infections (Becker et al. 2010).

Additionally I show that the regulation of AMPs dependent on FKH occurs

independently from the classical innate immune pathways. Thereby, reveals the fact that FKH and NF-κB signaling work in parallel to monitor AMP induction. This is similar to the FOXO-dependent activation of AMPs under fasting or starvation conditions (Becker et al. 2010).

To exclude that FKH activation had an influence on Toll and Imd immunity pathways, experiments were done in immune deficient larvae. Double mutants for *Relish* and *Dif* (Dorsal-related immunity factor) have been shown to fail AMP expression in a NF-κB dependent manner, resulting in quick lethality after bacterial infection (Hedengren-Olcott et al. 2004). For this purpose, Dif;Relish double mutant larvae were subjected to rapamycin feeding experiments. Induction of Dpt and Mtk in rapamycin fed larvae indicate that a FKH dependent expression of AMP genes can be achieved independently of NF-κB like innate immune pathways. Together, our results further demonstrate the existence of a dFOXO and FKH-dependent crossregulation of metabolism and innate immunity that seems to act under normal physiological conditions of oscillating energy levels and in parallel to the infection-triggered classical immunity pathways on AMP regulation. This strengthens a theory of dFOXO and FKH dependent AMP regulation in non-infected animals, for example it acts as a mechanism to prevent infection when the animal is suffering from food and energy shortage or in context of ageing, when general fitness and physiological functions decline.

5.2.3 Forkhead and dFOXO: semi-communal functions

FKH and dFOXO are regulated by discrete branches of the IIS/TOR pathway. FKH is regulated by the TOR pathway and dFOXO by the insulin/PI3K pathway, two signaling systems that are already interwoven at various levels such as TSC2 and 4E-BP. The fact that FKH and dFOXO share the conserved FKH DNA binding domain may suggest at least partially overlapping target gene populations. It has been suggested that *CG6770* is another common target gene of dFOXO and FKH and that dFOXO knock-down has the same impact on cell size upon starvation like knock-down of FKH and *CG6770* (Bülow et al. 2010). On the level of transcription factors, there seems to be differential regulation: FKH is activated under conditions of protein deprivation and low TOR signaling, while dFOXO is activated by complete starvation and low insulin signaling (Bülow et al. 2010). However, there is also a downstream node of convergence. The expression of the translational inhibitor 4E-BP, which has been established as a dFOXO target and is

transcriptionally induced by protein deprivation as well as complete starvation (Zinke et al. 2002) is induced under conditions of low TOR signaling by FKH. This emerging molecular scenario outlined by these observations would allow cells and organisms to react specifically to different conditions of nutrient availability and food composition. dFOXO regulates broader set of AMPs, whereas, FKH regulates Dpt and Mtk (Figure 5.2).

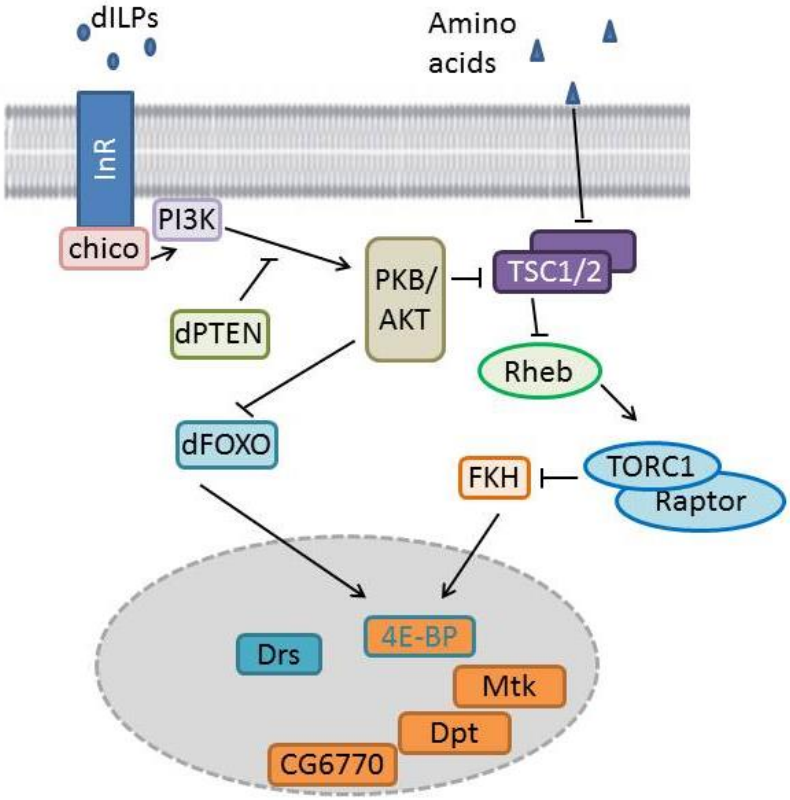

Figure 5.2. FKH regulates Dpt and Mtk.

dFOXO and FKH both are known to play a pivotal role in the longevity aspect. In *C. elegans*, the different functions of DAF-16/FoxO and pha-4/Forkhead can be distinguished by their role in longevity: while increased lifespan in insulin signaling mutants is dependent on FoxO, increased lifespan upon dietary restriction is dependent on FKH (Panowski et al. 2007). Thereby, the collaborative functions of both dFOXO and FKH on regulation of AMPs may in-turn play a key role in longevity and aid the body against infection at all time in different given scenarios of food and energy deprivation via regulation of different AMPs as well.

6 OUTLOOK

While the regulation of AMPs by IlS and TOR pathways in *Drosophila* has been presented here, and possible mechanisms of how it could cooperate with dFOXO and FKH to regulate genes in response to specific nutrient stimuli have been discussed, several open questions remain. The specific conditions under which dFOXO and FKH regulate their targets together, or when only one of them is active, are not completely elucidates yet. It remains to be examined if dFOXO and FKH regulate the AMPs directly. The function of AMPs downstream of dFOXO, FKH and TOR signaling raises a new interesting topic, because CecC and Dro are active upon starvation or through activation of dFOXO but not upon complete amino acid starvation, whereas Dpt and Mtk are induced upon amino acid starvation and starvation as well. These two AMPs could be responsible for protecting the organism during the oscillatory energy status, fortifying against stress and play a key role in prolonged life. Biochemical evidence for the interaction of dFOXO and FKH with the AMPs could be obtained by the analysis of possible binding sites that would be responsible for the regulation of AMPs and also by creating point mutations in those specific binding sights to ensure the function of the chosen binding sites. To ascertain the specific regulatory region, where the activation of dFOXO and FKH is necessary for the induction of AMPs *in vivo*, the reporter constructs of Dpt, Mtk, CecC and Dro can be crossed with various tissue specific driver lines. With the help of the UAS-GAL4 system driver lines like *Cad*-GAL4, CG-GAL4, *bagpipe*-GAL4 for expression in gut, fatbody and salivary glands, respectively, expressed in the background of dFOXO or FKH overexpression fly lines. Furthermore, the different tissues can be visualized with β-gal stainings or immunohistochemistry can be performed as well. To study further in detail, when the regulatory region element has been ascertained, the specific cell type in that element can be discovered with the help of different specific cell marker lines. E.g. the marker line *esg-lacZ* expresses gastric stem cells and *Dve-lacZ* line expresses interstitial cells in the gut of *Drosophila* (Marianes et al. 2013). The usage of these markers to drive expression of AMPs in dFOXO or FKH overexpression background will help visualize the cell types in which the AMPs are expressed upon activation of the TFs. The minimal inhibitory concentration, which is the lowest concentration of drug that inhibits bacterial growth can be performed, which will further enable to study the precise minimum concentration of AMPs required to protect the

organism. Further, it would be interesting to study the role of AMPs in longevity under the control of dFOXO and FKH, as both the TFs contribute significantly to longevity. To examine whether activation of dFOXO- and FKH-dependent AMPs ubiquitously or in different tissues contributes to lifespan extending effects, strains can be generated where ubiquitous and tissue restricted induction of dFOXO, FKH and AMPs dependent on both the TFs could be triggered by the RU486 inducer in either wild-type or a dFOXO, FKH mutant background. For the study of lifespan, food with optimal amount of dietary yeast (10% weight/volume) can be used (Bass et al. 2007). This would thereby maximize lifespan so that the effects of dFOXO, FKH and AMPs can be studied in addition to the beneficial effects of the diet.

7 SUMMARY

"Metabolism" and "Immunity" are two essential, recognized and independent functions of animal kingdom. They have their own biological cycle independent of each other. While metabolism contributes, amongst other things, to the growth and size of the organism; immunity on the other hand defends and is responsible for the health and longevity of the organism. If immunity is enhanced then an organism becomes stronger and may live longer.

In my thesis, I analyzed the role of antimicrobial peptides (AMPs) in metabolism and innate immunity in *D. melanogaster*. The cross regulation mechanism has been shown in larvae, wherein, dFOXO regulates AMPs, namely Drs independent of the Toll and IMD pathways. It unveiled a new mechanism for the regulation of AMPs by Insulin-like signaling pathway (IlS), known to regulate growth, energy homeostasis and life-span.

For the first time I examined whether such a cross regulation also occurs in the *D. melanogaster* adult flies. The adult flies, unlike the larvae that feed continuously, come in contact with various pathogens and have a complex metabolism. Thereby, it was interesting to study the regulation of AMPs in this stage where the fluctuation in expression levels based on development was absent. With an amalgamation of genetic and biochemical experiments I observed a similar mechanism in the adult flies, highlighting the existence of the fact that metabolism can regulate immunity through the dFOXO - AMP relationship via the key transcriptional factor dFOXO. I observed that ubiquitous overexpression or a tissue specific overexpression of dFOXO resulted in a distinct induction of the AMPs Dro, CecA1 and CecC. Furthermore, I determined that downregulation of dFOXO led to repression in those AMPs. Addditonally the regulation of CecC by dFOXO via overexpression and its induction through starvation was demonstrated. Thereby, a dFOXO dependent mode of AMP expression probably aids the organism to defend against microbial invasion and oscillatory energy status without the severe side effects induced by activation of NF-κB-like signaling pathways.

Furthermore, I analyzed whether target of rapamycin (TOR), another major regulator of growth and metabolism, also modulates AMP responses in the fly. The downregulation of TOR resulted in a specific induction of the AMPs Dpt and Mtk. In contrast, activation of TOR led to a repression of the two AMPs. The main transcription factor which is regulated by the TOR signaling is Forkhead (FKH), a member of the FoxA subfamily of Forkhead proteins. Further, Dpt and Mtk

activation is controlled by the transcription factor FKH, the founding member of the Forkhead box transcription family. Shuttling of FKH from the cytoplasm to the nucleus is induced in the fat body and in the posterior midgut in response to TOR downregulation. The FKH-dependent induction of Dpt and Mtk can be triggered in dFOXO null mutants and in immune-compromised Toll and IMD pathway mutants indicating that FKH acts in parallel to these regulators.

Together, this work has discovered that FKH is the second conserved member of the Forkhead family cross-regulating metabolism and innate immunity. dFOXO and FKH, which are activated upon downregulation of insulin or TOR activities, respectively, act in parallel to induce different sets of AMPs, thereby modulating the immune status of metabolic tissues such as the fatbody or the gut in response to the oscillating energy status of the organism.

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9 ABBREVIATIONS

°C	Degree Celsius
A.bidest	Aqua bidistilled
AP	Alkaline phosphatise
bp	Base pairs
BSA	Bovine Serum Albumin
cDNA	Complementary DNA
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	Deoxynucleotide triphosphates
E.coli	*Escherichia coli*
EDTA	Ethyldiamin-N,N-N',N'-tetraacetate
et al.	And others
g	Gramme
h	Hour
kB	Kilo bases
kg	Kilogramme
l	Liter
LB	LuriaBertani
M	Molar
m	Milli
Min	Minute
mRNA	Messenger RNA
NBT	Nitro blue tetrazolinum chloride
o/n	Over night
OD	Optical Density
pH	pHvalue
RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	Rotations per minute
RT	Room temperature
sec	Second
tRNA	Transfer RNA
U	Unit
UAS	Upstream activating sequence
UV	Ultraviolet
V	Voltage
v/v	Volume to Volume
vol.	Volume
w/v	Weight to volume
wildtype	white^{-}
µ	Micro