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Forkhead, a new cross regulator of metabolism and innate immunity downstream of TOR in *Drosophila*

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ABSTRACT

Antimicrobial peptides (AMPs) are conserved cationic peptides which act both as defense molecules of the host immune system and as regulators of the commensal microbiome. Expression of AMPs is induced in response to infection by the Toll and Imd pathway. Under non-infected conditions, the transcription factor dFOXO directly regulates a set of AMP expression at low levels when nutrients are limited.

Here we have analyzed whether target of rapamycin (TOR), another major regulator of growth and metabolism, also modulates AMP responses in *Drosophila*. We found that downregulation of TOR by feeding the drug rapamycin or by overexpressing the negative TOR regulators TSC1/TSC2, resulted in a specific induction of the AMPs Diptericin (Dpt) and Metchnikowin (Mtk). In contrast, overexpression of Rheb, which positively regulates TOR led to a repression of the two AMPs. Genetic and pharmacological experiments indicate that Dpt and Mtk activation is controlled by the transcription factor Forkhead (FKH), the founding member of the FoxO 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, we have discovered that FKH is the second conserved member of the FoxO 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 fat body or the gut in response to the oscillating energy status of the organism.

1. Introduction

Drosophila melanogaster is exposed to a variety of pathogens including viruses, bacteria, fungi and parasites, but the fly is lacking an adaptive immune system, which has only been described for vertebrate species so far. To recognize and fight infections *Drosophila* relies therefore on an innate immune system, which is phylogenetically highly conserved throughout the animal kingdom. Main effector molecules of the innate immunity are the antimicrobial peptides (AMPs), a group of structurally diverse, small and cationic peptides. Until today eight classes of AMPs have been described, and seven of them have been shown to be inducible (Lemaitre and Hoffmann, 2007). AMPs act as components of the systemic as well as the local immune response by fighting different classes of microorganisms including bacteria, yeasts and filamentous fungi, parasites and also some enveloped viruses (Imler and Bulet, 2005, for review). During the systemic response AMPs are expressed in the fatbody of the fly and released into the hemolymph, for example as the result of an invasion of microbes into the body cavity. The fatbody is therefore a central tissue of the immune system but at the same time the main metabolic organ of the fly, amongst others storing energy reserves and producing high numbers of metabolites. The local immune response takes place at barrier epithelia, which delimit the organism from the environment and form a surface where pathogenic and commensal microbes interact with their host, mainly at the epidermis, the reproductive, the respiratory and the digestive tract (Tzou et al., 2000). Upon an infection the expression of AMPs is mediated through the Toll (Lemaitre et al., 1996) and the Immune Deficiency (Imd) (Lemaitre et al., 1995) pathway, depending on the type of the attacking pathogen (Lemaitre et al., 1997) and the site of infection (Neyen et al., 2012; Ferrandon, 2013). Both signaling cascades activate transcription factors of the Nf-κB family such as Relish, Dif or Dorsal which bind to Nf-κB motifs in the regulatory region of AMP genes to activate their transcription

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(Silverman and Maniatis, 2001). Dif and Dorsal are the transcription factors controlled by the Toll pathway (Lemaitre et al., 1996), Relish is the signal transducer of the Imd cascade (Lemaitre et al., 1995).

While the properties of the immune system in *Drosophila* were mainly studied when challenged by an infection, we could demonstrate a new infection independent cross-regulation between the metabolism and the immune system of *Drosophila*. This mechanism works under normal physiological conditions dependent of the energy status of the organism and is controlled by the activity of the insulin/insulin-like growth factor signaling (IIS) pathway (Becker et al., 2010). The IIS cascade is a highly conserved, major regulator of energy homeostasis, growth and stress response (Brogiolo et al., 2001). A key factor of IIS is the transcription factor Forkhead box O (dFOXO) (Jünger et al., 2003; Puig et al., 2003). The phosphorylated and inactive form of the protein is retained in the cytoplasm. Reducing the activity of the IIS pathway by fasting wild type animals or by inactivating pathway components via mutation leads to the translocation of dFOXO from the cytoplasm into the nucleus and results in the transcriptional induction of AMP genes (Becker et al., 2010). Of note, the dFOXO-dependent expression of AMP genes can also be triggered in animals unable to respond to immune challenges due to defects in both the TOLL and IMD pathways. This indicates that the dFOXO-dependent regulation of AMPs is uncoupled of the Nf-κB-dependent regulation of AMPs triggered by infection (Becker et al., 2010). The dFOXO-dependent mechanism of AMP induction was shown to operate in the fatbody and in the barrier epithelia of *Drosophila* and it has been proposed that it is used to modulate defense reactions and the composition of microbial communities when animals are suffering from energy shortage or stress. Experiments in human cell culture proved the conservation of the mechanism and its overall importance (Becker et al., 2010).

Another major and conserved nutrient dependent pathway in *Drosophila* is constituted by the target of rapamycin (TOR) cascade (Oldham et al., 2000). TOR signaling responds specifically to the availability of proteins but in addition IIS and TOR signaling are linked via the interaction of the PKB/Akt kinase of the IIS pathway with TSC1/TSC2 (Tuberous Sclerosis Complex) (Inoki et al., 2002), a component of the TOR pathway. The transcription factor which is regulated by the TOR signaling is Forkhead (FKH), a member of the FoxA subfamily of Forkhead proteins. Similar to dFOXO, FoxA is translocated to the nucleus upon low TOR signaling causing the expression of its target genes. FoxA and dFOXO have both distinct but also common target genes such as the translational repressor d4E-BP (*Drosophila* 4E-binding protein) (Bülow et al., 2010). Due to the fact that we found numerous predicted FKH binding sites in the regulatory region of several AMPs we were interested in the potential impact of TOR signaling on the innate immunity and the expression of AMPs.

2. Results

2.1. Regulation of antimicrobial peptide (AMP) expression by the TOR pathway

Drosophila responds to immune challenges by upregulating antimicrobial peptide (AMP) expression in the fatbody and gut via the Toll- and the Imd signaling pathways (Fig. 1A) (Lemaitre and Hoffmann, 2007, for review). Previous work from our laboratory has shown that AMPs are not only regulated by immune pathways, but also by the insulin pathway (Fig. 1B) and that the transcription levels 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) (Bülow et al., 2010), which acts downstream of TOR signaling, we tested the hypothesis whether AMPs are regulated by the TOR pathway. We quantified the transcription levels 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 and found *Cecropin C* (*CecC*), *Diptericin* (*Dpt*) and *Metchnikowin* (*Mtk*) significantly upregulated, while *Defensin* (*Def*) and *Drosocin* (*Dro*) were downregulated (Fig. 1C). Since *Dpt* and *Mtk* were the two genes which were the most prominently upregulated AMPs, we decided to focus on these two in our further studies. Mtk is a proline-rich peptide (Levashina et al., 1998) which was found to inhibit the growth of filamentous fungi and Dpt is O-Glycopeptide directed against Gram-negative bacteria (Imler and Bulet, 2005, for review). *Drosomycin* (*Drs*) is an established dFOXO target gene (Becker et al., 2010) and we used it as a control in every experiment.

2.2. Genetic and pharmacological 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 TSC1/TSC2 (tuberous sclerosis) complex (Tapon et al., 2001) and Rheb (Ras homology enriched in brain) (Stocker et al., 2003; Saucedo et al., 2003). The TSC1/TSC2 complex is a negative regulator of TOR signaling which inhibits its growth. It acts inhibitory on Rheb, a positive regulator of TOR and of growth (Fig. 2A). We overexpressed TSC1/TSC2 using the ubiquitous, mifepristone (RU486)-inducible driver Tubulin-GeneSwitch-Gal4 (Osterwalder et al., 2001) and found that *Dpt* and *Mtk* as well as the positive control *CG6770* are upregulated, while the dFOXO target *Drs* is not regulated (Fig. 2B). Since TSC1/TSC2 suppresses TOR signaling, our result indicates in line with the result from Fig. 1C that inhibition of TOR signaling upregulates the transcript levels of AMPs and that *Drs* is not regulated by this mechanism. To further narrow down the impact of the TOR pathway on AMP transcript levels, we overexpressed Rheb, which hyperactivates the TOR pathway. We found that *Dpt*, *Mtk* and *CG6770* are downregulated while *Drs* is insignificantly upregulated (Fig. 2C). This further demonstrates 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). We fed white- larvae for 24 h with 50 μM Rapamycin which inhibits TOR signaling. In line with our results from Figs. 1C and 2B, we found that *Dpt*, *Mtk* and the positive control are upregulated. *Drs* was not regulated under this condition (Fig. 2D). Taken together, our findings demonstrate that regulation of AMP transcript levels occurs downstream of TOR signaling.

2.3. The transcription factor Forkhead regulates Dpt and Mtk

The TOR pathway controls growth by phosphorylation of d4E-BP (Gingras et al., 1999), but also by controlling gene expression via the transcription factor Forkhead (FKH). The IIS 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, we analyzed their transcript levels 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 (Fig. 3A), while upon FKH knock-down, reflecting high TOR signaling, *Dpt* and *Mtk* are downregulated and *Drs* expression is unchanged (Fig. 3B). We analyzed the promoter regions of *Dpt* and *Mtk* and found numerous FKH binding sequences in both