School of Doctoral Studies in Biological Sciences

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Faculty of Science

Role of the bZIP protein Atf3 in metabolic and immune homeostasis

and

Molecular interactions of the insect juvenile hormone receptor Met

Ph.D. Thesis

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Annotation

This thesis comprises two parts:

I. Role of the bZIP protein Atf3 in metabolic and immune homeostasis

This part of the thesis is based on analysis of loss-of-function phenotypes of the bZIP transcription factor Atf3 in *Drosophila* larvae. By employing genetic, biochemical, molecular and microscopic techniques, metabolic and immune disorders caused by *atf3* mutation have been characterized, suggesting a new role of Atf3 as a common regulator of metabolism and immunity in *Drosophila*.

II. Molecular interactions of the insect juvenile hormone receptor Met

In the second part of the thesis, ligand-binding properties of the candidate insect juvenile hormone (JH) receptor, the bHLH PAS transcription factor Met, have been investigated. It has been proven that Met binds JH with high affinity and point mutations disrupting JH binding have been identified. By using biochemical tools, JH-dependent protein-protein interactions of Met have been tested. The results show that Met senses the JH signal by specifically binding the hormonal ligand, thus establishing the function of Met as a JH receptor.

Declaration [in Czech]

Prohlašuji, že svoji disertační práci jsem vypracoval samostatně pouze s použitím pramenů a literatury uvedených v seznamu citované literatury.

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České Budějovice, 24.8.2012 .....

Jan Ryneš

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A substantial part of the research presented in the chapter „Role of the bZIP protein Atf3 in metabolic and immune homeostasis“ was conducted during my stay at the Institute for Genetics, University of Cologne, Cologne, Germany, under the supervision of **Dr. Mirka Uhlířová**.

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I would like to thank my supervisor, Dr. Marek Jindra, for scientific guidance, support throughout my PhD. studies and help with writing this thesis. My special thanks go to Dr. Mirka Uhlířová for supervising my research and for help during my stay in her lab at the University of Cologne, Germany. I would also like to thank Dr. Masako Jindrová and all members of her and Marek´s lab for friendly working atmosphere. I also thank my family and friends for their support during my studies.

List of published papers and the author's contribution

The thesis is based on the following papers (listed chronologically):

I. Charles, J.P., Iwema, T., Epa, V.C., Takaki, K., **Rynes, J.** and Jindra, M. 2011. Ligand-binding properties of a juvenile hormone receptor, Methoprene-tolerant. *Proc. Natl. Acad. Sci. USA.* 108(52): 21128-21133 (IF = 9.681).

Jan Ryneš contributed by preparing plasmid constructs, expressing recombinant proteins in mammalian cell cultures and testing JH-dependent protein-protein interactions using immunoprecipitation from transfected HEK293 cells.

II. **Rynes, J.,** Donohoe, C.D., Frommolt, P., Brodesser, S., Jindra, M. and Uhlirova, M. 2012. Activating Transcription Factor 3 Regulates Immune and Metabolic Homeostasis. *Mol. Cell. Biol.* [in press] (IF = 5.527).

Jan Ryneš was responsible for colorimetric measurements of saccharides and triacylglycerols in Drosophila larvae, cultivation and identification of bacterial microflora, respirometry, electron microscopy imaging and luciferase reporter assays. He also participated in analysing gene expression using RNA isolation, cDNA synthesis and qRT-PCR, in conducting genetic survival, rescue and starvation experiments in mutant Drosophila and in data analysis.

Contents

List of abbreviations

AhR – Aryl hydrocarbon receptor

AhRR - Aryl hydrocarbon receptor repressor

AMP – Antimicrobial peptide

AMPK – Adenosine monophosphate–activated kinase

AP-1 – Activator protein 1

APC – Antigen presenting cell

Arnt - Aryl hydrocarbon receptor nuclear translocator

ATF – Activating transcription factor

bHLH – basic helix-loop-helix

BRG-1 – Brahma-related gene 1

bZIP – basic region-leucine zipper

C/EBP – CAAT/enhancer binding protein

CD14 – Cluster of differentiation 14

CREB – cAMP response element-binding

CRM-1 - Chromosome region maintenance protein 1

DBD – DNA-binding domain

Dilp – Drosophila insulin-like peptide

EcR – Ecdysone receptor

EGFP – Enhanced green fluorescent protein

ER – Endoplasmatic reticulum

FFA – Free fatty acids

Fiaf - Fasting-induced adipocyte factor

FIH - Factor inhibiting HIF 1

FISC – βFtz-F1 interacting steroid receptor coactivator

FOXO – Forkhead box O

Ftz-F1 – Fushi tarazu factor 1

Gce – Germ cells expressed

Gpr – G-protein coupled receptor

HDAC – Histone deacetylase

HEK293 – Human embryonic kidney 293

HIF – Hypoxia inducible factor

HRE – Hypoxia response element

Hsp90 - Heat-shock protein 90

ICAM - Intercellular adhesion molecule

IGF – Insulin-like growth factor

IIS – insulin/IGF signalling

IKK – IκB kinase

IL - Interleukine

Imd – Immune deficiency

InR – Insulin receptor

IPC – Insulin producing cell

IRS – Insulin receptor substrate

JDP2 – Jun-dimerizing protein 2

JH – Juvenile hormone

JHRE - Juvenile hormone response element

Jnk – Jun N-terminal kinase

Kr-h1 - Kruppel-homolog 1

LB – Luria-Bertani

LPL – Lipoprotein lipase

LPS - Lipopolysaccharide

Lys - Lysozyme

Maf - Musculoaponeurotic fibrosarcoma oncogene homolog

MCP-1 - Monocyte chemotactic protein 1

Met – Methoprene tolerant

NES – Nuclear export signal

NF-kB – Nuclear factor kB

ODDD - Oxygen-dependent degradation domain

PAS – Per-Arnt-Sim

PDK-1 - 3-phosphoinositide dependent protein kinase 1

PHD - Prolyl hydroxylase domain-containing protein

PIP3 - Phosphatidylinositol (3,4,5)-triphosphate

PI3K - Phosphoinositide 3-kinase

PYY - Peptide YY

RAR – Retinoic acid receptor

Rel - Relish

RNAi – RNA interference

RXR – Retinoid X receptor

SCFA – Short chain fatty acid

SOCS - Suppressor of cytokine signalling

SRC – Steroid receptor coactivator

Ss - Spineless

T2D – Type 2 diabetes

Tai - Taiman

Tak1 – TGF-β activated kinase 1

TCDD - 2,3,7,8-tetrachlorodibenzo-p-dioxin

Tgo - Tango

TLR – Toll-like receptor

TNF-α – Tumor necrosis factor α

UAS – Upstream activating sequence

UPR – Unfolded protein response

Usp - Ultraspiracle

VCAM - Vascular cell adhesion molecule

XAP2 - Hepatitis B virus X-associated protein 2

XRE – Xenobiotic response element

Part I.

Role of the bZIP protein Atf3 in metabolic and immune homeostasis

Research objectives

Activating Transcription Factor 3 (ATF3) is a transcriptional regulator from the family of basic region-leucine zipper (bZIP) proteins. The main portion of knowledge about the function of ATF3 comes from mammalian model systems, where ATF3 has a paralog, Jun dimerizing protein 2 (JDP2). Neither ATF3, nor JDP2 are essential in mice, as null-mutants for either gene are viable. However, *Jdp2-/-* mice show signs of obesity. Mammalian ATF3 has been characterized as a negative regulator of innate immunity, and *Atf3*-deficient mice suffer from chronic activation of a TLR-dependent immune response.

Recently, we have identified a single ortholog of ATF3 in *Drosophila*. In the fly, loss of *atf3* function severely impaired viability, as only a small proportion of the mutant animals survived to adulthood. Preliminary observations indicated elevated expression of antimicrobial peptides and fat accumulation in the *atf3* mutant larvae.

The goal of my study was to elucidate the function of the ATF3 ortholog as a common regulator of immune and metabolic homeostasis in the *Drosophila* model, and thus contribute to our understanding of the ancient role of the duplicated and functionally specialized mammalian proteins. The aims of the work were to characterize the metabolic defect caused by loss of *atf3* function by determining the nutrient stores and ability of the larvae to utilize nutrient energy, and to identify the pathways responsible for the chronic stress and immune response activation and their contribution to the *atf3* phenotype. Transcriptome sequencing yielded information on gene expression patterns of *atf3* mutants and enabled selection of candidate genes for more detailed examinations. The UAS-Gal4 system enabled us to address the tissue-specific requirements for Atf3 function and to test the capacity of human ATF3 to substitute for its fly counterpart.

1. Introduction

1.1. Coordination of immunity and metabolism in mammals

1.1.1. Obesity, inflammation and insulin resistance

Integration of metabolic, stress and immune response pathways plays a crucial role in maintenance of tissue homeostasis. Disruption of the balance between metabolic pathways and stress signalling causes serious metabolic diseases, such as obesity that is linked to other pathological conditions, including cardiovascular disease or metabolic syndrome (pre-diabetes), which can progress to type 2 diabetes (T2D). Upon sustained overnutrition, lipids accumulate in the white adpose tissue, causing hypertrophy of adipocytes and expansion of the fat mass. Excessive fat deposition is linked to a low-grade chronic pro-inflammatory signalling, which can be causative to insulin resistance (Shoelson *et al.,* 2007; Figure 1.1.).

Mammalian white adipose tissue is a dynamic and heterogenous endocrine organ. It is vascularized and inervated, contains extracellular matrix and besides the fat storage cells, adipocytes, it is comprised of numerous cell types, such as preadipocytes, fibroblasts, stem cells and immune cells, including macrophages or T-cells (Kalupahana *et al.,* 2012; Shoelson *et al.,* 2007). Adipose tissue is not only a passive depot of fat, but it also secretes a variety of substances known as adipokines: metabolic homeostasis regulators, such as leptin, adiponectin, resistin, apelin and visfatin; chemokines and cytokines such as monocyte chemotactic protein 1 (MCP-1), IL-8, IL-6, IL-1, angiotensin II, TNF-α, and anti-inflammatory peptides such as IL-10 (Kalupahana *et al.,* 2012). Excessive fat accumulation, adipocyte damage and growth of the fat mass dysregulates the adipokine secretory patterns and results in predominating production of pro-inflammatory molecules (Figure 1.1.). In response to increased secretion of the monocyte chemoattractant MCP-1, adipose tissue becomes infiltrated by a monocyte-derived macrophages (Kanda *et al.,* 2006). Production of adhesive molecules (ICAM-1, VCAM-1) by endothelial cells helps to recruit immune cells to the adipose tissue that further contribute to the secretion of

pro-inflammatory cytokines. Inflammatory signals promote insulin resistance locally and also systemically, by entering the circulation (Shoelson *et al.*, 2007).

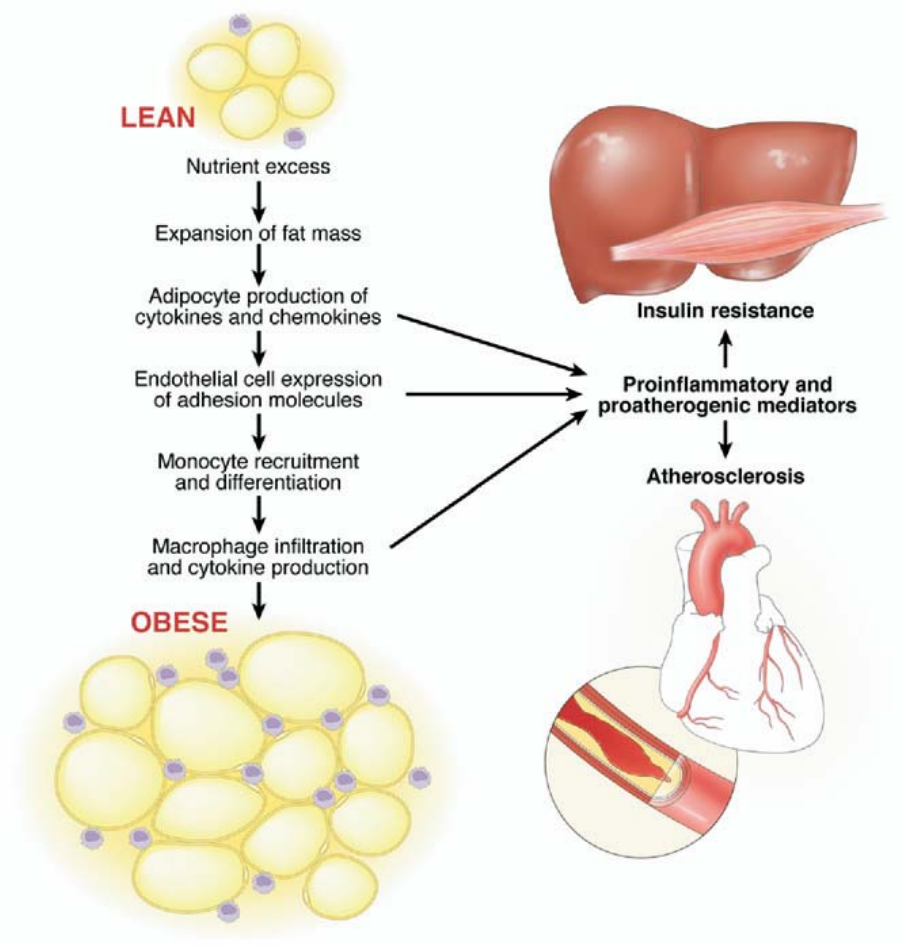

Figure 1.1. Model for obesity-induced inflammation. Accumulation of lipids, adipocyte hypertrophy and expansion of the fat mass initiate secretion of pro-inflammatory cytokines and chemokines, which leads to recruitment of immune cells to the adipose tissue. Infiltrated macrophages contribute to the pro-inflammatory signalling by cytokine production. Pro-inflammatory and pro-atherogenic mediators enter the circulation to promote systemic insulin resistance and increase risk for atherosclerosis. After (Shoelson *et al.*, 2007).

Adipose tissue, together with skeletal muscles and liver are the main mammalian organs sensitive to nutrient storage regulation by the hormone insulin. During fasting conditions, the liver participates in maintaining of normoglycemia by

releasing glucose, produced via glycogenolysis and gluconeogenesis, to the blood stream. Upon feeding, glucose is supplied to the circulation through the intestine, and increasing blood glucose concentration stimulates insulin secretion by the pancreas. As a reaction to hyperglycemia, insulin suppresses glycogenolysis and gluconeogenesis in the liver and increases hepatic glycogen synthesis. At the same time, insulin stimulates uptake and utilization of glucose by the skeletal muscles. Finally, insulin also inhibits lipolysis and upregulates lipogenesis in the adipose tissue (Kalupahana *et al.,* 2012). Thus, insulin resistance (i.e. insensitivity of the tissues to insulin) is connected to hyperglycemia caused by ineffective glucose utilization and increased glucose output from the liver. Misregulated lipid storage in the adipose tissue results in high plasma levels of free fatty acids (FFA). In order to compensate hyperglycemia, pancreatic β cells overproduce insulin. Hypersecretion of insulin, together with the lipotoxic effect of FFA leads to the β cells failure and T2D development (Cusi, 2010; Stumvoll *et al.,* 2005).

In the target tissues, insulin signalling occurs through activation of transmembrane dimeric receptor tyrosine kinase (Insulin receptor, InR), which in turn leads to phosphorylation of downstream substrates, including the insulin-receptor substrates (IRS). Phosphorylated IRS recruits phosphoinositide 3-kinase (PI3K) that activates serine/threonine protein kinase Akt/PKB via the lipid second messenger, PIP_3, and serine/threonine kinase PDK-1. The active Akt kinase targets several downstream proteins, controlling glucose transport and metabolism, or transcriptional regulation (Figure 1.2.).

At the molecular level, inflammatory signalling interferes with the insulin action by inhibiting the relay of signal via IRS (Figure 1.2.). Cytokine signalling pathways activate the stress-response Jun N-terminal kinase (Jnk) that mediates an inhibitory serine phosphorylation of IRS (Aguime *et al.,* 2000). Free fatty acids, whose plasmatic level is elevated in obesity, can also serve as pro-inflammatory molecules, acting through the Toll-like receptors (TLRs) (Shi *et al.,* 2006). TLR signalling activates IKK kinase within the NF-kB pathway, which leads to the inhibitory serine phosphorylation of IRS (Gao *et al.,* 2002). Regulators involved in negative feedback loops of cytokine signalling, suppressors of cytokine signalling (SOCS), can also block the insulin action, either by degradation of IRS (SOCS-1 and 3) (Rui *et al.,* 2002), or by impairing the interaction between InR and IRS (Emanuelli *et al.,* 2001).

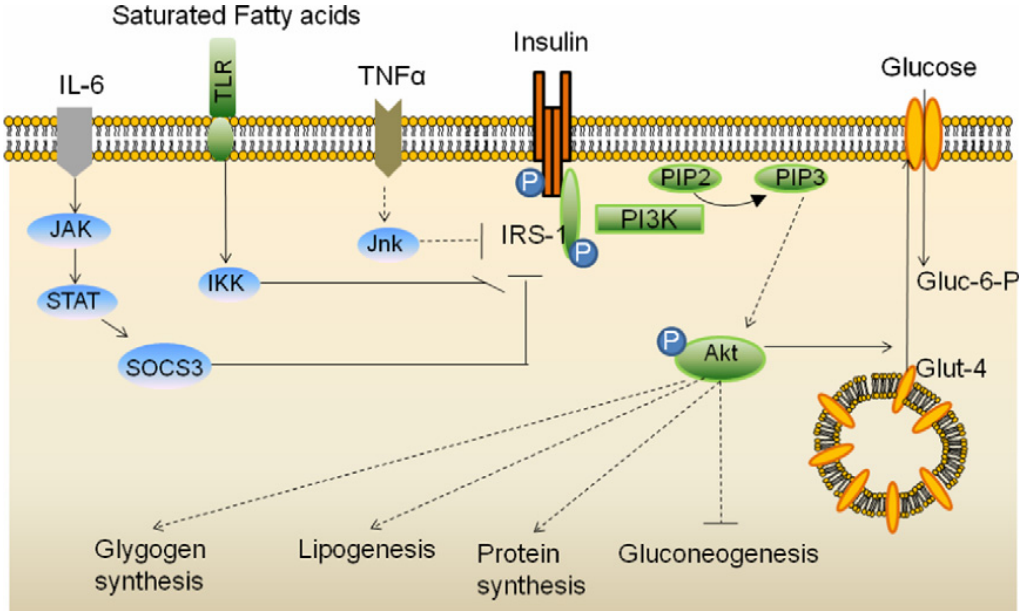

Figure 1.2. Molecular mechanisms of inflammatory-induced insulin resistance. Activated insulin receptor triggers a cascade of phosphorylation events, resulting in activation of Akt. Active Akt inhibits gluconeogenesis, promotes glycogen synthesis and regulates transcription factors. Pro-inflammatory cytokines and FFA activate intracellular serine/threonine kinases such as Jnk and IKK which block IRS by inhibitory serine phosphorylation. SOCS-3 downregulates insulin pathway by impairing IR and IRS interactions. Reprint from (Kalupahana *et al.*, 2012).

Moreover, insulin resistance can also be promoted via activation of inflammatory signalling (Jnk, NF-kB), triggered by endoplasmatic reticulum (ER) stress and resulting unfolded protein response (UPR) (Hotamisligil, 2010).

1.1.2. Role of gut microflora in low-grade systemic inflammation and energy storage

Recently, increasing number of studies have implicated the commensal gut bacteria in regulation of the host immunity and metabolism. The initial link between

obesity and altered gut microflora comes from the leptin-deficient, genetically obese (*ob/ob*) mice (Ley *et al.,* 2005). 16S rRNA analysis revealed that compared to lean controls (+/+ and *ob/*+), obese mice (*ob/ob*) have different proportion of the two dominant bacterial strains in their guts: about 50% reduction in the abundance of *Bacteroidetes* and a proportional increase in *Firmicutes* (Ley *et al.,* 2005). Several mechanisms have been proposed for how the gastrointestinal microbiota impact inflammation, insulin resistance and adiposity of the host organism.

Recent studies have demonstrated that lipopolysaccharide (LPS), a component of the Gram-negative bacterial cell walls, can initiate the low-grade inflammation, connected with obesity and insulin resistance in mice (Cani *et al.,* 2007a; Cani *et al.,* 2007b). LPS is absorbed from the gut more efficiently upon high-fat diet feeding, increasing the plasma levels of LPS in obese animals (Cani *et al.,* 2007a), probably due to the changes of intestinal microflora composition (decrease of *Bifidobacterium spp.*), linked to increased gut permeability (Cani *et al.,* 2007b; Cani *et al.,* 2008). LPS triggers innate immune response via TLR-4 activation, since mice lacking CD14 (a co-receptor of TLR-4) do not develop the LPS-induced inflammation (Cani *et al.,* 2007a).

The gut microbiota play a role in harvesting of energy from the diet. It has been shown that germ-free mice have 40% less total body fat than conventionally raised controls, even if the caloric intake of the germ-free animals is higher. Colonization of the gut with normal bacterial community produces dramatic increase in the body fat (Backhed *et al.,* 2004). The microbiota promote absorption of monosaccharides from the gut, resulting in increased hepatic lipogenesis, and also induce lipid storage in the adipocytes by suppression of fasting-induced adipocyte factor (Fiaf) in the intestinal epithelium. Fiaf is a negative regulator of a circulating lipoprotein lipase (LPL) that promotes lipid storage in the adipocytes (Backhed *et al.,* 2004; Figure 1.3.).

The germ-free mice also show increased activity of AMP-activated protein kinase (AMPK) that positively regulates enzymes involved in fatty acid oxidation (Backhed *et al.,* 2007). The gut bacteria downregulate the levels of phosphorylated AMPK in the liver and skeletal muscles and thereby prevent lipid catabolism in a Fiaf/LPL independent manner (Backhed *et al.,* 2007; Figure 1.3.).

Another pathway involves products of polysaccharide fermentation, the short chain fatty acids (SCFA). SCFA act not only as substrates for lipogenesis in the host

liver, but also as signalling molecules, recognized by G-protein coupled receptors Gpr41 and Gpr43 (Le *et al.,* 2003; Figure 1.3.). It has been shown that Gpr41-defficient (*Gpr41-/-*) mice colonized with a fermentative microbiota (*Bacteroides thetaiotaomicron* and *Methanobrevibacter smithii*) accumulate less lipids than wild-type controls (Samuel *et al.,* 2008). Colonization of wild-type germ-free mice results in increased plasma levels of an enteroendocrine hormone, peptide YY (PYY), which has not been observed in (*Gpr41-/-*) mutants. Since PYY normally inhibits gut motility and thus increases absorption of SCFA from the diet, it may contribute to fat deposition in the host organism (Samuel *et al.,* 2008).

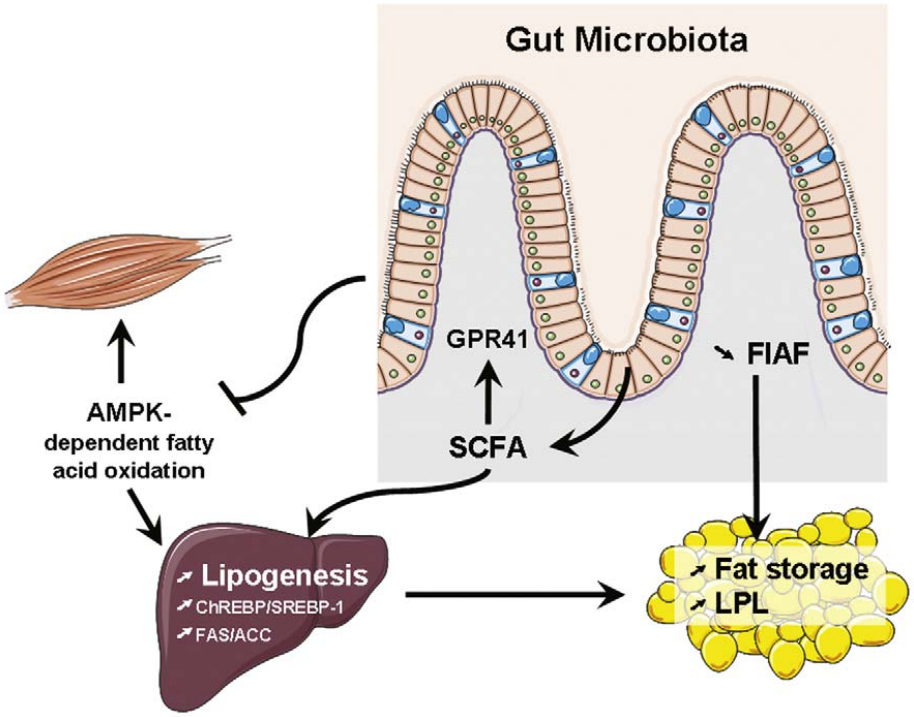

Figure 1.3. Commensal gut microbiota promote energy storage. The intestinal microflora regulates fat accumulation by increasing nutrients absorption (monosaccharides, SCFA), derepression of LPL function, or inhibition of AMPK-dependent fatty acid oxidation. Adapted from (Cani *et al.,* 2009).

1.2. Crosstalk of insulin action, stress and immune response in *Drosophila*

In *Drosophila*, like in mammals, the metabolic and immune system responses are tightly interconnected. Also in the fly, metabolic homeostasis is primarily controlled by the conserved insulin/IGF signalling pathway (IIS). *Drosophila* insulin-like peptides (Dilps), secreted by the insulin-producing cells (IPCs) in the brain, activate the insulin receptor (InR), which triggers a signalling cascade resulting in phosphorylation of serine/threonine kinase Akt. Activated Akt phosphorylates downstream substrates to promote growth and energy storage. An important target of Akt is the transcription factor Forkhead box O (FOXO). Phosphorylation of FOXO by Akt leads to its retention in the cytoplasm and thereby inhibition of its nuclear transcriptional activity (Teleman, 2010). Nuclearly localized, transcriptionally active FOXO regulates large amount of target genes, among others stress response genes and antimicrobial peptides (Becker *et al.,* 2010; Wang *et al.,* 2005; Figure 1.5.).

Similar to mammals, stress-activated Jnk pathway antagonizes insulin signalling in the fly. In peripheral tissues, stress stimuli causes cell-autonomous activation of the Jnk pathway. Jnk then suppresses IIS and causes activation of FOXO, resulting in upregulation of the FOXO target genes, which mediate local stress response and growth arrest. Activation of Jnk in IPCs causes FOXO-dependent repression of *dilp2* gene and thereby promotes systemic downregulation of IIS (Wang *et al.,* 2005; Figure 1.4.).

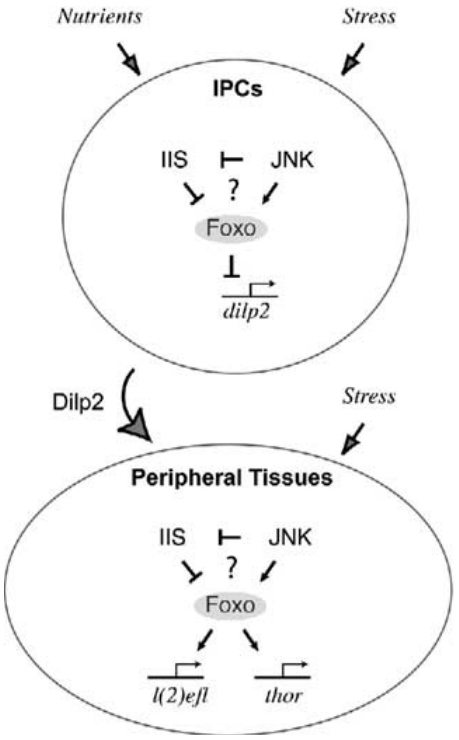

Figure 1.4. Model for antagonistic action of Jnk and insulin/IGF signalling in *Drosophila*. Jnk and insulin/IGF signals converge on FOXO and regulate the balance between growth and stress protection at the systemic and cell autonomous level. Adapted from (Wang *et al.,* 2005).

The innate immune response of *Drosophila* is regulated by the Immune deficiency (Imd) and the Toll receptor pathways. These signalling cascades are activated by bacterial or fungal infections and the signal is relayed to the nucleus through transcription factors of the NF-κB-like family (Relish, Dorsal, Dif), which results in a massive induction of the antimicrobial peptide (AMP) genes (Lemaitre and Hoffmann, 2007; Figure 1.5.). It has been demonstrated that selective activation of the Toll signalling in the *Drosophila* fat body suppresses IIS both cell autonomously and systemically, leading to growth arrest and decrease of nutrient stores (DiAngelo *et al.,* 2009). The Imd signalling does not attenuate IIS, although it has been previously shown that the Tak1 kinase, involved in the Imd pathway, activates also Jnk (DiAngelo *et al.,* 2009; Silvermann *et al.,* 2003).

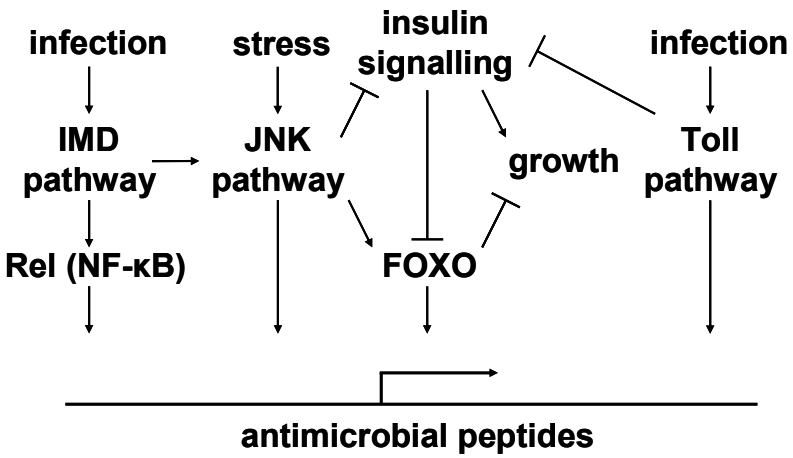

Figure 1.5. Coordination of stress and immune responses with insulin action in *Drosophila*.

1.3. The bZIP transcription factor ATF3

The Activating Transcription Factor 3 (ATF3) is a member of a large group of basic region-leucine zipper (bZIP) transcription regulators. ATF3 belongs to the ATF/cAMP response element-binding (CREB) protein family of transcription factors that share the basic region-leucine zipper domain. The N-terminal basic region of the bZIP domain is responsible for specific DNA binding and the leucine zipper serves as interface for homodimerization or heterodimerization with other bZIP proteins, such as AP-1, C/EBP or Maf. ATF/CREB proteins bind to the same consensus sequence TGACGTCAC *in vitro* (Hai *et al.,* 1999). ATF3 either homodimerizes or heterodimerizes with other ATF/CREB proteins, including ATF2, c-Jun, JunB and JunD. Depending on the promoter context, these dimers either activate or repress transcription (Thompson *et al.,* 2009).

ATF3 is an adaptive response gene – its expression is low on normal conditions and is upregulated upon wide variety of stress signals, including cytokines, genotoxic agents or physiological stresses (Thompson *et al.,* 2009). *Atf3-/-* knockout mice are viable with defective insulin signalling due to impaired β-cells function and viability (Hartman *et al.,* 2004; Zmuda *et al.,* 2007). ATF3 also controls TLR-induced innate immune response. In mouse and human antigen presenting cells (APCs), ATF3 expression is induced in response to signalling via Toll-like receptors,

TLR2/6, TLR3, TLR4, TLR5, TLR7 and TLR9. Conversely, *Atf3*-deficient macrophages express elevated levels of IL-6 and IL-12p40 cytokines upon activation of various TLRs, and elevated amounts of TNF-α following TLR9 activation alone (Whitmore *et al.,* 2007). This is in agreement with previously published results that demonstrated ATF3 upregulation upon TLR4 stimulation, and increased IL-6 and IL-12b production in *Atf3*-deficient macrophages treated with LPS. Moreover, these genes contain ATF/CREB promoter binding sites within close proximity of NF-κB sites, suggesting that ATF3 may directly modulate NF-κB signalling, possibly by recruiting histone deacetylase 1 (HDAC1) (Gilchrist *et al.,* 2006).

Immune response and metabolic regulation are complex processes, interconnected to each other. Misregulation of either of them can disrupt tissue homeostasis and result in development of deleterious pathologies. Since regulation of metabolism and immunity is highly synchronized, involving also commensal bacteria in the gut, we have to view the processes in context rather than separately. In *Drosophila*, the main metabolic, stress response and innate immune pathways are conserved, therefore the fly provides us with a simpler model for unraveling the basic principles of the tissue homeostasis regulation.

In mammals, ATF3 has a paralog, Jun-dimerizing protein 2 (JDP2). Similar to *Atf3-/-* mutants, *Jdp2-/-* mice are viable but they accumulate extra fat in the white adipose tissue (Nakade *et al.,* 2007). Recently, a single ortholog of ATF3 in *Drosophila* has been identified (Sekyrova *et al.,* 2010). Loss of *atf3* function in the fly combines phenotypes of both *Atf3-/-* and *Jdp2-/-*: dysregulated innate immune response and accumulation of surplus fat, leading to mortality during larval and pupal stages. Thus, *Drosophila* enables us to establish a model for Atf3 function in immune and metabolic homeostasis with the advantage of availability of many genetic and molecular tools.

2. Results

Research article:

***Mol. Cell. Biol.* (2012); [in press]**

Activating Transcription Factor 3 Regulates Immune and Metabolic Homeostasis

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Abstract

Integration of metabolic and immune responses during animal development ensures energy balance, permitting both growth and defense. Disturbed homeostasis causes organ failure, growth retardation, and metabolic disorders. Here, we show that the *Drosophila melanogaster* activating transcription factor 3 (Atf3) safeguards metabolic and immune system homeostasis. Loss of Atf3 results in chronic inflammation and starvation responses mounted primarily by the larval gut epithelium, while the fat body suffers lipid overload, causing energy imbalance and death. Hyperactive proinflammatory and stress signaling through NF-κB/Relish, Jun–N-terminal kinase, and FOXO in *atf3* mutants deregulates genes important for immune defense, digestion, and lipid metabolism. Reducing the dose of either FOXO or Relish normalizes both lipid metabolism and gene expression in *atf3* mutants. The function of Atf3 is conserved, as human ATF3 averts some of the *Drosophila* mutant phenotypes, improving their survival. The single *Drosophila* Atf3 may incorporate the diversified roles of two related mammalian proteins.

3. Unpublished results

3.1. Intestinal lysozymes may regulate gut microbiota in *Drosophila*

Lysozymes (EC 3.2.1.17) are hydrolytic enzymes that cleave the *β*-(1,4)-glycosidic bond between *N*-acetylmuramic acid and *N* acetylglucosamine in peptidoglycan, the major bacterial cell wall polymer, thus having antibacterial and digestive function (Callewaert and Michiels, 2010). *Drosophila* possesses the c-type lysozymes, expressed in a specific pattern: four closely related genes, LysB, C, D and E, are strongly expressed in the midgut of larvae and adults; LysP is expressed in the adult salivary gland; LysS is expressed mainly in the gastric caeca of larvae; and LysX is primarily expressed in the metamorphosing midgut of late larvae and early pupae (Daffre *et al.,* 1994). All lysozymes from the fly, except LysP, are acidic lysozymes, suggesting their function in digestion of the gut bacteria, rather than anti-pathogenic defence (Callewaert and Michiels, 2010). Consistently, none of the lysozymes is induced upon infection (Daffre *et al.,* 1994). In the *atf3* mutants, the intestinally-expressed cluster of lysozymes LysB, C, D and E (*LysBCDE*) is significantly suppressed, together with increased amounts of the gut microbiota (*Acetobacter sp.* and *Lactobacillus sp.*). Therefore we asked whether the lowered expression of lysozymes is caused by increase of the bacterial amount in the gut, or whether the lysozymes regulate the microflora.

To address this question, we prepared cDNA from the third instar *atf3* mutant larvae and *yw* controls that were reared either on standard diet, or diet supplemented with antibiotics to eliminate bacteria. Indeed, in contrast to antibiotic-free diet, no bacteria from the larvae reared on the antibiotics grew upon cultivation on LB plates (data not shown). Quantitative RT-PCR analysis using primers common to all genes in the *LysBCDE* cluster revealed that the lysozymes were downregulated in the *atf3* mutant larvae also upon the antibiotic treatment, suggesting that *Acetobacter* and *Lactobacillus* do not affect the intestinal lysozyme expression (Figure 3.1.).

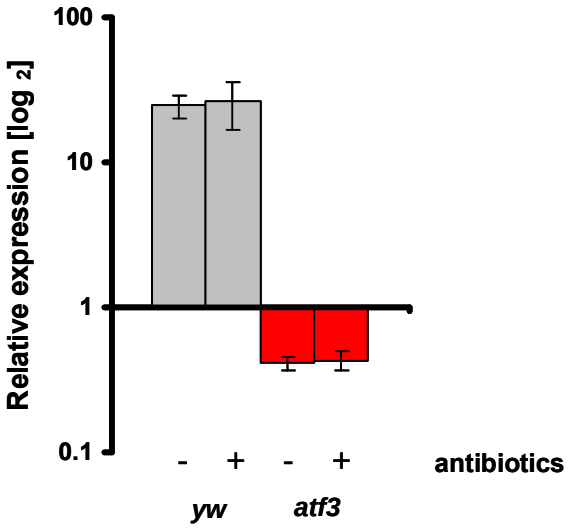

Figure 3.1. Expression of *LysBCDE* in the third instar larvae is not affected by intestinal microbiota. Larvae were grown on standard food and food supplemented with antibiotics (combination of Carbenicilin, Tetracycline and Rifampicin). Expression of *LysBCDE* was quantified by qRT-PCR. Rp49 was used for normalization.

We also noticed that both *Acetobacter* and *Lactobacillus* were eliminated from the wild-type and *atf3* mutant larvae upon starvation. Our question was, whether this clearance of bacteria is caused by induction of lysozymes. Whole DNA was isolated from fed larvae and larvae upon 12-h starvation, both *yw* controls and *atf3* mutants. Using primers specific for amplification of 16S rDNA from *Lactobacillus* or *Acetobacter*, the amount of bacterial DNA in the larvae was quantified by qRT-PCR. In parallel, cDNA was prepared from the fed larvae and larvae upon 12 h and 24 h starvation and the level of *LysBCDE* expression was determined by using qRT-PCR. The results confirmed that *yw* larvae expressed higher levels of lysozymes than the *atf3* mutants and also that the amount of the intestinal microbiota was lower in *yw* larvae (Figure 3.2.). Surprisingly, levels of lysozymes were decreasing during starvation in *atf3* mutant larvae and *yw* controls alongside with the bacteria (Figure 3.2.), suggesting that the elimination of intestinal microflora upon starvation is probably caused by simple removal of the food from the gut.

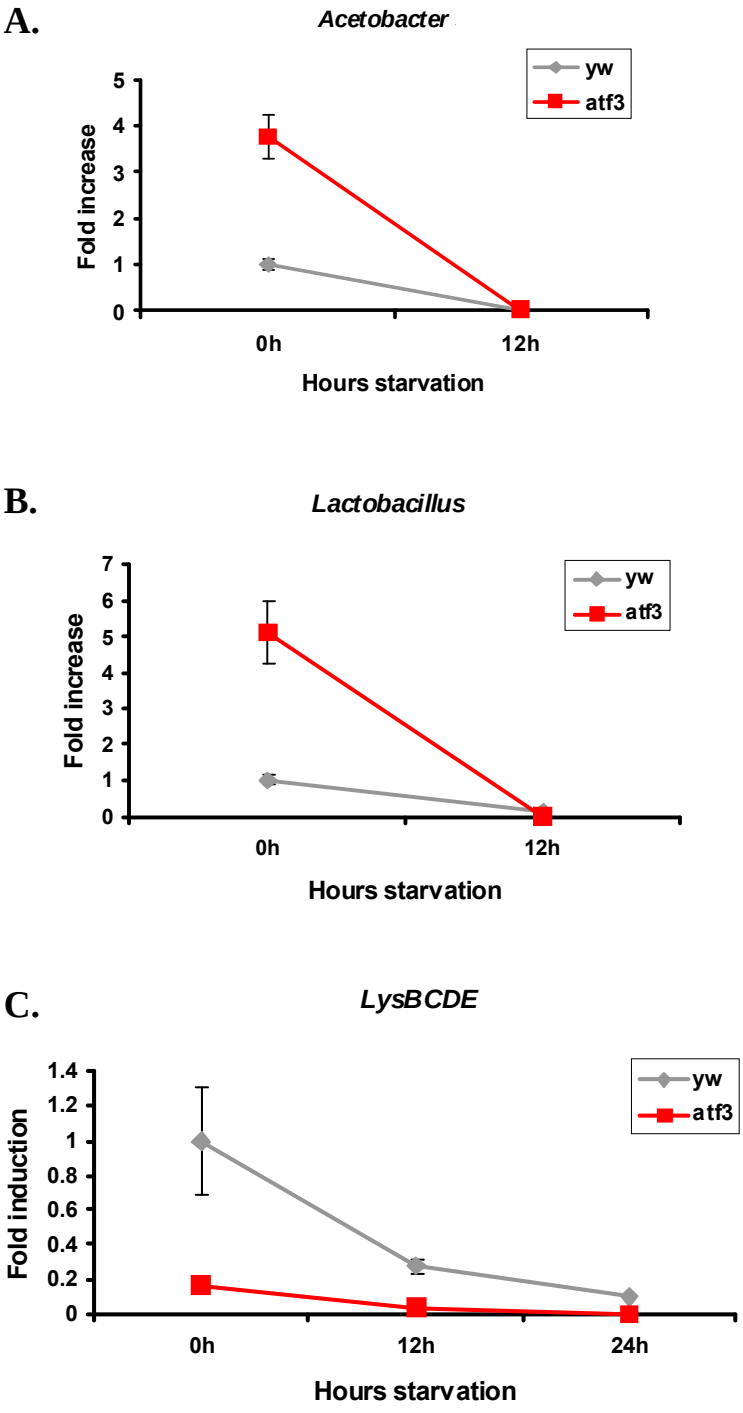

Figure 3.2. Amounts of *Acetobacter* **(A.)** and *Lactobacillus* **(B.)** in the third instar larvae decrease upon starvation, as well as expression of *LysBCDE* **(C.)**. Bacterial 16S rDNA or *LysBCDE* cDNA was quantified by qRT-PCR. Rp49 was used for normalization. The values are expressed as fold increase (induction) relative to the *yw* control sample at time-point 0h, which was arbitrary set to 1.

The decrease of *LysBCDE* expression during starvation is probably not caused by the elimination of bacteria, since removing of bacteria with antibiotics did not affect the lysozyme levels. Most likely, downregulation of the gut lysozymes was triggered by starvation stress. Similarly to the starvation-induced genes *thor*, *rel* and *drs*, expression of *LysBCDE* was downregulated even in the fully fed *atf3* mutant larvae and this effect was further enhanced by food deprivation.

Lysozymes catalyze cleavage of peptidoglycan, which leads to desintegration of the bacterial cell walls and makes the cells sensitive to osmotic stress. In Gram-negative bacteria, the peptidoglycan layer is surrounded by lipopolysaccharide-containing outer membrane and is thus inaccessible to lysozymes. However, this barrier can be permeabilized by the antimicrobial peptides, such as cecropins, defensins or attacins. Moreover, some insect c-type lysozymes are antibacterial against Gram-negative bacteria (Callewaert and Michiels, 2010). It is therefore possible that the fly intestinal lysozymes can digest both Gram-positive *Lactobacillus* and Gram-negative *Acetobacter*.

4. Conclusions

1. ***Atf3 has an essential function in the growth of Drosophila larvae.***
   Loss of *atf3* function severely impairs the larval viability. Compared to the wild-type controls, the *atf3* mutant larvae are smaller, although capable of feeding. Their pupariation and eclosion is delayed and only about 14% adults emerge when nurtured under reduced competition from *atf3*+ siblings.

2. ***atf3 mutant larvae suffer from chronic inflammation and stress.***
   Transcriptome analyses have revealed that *atf3*-deficient larvae show altered transcriptional program, primarily in their gut, that resembles transcriptome changes observed during intestinal bacterial infection or gut damage.

3. ***atf3-deficient larvae have altered intestinal microflora.***
   atf3 mutants have increased amounts of commensal bacteria in their guts. Changes in composition of gut microbiota linked to metabolic disorders and chronic inflammation have also been reported from mammals, suggesting importance of the microbiota in the regulation of immune and metabolic homeostasis.

4. ***atf3 mutants are obese while experiencing starvation.***
   Compared to controls, *atf3* mutants accumulate significantly higher amounts of stored fat (TGA) and also DAG and FFA. Adipose cells in the fat body of *atf3*-deficient larvae contain large lipid droplets. Despite increased energy storage, their gene expression profile resembles chronic starvation.

5. ***Human ATF3 normalizes fat content and gene expression in atf3 mutants.***
   Despite a limited degree of sequence conservation between the Drosophila and human ATF3 proteins, expression of hATF3 in *atf3* mutant larvae reduces fat levels and normalizes expression of some misregulated genes, indicating that at least some functions of Atf3 are evolutionarily conserved.

6. ***Attenuation of immunity improves lipid metabolism and survival of atf3 mutants.***

   Suppression of immune response by reducing gene dose of Relish or FOXO decreases the amounts of TGA and DAG in *atf3* mutants. Moreover, Relish heterozygosity also reduces levels of FFA. Reduced Relish (but not FOXO) function significantly rescues adult viability**.**

7. ***Drosophila Atf3 is a common regulator of immune and metabolic homeostasis, integrating both functions of mammalian orthologs, ATF3 and JDP2.***

   The complex role of the single *Drosophila* atf3 gene has been apparently divided between two duplicated mammalian orthologs.

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Part II.

Molecular interactions of the insect juvenile hormone receptor Met

Research objectives

Juvenile hormone (JH) is an essential regulator of insect development, which controlls the entry into metamorphosis. Despite the effect of JH has been known for decades, the molecular mechanisms of JH signalling remain largely unknown, mainly because a JH receptor has not been identified. The best candidate for the JH receptor is the Methoprene-tolerant (Met) basic helix-loop-helix (bHLH)- Per-Arnt-Sim (PAS) protein, which is an essential mediator of the anti-metamorphic JH signal. Nevertheless, its function as the bona fide JH receptor has not been established, since the specific high-affinity JH binding has not been sufficiently proven and the ligand-binding domain of Met has not been characterized.

The goal of our study was to prove specific JH binding to Met and identify the ligand-binding domain, and thus provide the missing piece of evidence that Met is the JH receptor. JH binding was determined with Dextran-Coated Charcoal assay, using radioactively-labeled JH and *in vitro* transcribed and translated Met. Based on homology with other members of the bHLH PAS protein family, we proposed a structural model of the putative ligand-binding domain of Met, which enabled us to specifically disrupt JH binding by single amino acid substitutions within the ligand-binding pocket. We also tested the effects of specific ligand binding on JH-dependent homo- and heterodimerization of Met. Using luciferase reporter assays, we examined the transcriptional activation properties of Met.

1. Introduction

1.1. Overview of JH role in insect metamorphosis

Insect development from the embryo to the adult stage consists of a series of larval instars, terminated with metamorphosis into a winged adult. In holometabolous species such as moths, flies or beetles, metamorphosis involves an intermediary stage, known as the pupa (Figure 1.1.). Entry into metamorphosis is controlled by two types of insect hormones: steroid hormones (ecdysteroids), acting via classical nuclear receptors (Thummel, 1996), which trigger and coordinate molts between the larval stages and the metamorphic changes, and the sesquiterpenoid juvenile hormone (JH), which specifies the character of the next stage. In the presence of JH, continuously secreted from the corpora allata, a larva molts to another larval instar (Figure 1.1.). After a larva reaches the final instar and achieves a certain size, corpora allata cease to produce JH, which allows the tissues to make adult (or first pupal and then adult) structures during metamorphosis (Riddiford, 1994).

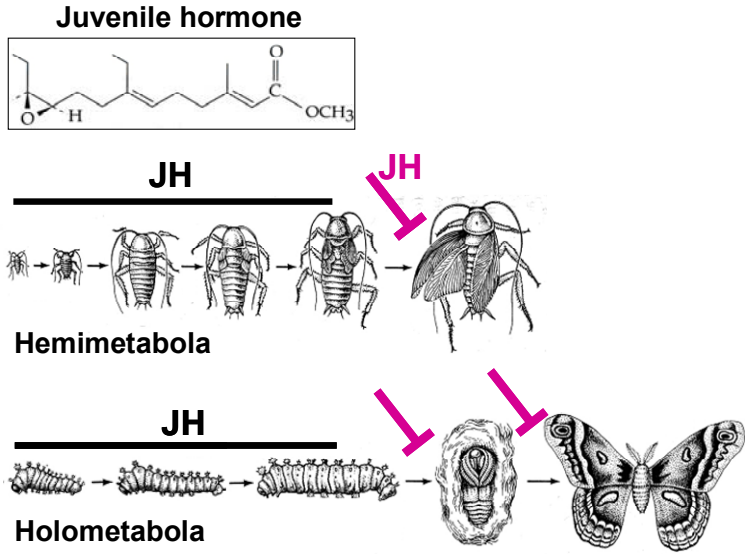

Figure 1.1. Presence of JH (black bars) keeps an insect in a juvenile stages. In the final larval instar, JH level drops, which allows entry to the metamorphosis. Ectopic JH (purple lines) can prevent adult or pupal development, resulting in extra larval or pupal stages. The structure of JH I (Röller *et al.,* 1967) is shown.

Experimental ectopic application of JH or its biologically active mimics, such as methoprene, confirms the anti-metamorphic function of JH. Treatment of final larval instars with JH prevents transition to the adult or pupal stages and causes repetition of larval instars. Similarly, ectopic JH in the pupal stages prevents development of adult structures and results in another pupal stage (Figure 1.1.). On the other hand, removing of JH during early larval stages causes premature development of pupal or adult structures. Surgical (Wigglesworth, 1936; Fukuda, 1944) or chemical (Bowers et al., 1975) ablation of larval corpora allata, degradation of JH by overexpressing JH esterase (Tan *et al.,* 2005), and disruption of JH synthesis by mutation (Daimon *et al.,* 2012) of RNAi (Minakuchi *et al.,* 2008) of key biosynthetic enzymes all lead to precocious metamorphosis.

1.2. Candidate JH receptor, a bHLH PAS protein Methoprene- tolerant

Despite the anti-metamorphic effect of juvenile hormone has been known since 1934 (Wigglesworth, 1934) and the structure of JH was resolved in 1967 (Röller *et al.,* 1967), JH signalling pathway still remains largely unknown. The key step to uncover JH signalling at molecular level is identification of a JH receptor. A screen for *Drosophila* mutants resistant to lethal doses of a JH analog insecticide, methoprene, revealed that mutation in the *Methoprene-tolerant* (*Met*) gene confers tolerance to JH, indicating a role of *Met* in JH signal transduction (Wilson and Fabian, 1986). Moreover, tissues of Met^{-} flies have reduced JH-binding ability (Shemshedini and Wilson, 1990). However, Met^{27} null mutant flies are viable and develop normally without signs of precocious metamorphosis, which would be expected for defective JH signalling (Wilson and Ashok, 1998). This discrepancy has been recently explained by identifying and characterizing a gene paralogous to *Met* in *Drosophila*, g*erm cells expressed* (g*ce*). Functions of Met and Gce overlap, since overexpression of Gce in *Met* null mutants increases sensitivity to methoprene and the *Met gce* double mutant dies during the larval-pupal transition, whereas both *Met* and *gce* null single mutants are fully viable (Abdou et al., 2011; Baumann et al.,

2010). Also studies in other insects have confirmed the role of Met in juvenile hormone sensing. The beetle *Tribolium* possesses a single ortholog of *Drosophila Met* and *gce*. RNAi knockdown of Met in *Tribolium* prevented inhibition of adult development by ectopic JH and injecting Met double-stranded RNA in 3rd or 4th larval instar causes precocious metamorphosis after 5th or 6th larval stage (Konopova and Jindra, 2007), which phenocopies the effect of lacking JH (Minakuchi et al., 2008). Also in the hemimetabolous bug, *Pyrrhocoris apterus*, Met mediates the anti-metamorphic effect of JH, as Met RNAi knockdown induces precocious metamorphosis (Konopova et al., 2011).

Met encodes a nuclearly localized transcriptional regulator of the basic helix-loop-helix (bHLH)- Per-Arnt-Sim (PAS) domain family (Ashok et al., 1998; Pursley et al., 2000) and it is therefore possible that Met acts as an intracellular receptor for the small lipophilic JH molecule, in a manner similar to steroid-hormone nuclear receptors. Indeed, it has been shown that *in vitro* synthesized *Drosophila* Met directly binds JH III in physiological (nanomolar) concentrations and fusion of Met with the DNA-binding domain of the Gal4 (Gal4 DBD) activate transcription from the UAS-*luciferase* reporter in response to JH in *Drosophila* S2 cells (Miura *et al.,* 2005). Consistently with the JH binding capability, Met can interact with its protein partners in a JH-dependent manner. *Drosophila* Met can form a homodimer or a heterodimer with Gce. Both dimers are JH-sensitive, the complexes dissociate in the presence of methoprene or JH III (Godlewski *et al.,* 2006) and this observation has been confirmed with Met proteins from the mosquito *Aedes aegypti* (Li *et al.,* 2011). Upon JH-binding, Met associates with another bHLH PAS protein, Taiman, also known as Steroid receptor coactivator (SRC) in *Tribolium* (Zhang *et al.,* 2011), or FISC in *Aedes* (Li *et al.,* 2011). The complex of Met and Taiman has been detected by chromatin immunoprecipitation near the promoter of the *Aedes early trypsin* gene where a putative JH response element (JHRE; CCACACGCGAAG) occurs, and to activate transcription from this site upon JH addition *in vitro* (Li *et al.,* 2011). A similar interaction between a Met homolog (Met2) and Taiman/SRC has been reported from *Bombyx*, where Met2 activates the *Kr-h1* gene from a similar JHRE containing a canonical E-box motif, CACGTG (Kayukawa et al., 2012). Although bHLH-PAS proteins are known to bind to E-boxes, evidence for direct binding of Met to any DNA is still missing.

1.3. Transcription regulators of the bHLH PAS protein family

1.3.1. Structural characteristics of the bHLH PAS proteins

The bHLH PAS (basic helix-loop-helix Per-Arnt-Sim) family involves proteins with common arrangement of shared structural motifs, functioning as dimeric transcriptional regulators. The members of this family control many physiological and developmental processes such as toxin metabolism, response to hypoxia, neurogenesis, tracheal and salivary duct formation or circadian rhythms. The domains in the bHLH PAS proteins are typically organized, starting from the N-terminus: the helix-loop-helix containing a basic part within the N-terminus of the first helix, followed by the PAS-A and PAS-B domains and a non-conserved C-terminal part, responsible for transactivation or transrepression properties (Figure 1.2.). The basic region mediates direct contact with DNA and the HLH part serves for dimerization with interaction partners from the bHLH PAS family. The PAS domains also contribute to dimer formation by providing a secondary interaction interface that ensures partner specificity and that is necessary for full interaction strength (Lindebro *et al.,* 1995; Reisz-Porszasz *et al.,* 1994). Based on structural analysis of PAS-B domains of the Hypoxia inducible factor 2α (HIF-2α) and Aryl hydrocarbon receptor nuclear translocator (Arnt) and homology of the bHLH motif with resolved structure of the transcription factor Max bound to its cognate DNA, a model for dimerization and DNA-binding of bHLH PAS proteins has been proposed (Card *et al.,* 2005; Figure 1.3.). Moreover, the PAS domains of Aryl hydrocarbon receptor (AhR) and Arnt contribute to the DNA-binding affinity, probably by forming intramolecular contacts with the bHLH regions (Chapman-Smith and Whitelaw, 2006). In AhR and HIF-2α, the PAS-B domains are the ligand-binding sites (Coumailleau *et al.,*1995; Scheuermann *et al.,* 2009).

According to the selective binding of dimerization partners, the bHLH PAS transcription factors can be divided into two classes: Class I bHLH PAS proteins neither homodimerize nor heterodimerize with other factors from the same class. Class II factors form either homodimers or promiscuously heterodimerize with Class

I factors to form transcriptionally active complexes (Kewley *et al.,* 2004; Figure 1.2.). In general, Class I members serve as the transcriptional regulatory unit (e.g. AhR or HIF-α factors), which senses stimulatory cues and transmits the signals to the nucleus. Class II factors are constitutively nuclear and are required to form active DNA-binding complexes.

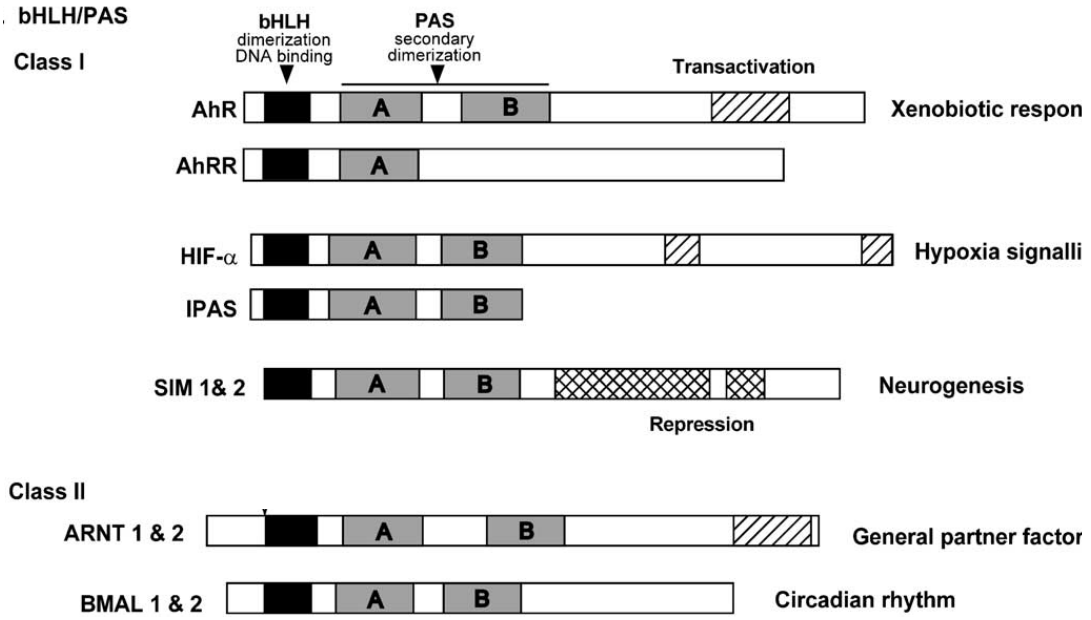

Figure 1.2. Schematic representation of domain organization of some bHLH PAS family members. The N-terminal bHLH DNA binding/dimerization domain is followed by the PAS domains and the C-terninal region, responsible for transactivation/transrepression properties of the transcription factors. Based on dimerization properties, the bHLH PAS transcription factors are grouped into two classes. Adapted from (Kewley *et al.,* 2004).

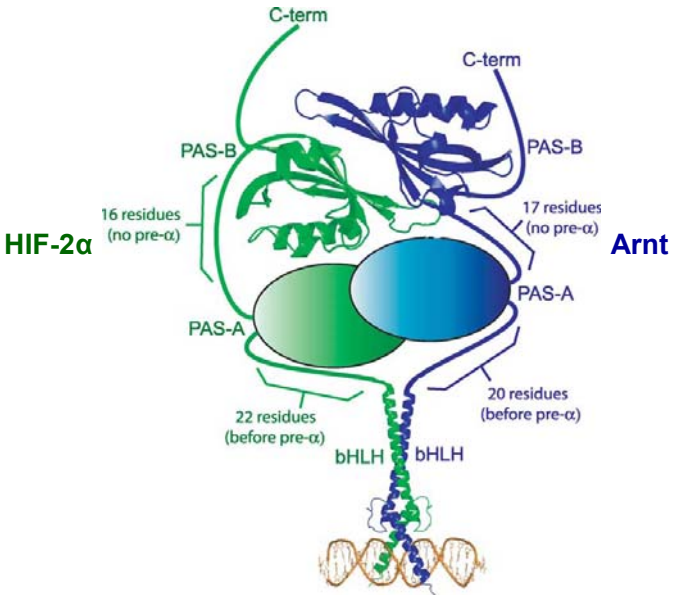

Figure 1.3. Model for the interaction of HIF-2α and Arnt. The bHLH domain binding DNA is represented by the crystal structure of the bHLH protein Max, bound to its cognate DNA, followed by a schematic representation of a putative PAS-A domains interaction (not drawn to scale). The antiparallel HIF-2a/ARNT PAS-B interaction is shown by the representative structure from the HADDOCK calculations. Adapted from (Card *et al.,* 2005).

1.3.2. Sensing of external stimuli by the bHLH PAS factors

1.3.2.1. Hypoxia inducible factor alpha

Delivery of oxygen to tissues and cells and maintaining oxygen homeostasis is essential for survival of multicellular organisms. Therefore, organisms have evolved mechanisms to sense the oxygen concentrations and adapt to hypoxic conditions. Response to low oxygen tension is mediated through an oxygen-sensitive transcription regulator, Hypoxia inducible factor alpha (HIF-α). In mammals, three paralogs of HIF-α are present: HIF-1α, which is ubiquitously expressed in human and mouse tissues and plays a role in many physiological adaptations to hypoxia, such as metabolic changes, erythropoiesis or angiogenesis; HIF-2α that is tissue specific; and HIF-3α, alternatively spliced variant of which can inhibit HIF-1α signalling (Ke and Costa, 2006).

Regulation of HIF-α occurs at the level of proteasomal degradation. HIF-α mRNA is constitutively expressed and the protein synthesized, but during normoxia

is rapidly degraded by the ubiquitin-proteasome pathway. HIF-α is hydroxylated on two prolines within the oxygen-dependent degradation domain (ODDD) by an oxygen and iron dependent prolyl hydroxylases (PHD). This hydroxylation serves as a docking site for von Hippel-Lindau ubiquitin E3 ligase complex, which in turn ubiquitinylates HIF-α and thereby marks it for degradation by the 26S proteasome. Moreover, the asparaginyl hydroxylase factor inhibiting HIF 1 (FIH-1), mediates hydroxylation of an asparagine residue within the C-terminal transactivation domain of HIF-α that prevents the recruitment of coactivator CBP/p300 and subsequent transactivation of target genes. Under hypoxic conditions, PHD and FIH-1 are inactivated by low oxygen level, which allows HIF-α to accumulate and heterodimerize in the nucleus with Arnt (also termed HIF-β in the context of hypoxia signalling). The dimeric transcription factor binds to the hypoxia response elements (HRE; core consensus 5´-G/ACGTG-3´) in the regulatory regions of the target genes, recruits coactivators such as CBP/p300 and activates transcription (Ke and Costa, 2006; Kewley *et al.,* 2004; Figure 1.4.).

Although HIF-α senses oxygen levels via oxygen-dependent hydroxylation and degradation, crystallographic structure analysis of human HIF-2α PAS-B domain revealed an internal cavity, which accommodates a small-molecule artificial ligands (Scheuermann *et al.,* 2009; Figure 1.5.). Although a natural ligand is not known, artificial ligand binding to the cavity of HIF-2α PAS-B modulates affinity to the PAS-B domain of Arnt, suggesting a possible mechanism of HIF-2α activity regulation (Scheuermann *et al.,* 2009).

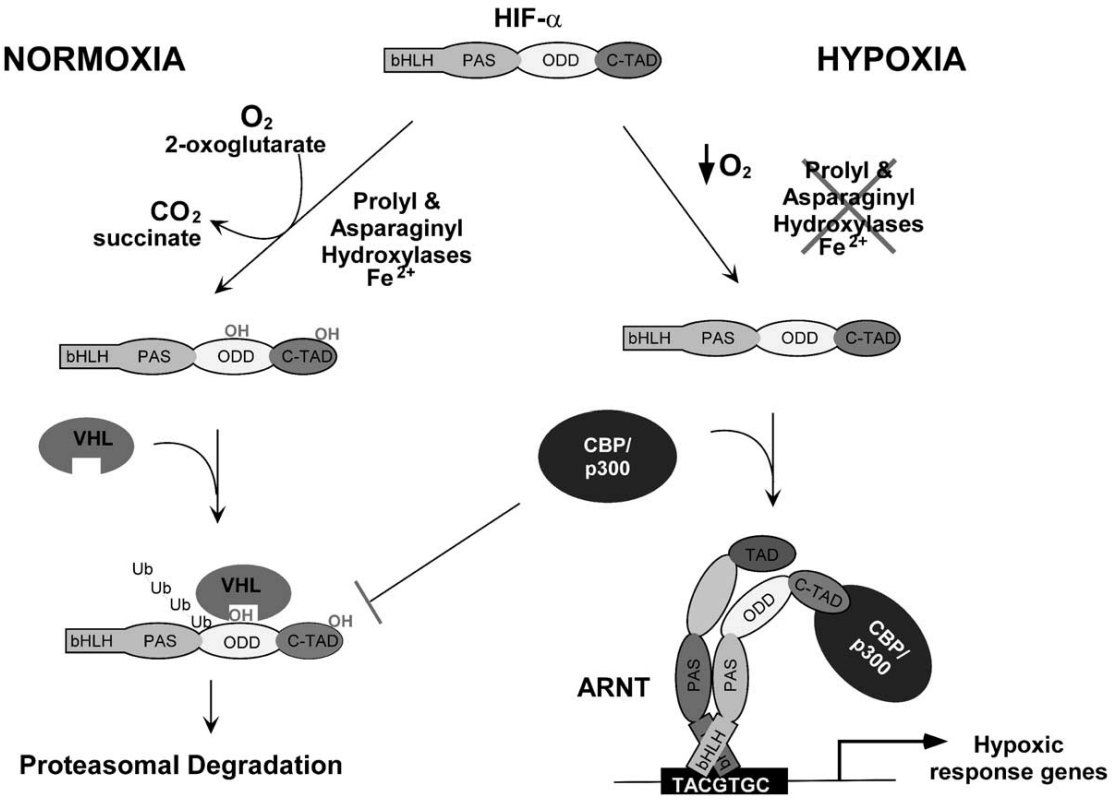

Figure 1.4. HIF-α signalling in response to hypoxia. Under normoxic conditions, HIF-α is hydroxylated at two proline residues within the oxygen-dependent degradation domain (ODD). This hydroxylation recruits the E3 ubiquitin ligase complex via interaction with von Hippel-Lindau protein (VHL) and subsequent polyubiquitinylation targets HIF-α for the proteasomal degradation. Hydroxylation on an asparagine residue within the C-terminal transactivation domain (C-TAD) of HIF-α interfere with binding of coactivator CBP/p300, which prevents transcriptional activation by any non-degraded HIF-α. During hypoxia, oxygen-dependent hydroxylases are inactive, which leads to HIF-α accumulation. In the nucleus, HIF-α interacts with Arnt. The active dimer binds HREs in the regulatory elements of the target genes and activate transcription via recruiting coactivator CBP/p300. Reprint from (Kewley *et al.,* 2004).

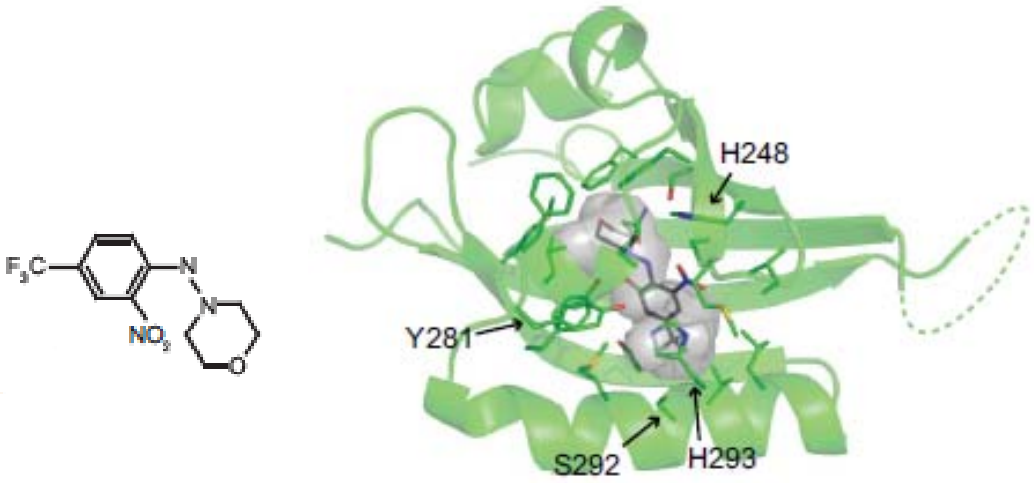

Figure 1.5. Structure of the HIF-2α PAS-B domain with a bound ligand. The ligand, THS-044 (gray sticks; chemical structure shown on left), occupies the HIF-2α PAS-B internal cavity (transparent gray surface). Indicated amino acid side chains mediate contacts with the ligand molecule. Adapted from (Scheuermann *et al.,* 2009).

1.3.2.1. Aryl hydrocarbon receptor

Whereas the activity of HIF-α factor is regulated by an oxygen-dependent hydroxylation and degradation, Aryl hydrocarbon receptor (AhR; also called Dioxin receptor) is a ligand-sensing molecule. Many planar polyaromatic xenobiotics (either synthetic or natural) can act as ligands and the prototypical agonist for the AhR is 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) (Denison and Nagy, 2003). In a non-active state, AhR is localized in the cytoplasm, bound to a complex of interaction partners, including a dimer of the molecular chaperone heat shock protein 90 (Hsp90), co-chaperone p23 and an immunophillin-like molecule, hepatitis B virus X-associated protein (XAP2) (Beischlag *et al.,* 2008). Hsp90 binding is mediated via the bHLH domain and the PAS-B (also ligand-binding) domain of AhR (Fukunaga *et al.,* 1995; Figure 1.6.). Interaction with the chaperone Hsp90 is crucial for the AhR signalling. In a state without a ligand bound, Hsp90 probably masks the nuclear localization signal in the N-terminal part of AhR and thereby ensures retention of the complex in the cytoplasm. Moreover, without the association with chaperones Hsp90, AhR is not able to effectively bind the ligand. Since the Hsp90 dimerization

site overlaps with the ligand-binding domain of AhR, Hsp90 may help to maintain a high affinity ligand binding conformation of the AhR (Furness *et al.,* 2007).

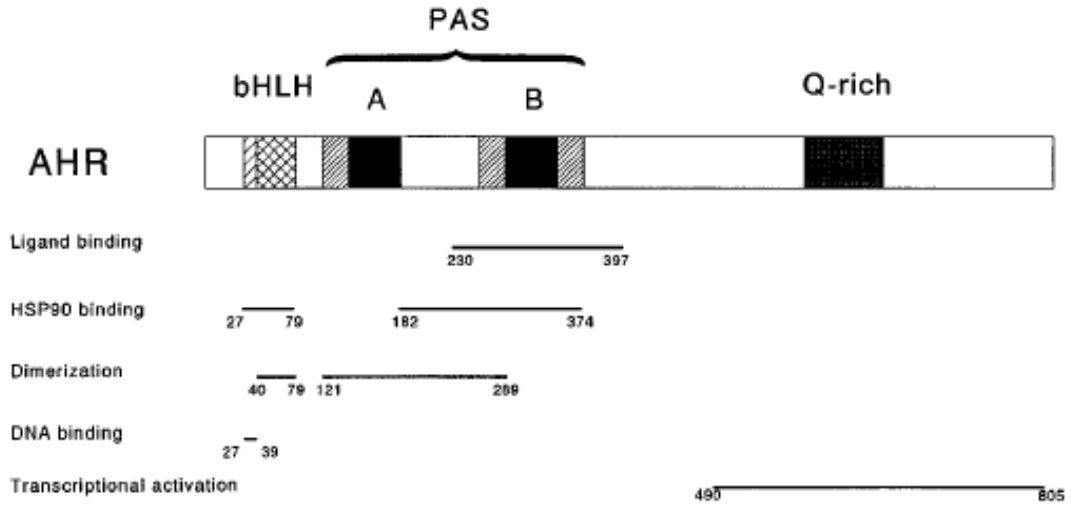

Figure 1.6. Functional domains of mouse AhR. Reprint from (Fukunaga *et al.,* 1995).

Upon ligand binding, the AhR/Hsp90 complex translocates to the nucleus, where Hsp90 is exchanged for Arnt (Figure 1.7.). The active heterodimer with the bound ligand recognizes xenobiotic response elements (XRE; core consensus 5´-T/GCGTG-3´) in the regulatory regions of a xenobiotic response genes, such as cytochrome P4501A1 and glutathione S-transferase. One of the AhR targets is a gene encoding a bHLH protein with homology to the N-terminal region of the AhR. This bHLH PAS factor, lacking the ligand binding and transactivation domains, heterodimerizes with Arnt and forms a transcriptionally inactive complex, recognizing the XRE. Thus, expression of this AhR repressor (AhRR) represents a negative feedback loop to regulate the AhR signalling (Kewley *et al.,* 2004).

The AhR/Arnt complex bound to the DNA recruits transcriptional coactivators to activate transcription of the target genes. These cofactors include histone acetyl transferase CBP/p300, the bHLH PAS coactivators SRC-1, NcoA2 (SRC-2) and p/CIP (SRC-3), components of ATP-dependent chromatin remodeling complexes, such as BGR-1, components of the mediator complex and the RNA elongation factor P-TEFb (Beischlag *et al.,* 2008).

AhR also contains two nuclear export sequences (NES), one is present in the PAS-A domain and the second within the N-terminal bHLH. Nuclear export of AhR that is at least partially dependent on the chromosome region maintenance protein 1 (CRM-1), together with ubiquitin-dependent degradation of AhR, ensures downregulation of the signalling (Furness *et al.,* 2007).

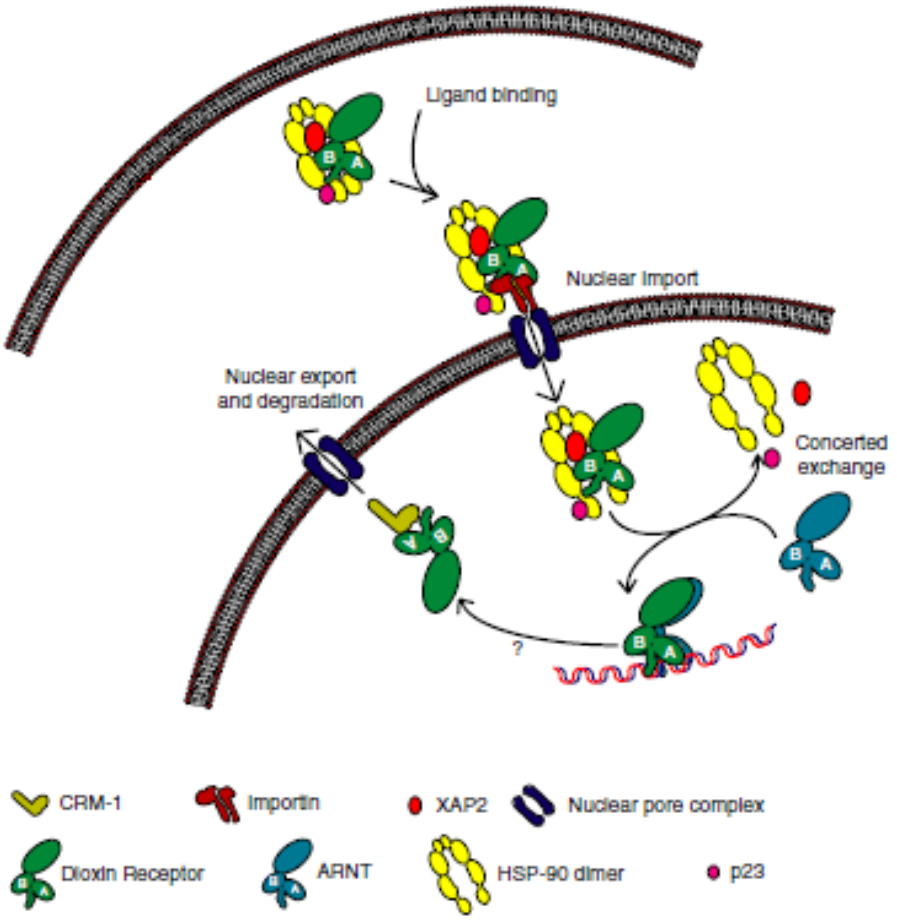

Figure 1.7. The AhR signalling pathway. The inactive AhR is localized in the cytoplasm, associated with molecular chaperones Hsp90, p23 and XAP2. Upon ligand binding, the complex translocates to the nucleus, where AhR dimerizes with Arnt. The active heterodimer recognizes XRE motifs in a gene regulatory regions , recruits coactivators and triggers transcription of the target genes. AhR signalling is downregulated by nuclear export and proteasomal degradation of the AhR. Reprint from (Furness *et al.,* 2007).

Capability of some bHLH PAS family members to sense external stimuli and transduce the signal to the nucleus indicates a potential of these transcription regulators to serve as the intracellular receptors. Based on the conserved domain organization, dimerization patterns, DNA-binding and ligand-binding properties of the bHLH PAS transcription factors, we can postulate hypotheses about structure and molecular functions of the candidate JH receptor, the bHLH PAS protein Met and test these predictions experimentally.

2. Results

Research article:

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Ligand-binding properties of a juvenile hormone receptor, Methoprene-tolerant

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Abstract

Juvenile hormone (JH) is a sesquiterpenoid of vital importance for insect development, yet the molecular basis of JH signaling remains obscure, mainly because a bona fide JH receptor has not been identified. Mounting evidence points to the basic helix–loop–helix (bHLH)/Per-Arnt-Sim (PAS) domain protein Methoprene-tolerant (Met) as the best JH receptor candidate. However, details of how Met transduces the hormonal signal are missing. Here, we demonstrate that Met specifically binds JH III and its biologically active mimics, methoprene and pyriproxyfen, through its C-terminal PAS domain. Substitution of individual amino acids, predicted to form a ligand-binding pocket, with residues possessing bulkier side chains reduces JH III binding likely because of steric hindrance. Although a mutation that abolishes JH III binding does not affect a Met–Met complex that forms in the absence of methoprene, it prevents both the ligand-dependent dissociation of the Met–Met dimer and the ligand-dependent interaction of Met with its partner bHLH-PAS protein Taiman. These results show that Met can sense the JH signal through direct, specific binding, thus establishing a unique class of intracellular hormone receptors.

3. Unpublished results

3.1. TcMet interacts with TcTgo

Transcription factors of the bHLH-PAS protein family form either homodimers or heterodimers with other members of this family. The Aryl hydrocarbon receptor nuclear translocator (Arnt) serves as universal interaction partner of other bHLH-PAS proteins. It binds for example the activated Aryl hydrocarbon receptor (AhR) through the HLH and PAS domains (Reisz-Porszasz *et al.,* 1994), the Hypoxia inducible factor α (HIF-α) (Wang *et al.,* 1995) or the Single minded (Sim) proteins (Ema *et al.,* 1996; Sonnenfeld *et al.,* 1997). It has been also shown that Arnt homodimerizes and the homodimer is able to bind the DNA sequence of adenovirus major late promoter, containing the core E box palindrome CACGTG (Sogawa *et al.,* 1995). Because Arnt is capable of forming functional complexes with multiple bHLH-PAS proteins, we decided to test interaction between the Arnt ortholog from *Tribolium castaneum*, Tango (TcTgo), and TcMet, although heterodimerization of homologous proteins from *Drosophila melanogaster* (DmTgo with DmMet) has not been detected (Godlewski *et al.,* 2006).

We coexpressed a Myc-tagged fragment of TcTgo(93-452), containing the HLH part and both PAS-A and PAS-B domains, together with EGFP-tagged full length TcMet(1-516) in human HEK293 cells. Using immunoprecipitation with *anti*-EGFP antibody and detection with *anti*-Myc antibody, we have found that TcMet and TcTgo dimerize (Figure 3.1.). To test specificity of the interaction, we cotransfected TcTgo(93-452) with EGFP alone, or with an EGFP-tagged unrelated protein (nuclear receptor NHR-25 from *C.elegans*). None of these combinations resulted in co-precipitation of Myc-TcTgo(93-452). Although there is a high degree of homology between *Tribolium* and *Drosophila* proteins Met and Tgo, our finding is in contrast with previously published results (Godlewski *et al.,* 2006). This could be explained by different stringency of the conditions (e.g. salt concentrations) used during co-immunoprecipitation.

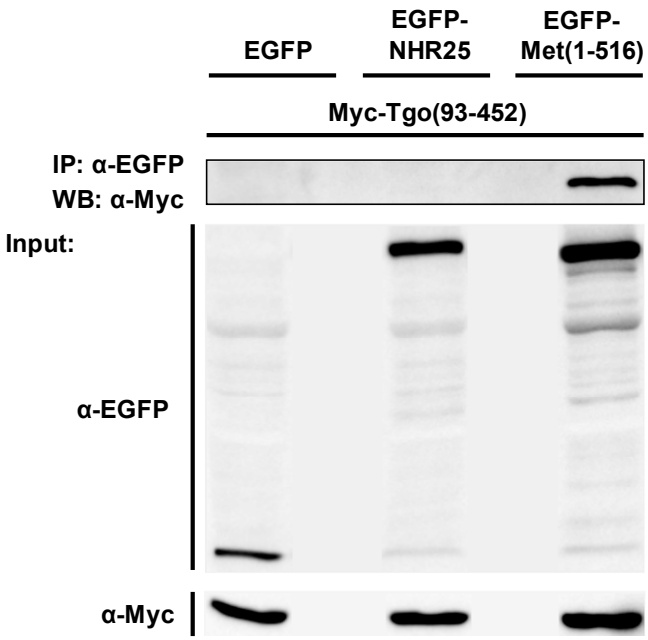

Figure 3.1. Met and Tgo from *Tribolium* form a dimer. Proteins, N-terminally tagged with EGFP or Myc, were coexpressed in human HEK293 cells. 36 hours after transfection, the cells were lysed and the proteins were immunoprecipitated with an *anti*-EGFP antibody. Interaction partner was detected on Western blot using an *anti*-Myc antibody. As a control, EGFP alone or EGFP-tagged unrelated protein (nuclear receptor from *C.elegans*, NHR-25), were coexpressed with Myc-tagged Tgo. Input represents 7% of the lysate used for immunoprecipitation.

3.1.1. Interaction of TcMet and TcTgo is methoprene – independent

If we consider Met to be a part of juvenile hormone sensing complex, it is reasonable to ask whether presence of the hormone affects composition of this complex. Previous experiments have demonstrated that Met from *Drosophila melanogaster* (DmMet) and also Met from *Aedes aegypti* (AaMet) are able to homodimerize. Moreover, DmMet can form dimer with its paralog from *Drosophila*, Gce. In the presence of JH III or its analogs, the complexes dissociate (Godlewski *et al.,* 2006; Li *et al.,* 2011). We have also confirmed dissociation of TcMet homodimer upon addition of methoprene. Presence of ligand (JH III, methoprene) induces heterodimerization of Met from *Aedes* and *Tribolium* with another member of the bHLH-PAS protein family, Taiman (also termed FISC or SRC) (Li *et al.,* 2011;

Zhang *et al.*, 2011; this thesis). Therefore we have examined effect of methoprene on heterodimerization of TcMet and TcTgo.

N-terminally tagged Met and Tgo were cotransfected into HEK293 cells. Before lysis, the cells were treated for 60 min. either with 1 μM methoprene dissolved in ethanol or ethanol alone as a control and the same substances were present also during immunoprecipitation. We have found no significant difference in the strength of interaction upon methoprene addition (Figure 3.2.). Although it may appear that the interaction between Met and Tgo is weaker in the presence of methoprene, this effect of hormone is negligible compared to Met-Met or Met-Taiman dimers.

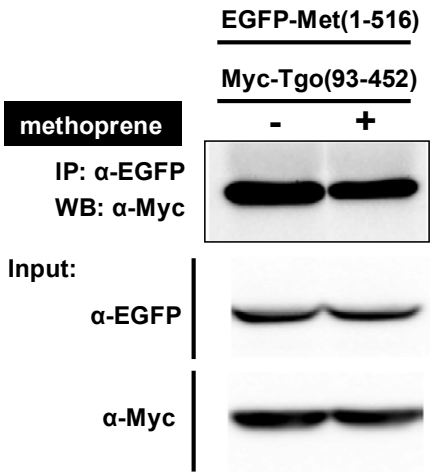

Figure 3.2. Methoprene does not interfere with dimerization of Met and Tgo. EGFP-tagged full length Met(1-516) and Myc-tagged Tgo(93-452) were coexpressed in human HEK293 cells. Before lysis, the cells were treated for 60 min. with 1 μM methoprene and the control cells were treated with ethanol. The same substances were added to the lysates. Proteins were immunoprecipitated from the lysates with an *anti*-EGFP antibody and interaction partner was detected on Western blot using an *anti*-Myc antibody. Input represents 7% of the lysate used for immunoprecipitation.

3.1.2. N- and C- terminal parts of TcTgo mediate interaction with TcMet

To identify the regions of TcTgo responsible for dimerization with TcMet, a series of truncated TcTgo versions have been prepared. Based on the homology with human Arnt and DmTgo (Sonnenfeld *et al.,* 1997), we have designed three constructs: Tgo(78-262), comprising bHLH and PAS-A domains; Tgo(132-452), conmprising PAS-A and PAS-B domains; Tgo(222-642), harboring PAS-B domain and C-terminal part of the protein (Figure 3.3.). The above described Myc-tagged constructs were cotransfected together with full-length EGFP-Met(1-516) into

HEK293 cells and subjected to immunoprecipitation using an *anti*-EGFP antibody. Polypeptides Tgo(78-262) and Tgo(222-642) co-precipitated with Met, indicating that dimerization occurs through the bHLH domain and the C-terminal segment of Tgo. The central part of Tgo, including both PAS domains, failed to interact with Met (Figure 3.4.).

Dimerization of bHLH-PAS proteins is usually mediated via both the HLH domains and PAS domains (Pongratz *et al.,* 1998; Card *et al.,* 2005). In agreement with the published results, we have found that the bHLH domain of Tgo represents the interaction interface also for dimerization with Met. Despite the fact that PAS domains play a role in dimerization of bHLH-PAS proteins, the polypeptide Tgo(132-452) comprising PAS-A and PAS-B domains, do not interact with Met. This result corresponds to previously observed inability of mouse Arnt fragment, consisting of PAS-A and PAS-B domains (amino acids 144-474), to dimerize with full length mouse AhR, with or without presence of the AhR ligand, TCDD (Reisz-Porszasz *et al.,* 1994). Interestingly, C-terminal part of Tgo, which has been described as transactivation domain in mammalian Arnt (Yamaguchi and Kuo, 1995), significantly contributes to interaction with Met.

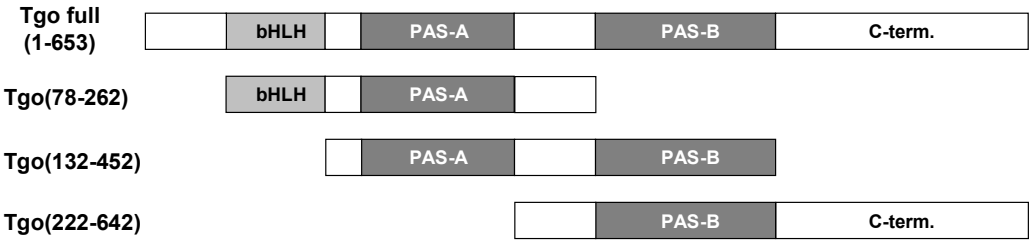

Figure 3.3. Truncated versions of TcTgo.

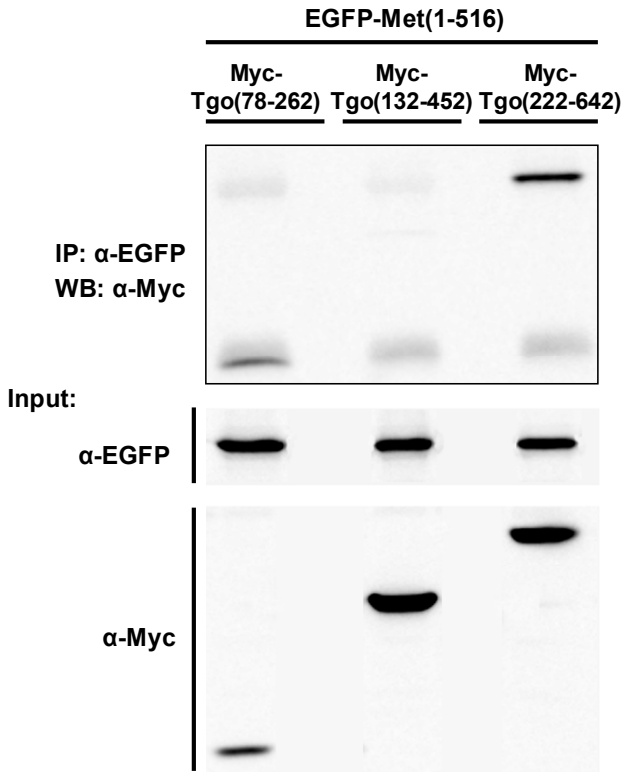

Figure 3.4. N- and C- terminal parts of Tgo mediate interaction with Met. EGFP-tagged full length Met(1-516) and Myc-tagged truncated versions of Tgo were coexpressed in human HEK293 cells. Proteins were immunoprecipitated from the lysates with an *anti*-EGFP antibody and interaction partners were detected on Western blot using an *anti*-Myc antibody. Input represents 7% of the initial material.

3.1.3. TcMet homodimer and heterodimer with TcTgo activate transcription in a luciferase reporter system

The bHLH-PAS proteins act as homo- or heterodimeric transcription regulators, binding DNA elements derived from the E-box enhancer sequence, containing the core consensus CANNTG (Kewley *et al.*, 2004). Because the DNA sequence recognized by Met is not known, we have employed a ligand sensor approach, which has been used to detect ligands of nuclear receptors (Palanker *et al.*, 2006). This method is based on fusion of ligand-binding domain of a putative receptor with the DNA-binding domain of the Gal4 (Gal4 DBD), which recognizes the UAS sequence and activates transcription of a reporter gene.

We have fused Gal4 DBD with TcMet and TcTgo and the constructs were cotransfected into *Drosophila* S2 cells and human HEK293 cells, together with the luciferase reporter vectors containing multilpe Gal4 binding sites, UAS-TATA-luc and UAS-TK-luc, respectively. Although this approach was successfully used for characterization of transactivation properties of DmMet (Miura *et al.,* 2005), in our hands neither Gal4 DBD-TcMet alone, nor its combination with Gal4 DBD-TcTgo activated transcription of the reporter gene, regardless of presence or absence of methoprene (data not shown).

Spacing and orientation of the DNA motifs recognized by dimeric transcription factors, such as nuclear receptors, is crucial for proper binding of the protein complex to the DNA. Therefore, UAS elements used in UAS-TATA-luc and UAS-TK-luc reporters, may not provide optimal binding sites for a dimer of bHLH-PAS proteins fused to the Gal4 DBD. On the other hand, Gal4 DBD dimerizes (Keegan *et al.,* 1986), which could affect the correct orientation of bHLH-PAS proteins in the complex and interfere with binding of putative cofactors. To overcome these potential problems, we have modified the experimental settings by using domain swapping approach. *Drosophila* homolog of Arnt, Tango (DmTgo) and homolog of AhR, Spineless (DmSs), form a heterodimer and activate transcription from Xenobiotic response element (XRE) – bearing reporter gene in *Drosophila* SL2 cells (Emmons *et al.,* 1999). We have swapped the N-terminal basic DNA-binding domains in TcMet, TcTgo and TcSs for the basic domains from DmTgo and DmSs, to obtain a chimeric bHLH-PAS proteins that are able to bind the XRE sequence (Figure 3.5. A). Transactivation properties of the chimeric transcription factors were examined using luciferase reporter vector containing 5x XRE element in transiently transfected HEK293 cells. Cotransfection of chimeras DmTgo x TcMet and DmSs x TcMet, as well as DmTgo x TcTgo and DmSs x TcMet activated transcription from the XRE-*luc* reporter, indicating that either Met-Met homodimer or Tgo-Met heterodimer can act as transcription activators (Figure 3.5. B). None of the chimeric proteins alone was able to transactivate. Cotransfections of DmTgo and DmSs or DmTgo x TcTgo and DmSs x TcSs were used as positive controls.

Addition of JH III or its analogs, methoprene or pyriproxyfen, has no effect on transactivation of the XRE-luc reporter by Met-Met homodimer or Tgo-Met heterodimer, either in HEK293 cells or S2 cells (data not shown).

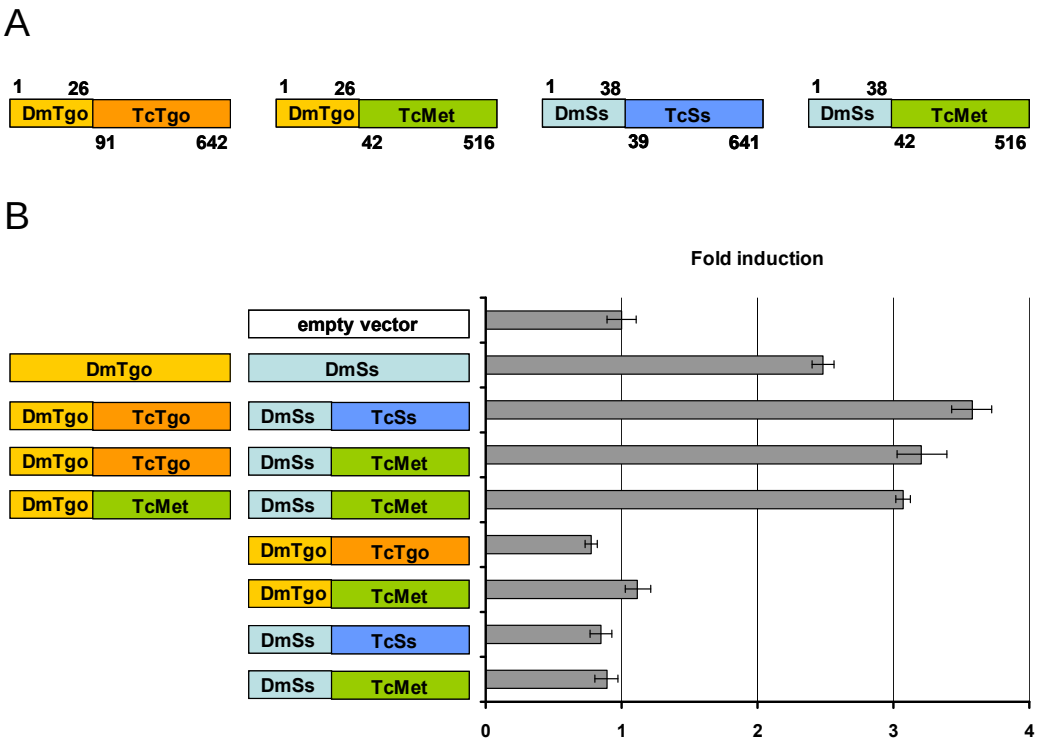

Figure 3.5. A. Chimeric bHLH-PAS proteins with the basic domain from *Drosophila* and the rest of the protein from *Tribolium*. Numbers indicate amino acid positions in *Drosophila* (upper) and *Tribolium* (lower) proteins. **B.** Cell culture experiments show that Met homodimer and Tgo-Met heterodimer are transcription activators. Human HEK293 cells were transiently transfected with the reporter plasmid, XRE-*luc*, which contains 5 copies of the cytochrome P450IA1 XRE element that drives firefly *luc* from pGL3, together with vectors expressing indicated bHLH-PAS proteins and empty vector as a negative control. The pRL0 plasmid that has a *renilla luciferase* gene was used to normalize transfection efficiency. Normalized luciferase units are expressed as fold induction relative to activity of the reporter, cotransfected with empty vector. Values are mean ± SEM of 3 independent transfections.

3.2. Mapping of the interaction between TcMet and TcTai

3.2.1. JH III induces dimerization of TcMet and TcTai

In our recently published study, we have shown that TcMet interacts with TcTaiman (TcTai) in a ligand-dependent manner. Dimerization of TcMet and TcTai occurs only in presence of the ligand and the mutants of TcMet, unable to bind JH III or JH analogs, fails to interact with Taiman. In these experiments, we have uniformly used methoprene in 1 μM concentration.

Therefore we have tested, whether TcMet and TcTai heterodimerize also in presence of JH III and how effective this treatment is, comparing to methoprene. EFGP-Taiman and full length, Myc-tagged Met(1-516) were coexpressed in human HEK293 cells and co-immunoprecipitated in the presence of 1 μM JH III, 1 μM and 1 mM methoprene (Figure 3.6.). Imunoprecipitation in absence of the ligand and coexpression of Taiman with Met V297F, incapable of binding juvenile hormone, were used as negative controls. The results have shown that strength of the interaction is dose-dependent on the ligand and that JH III is slightly more effective than methoprene, which corresponds to higher affinity of TcMet to JH III.

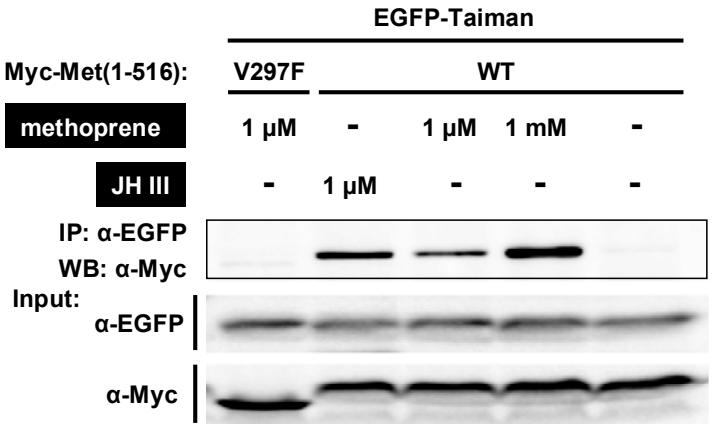

Figure 3.6. TcMet interacts with TcTai in presence of JH III and the complex formation is dose-dependent on ligand. EGFP-tagged Taiman and Myc-tagged either wild-type full length Met(1-516), or Met(1-516) V297F, which is incapable to bind JH, were coexpressed in human HEK293 cells. Before lysis, the cells were treated for 60 min. with 1 µM JH III, 1 µM or 1mM methoprene and the control cells were treated with ethanol. The same substances were added to the lysates. Proteins were immunoprecipitated from the lysates with an *anti*-EGFP antibody and interaction partners were detected on Western blot using an *anti*-Myc antibody. Input represents 7% of the initial material.

3.2.2. JH-dependent dimerization of TcMet and TcTai is mediated through the PAS-B domains

Our previous results have shown that fragment of TcMet(240-516), consisting of the PAS-B domain and C-terminus of the protein, is responsible for JH-dependent interaction with Taiman (Charles *et al.,* 2011). Based on structural data showing heterodimerization of PAS-B domains from mammalian proteins HIF-2α and Arnt (Erbel *et al.,* 2003; Scheuermann *et al.,* 2009), we propose a hypothesis where binding of JH into the ligand-binding pocket of Met induces conformational changes within the PAS-B domain, which allows interaction with the PAS-B domain of a heterodimeric partner. However, it has been recently shown that DmMet and DmGce interact with the nuclear receptor FTZ-F1 in a JH-dependent manner and this interaction requires a conserved motif LIXXL, located C-terminally from the PAS-B domain of DmMet or DmGce (Bernardo and Dubrovsky, 2012).

Therefore, we have tested whether the PAS-B domains of TcMet and TcTai are sufficient for mediating ligand-depentent dimerization. Truncated versions of TcMet, lacking the C-terminal part including LIXXL motif were coexpressed with Taiman in HEK293 cells and subjected to immunoprecipitation. Myc-Met(1-386) have co-immunoprecipitated with EGFP-Taiman (Figure 3.7. A) and also Myc-Taiman have dimerized with EGFP-Met(240-386) (Figure 3.7. B), both after treatment with 1 μM JH III. As a controls, we used coexpression of full length Met(1-516) or Met(105-516) with Taiman in absence or presence of JH III. Finally, we cotransfected constructs EGFP-Met(240-386) and Myc-Tai(394-510), representing the PAS-B domains of the proteins. Although the PAS-B domains without C-termini of the proteins are weakly expressed in HEK293 cells, Myc-Tai(394-510) have co-precipitated with EGFP-Met(240-386) in presence of 1 μM JH III (Figure 3.7. C). Together our results indicate that the PAS-B domain of Met is required and sufficient for heterodimerization with Taiman.

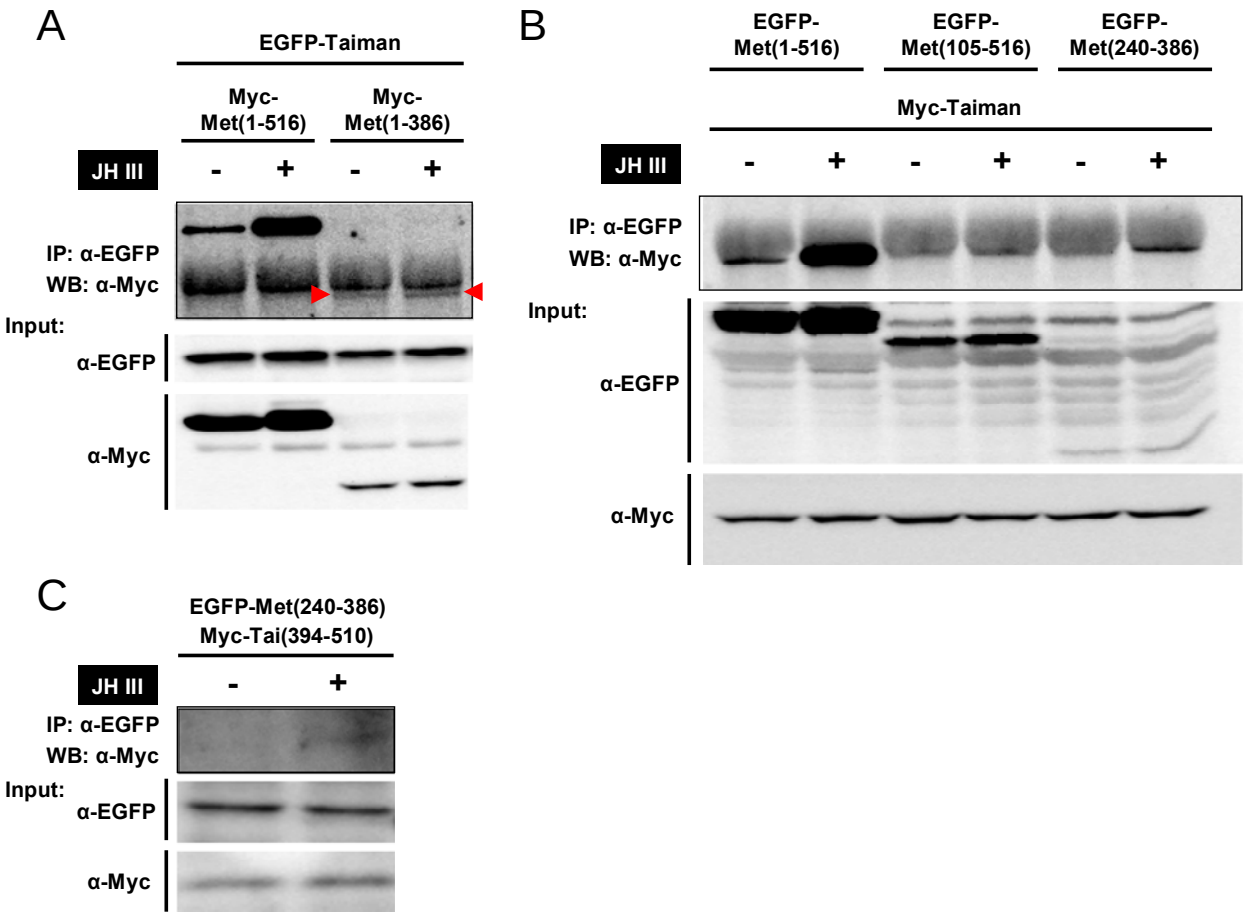

Figure 3.7. PAS-B domain of Met is sufficient for heterodimerization with Taiman. EGFP- and Myc-tagged proteins were coexpressed in human HEK293 cells. Before lysis, the cells were treated for 60 min. with 1 μM JH III and the control cells were treated with ethanol. The same substances were added to the lysates. Proteins were immunoprecipitated from the lysates with an *anti*-EGFP antibody and interacting partners were detected on Western blot using an *anti*-Myc antibody. Input represents 7% of the initial material. **A.** Myc-Met(1-386), containing the bHLH and PAS domains without C-terminal part of the protein interacts with EGFP-tagged Taiman, upon treatment with 1 μM JH III (arrowheads). **B.** Myc-Taiman coprecipitates with EGFP-tagged PAS-B domain of Met (residues 240-386) in JH-dependent manner. **C.** The PAS-B domains of Taiman (Myc-Tai(394-510)) and Met (EGFP-Met(240-386)) heterodimerize in presence of JH III.

3.2.3. Effects of point mutations within the PAS-B domain of TcMet on ligand-dependent dimerization with TcTai

Recently, we have characterized a series of amino acid substitutions within the PAS-B domain of TcMet in terms of JH binding and dimerization properties. Substitution of conserved valine 346 for phenylalanine (V346F) did not abolish either JH binding or interaction of Met with Taiman (Charles *et al.,* 2011). Based on the prediction of structure of TcMet PAS-B domain, the side chain of the amino acid residue V346 points towards putative interaction interface. To test the importance of V346 for dimerization, we substituted this residue with arginine (V346R). Whereas substitution of valine for bulkier phenylalanine does not change physicochemical properties of the side chain, arginine changes hydrophobic nature of the site by bringing a positive charge.

However, immunoprecipitation in transfected HEK293 cells has revealed that mutations of the conserved valine 346 in TcMet do not affect JH-dependent interaction with Taiman (Figure 3.8.). EGFP-Met(240-516) V346R and V346F, as well as wild type Met(240-516) dimerize with Myc-Taiman upon JH III treatment. Met(240-516) V297F, lacking the hormone binding capability, failed to heterodimerize.

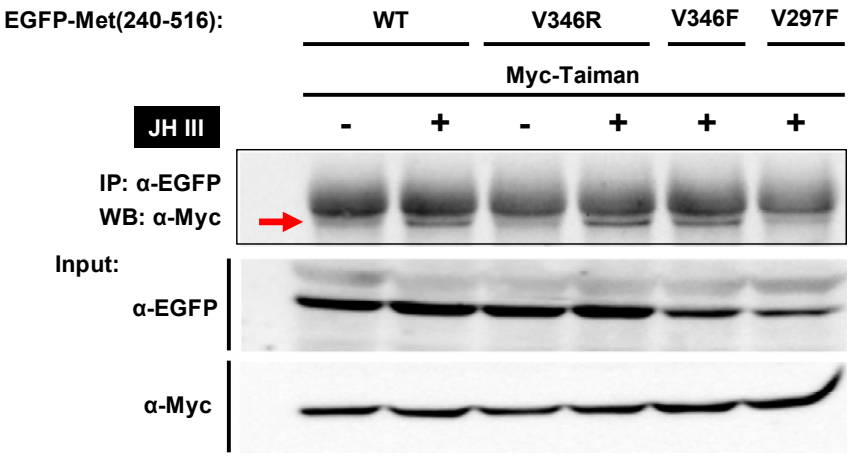

Figure 3.8. Mutations of the amino acid 346 from putative interaction interface of TcMet PAS-B domain do not affect heterodimerization with Taiman. Myc-tagged Taiman and EGFP-tagged wild-type Met(240-516), Met(240-516) V346R, V346F and V297F, which is incapable to bind JH, were coexpressed in human HEK293 cells. Before lysis, the cells were treated for 60 min. with 1 μM JH III and the control cells were treated with ethanol. The same compounds were added also to the lysates. Proteins were immunoprecipitated from the lysates with an *anti*-EGFP antibody and interaction partners were detected on Western blot using an *anti*-Myc antibody (the bands indicated by arrow). Input represents 7% of the initial material.

4. Conclusions

1. ***Met uses the ligand-binding pocket within its PAS-B domain to sense the JH signal.***

   Similarly to mammalian Aryl hydrocarbon receptor (AhR), the PAS-B domain of Met is necessary and sufficient for the ligand binding. Substitutions of several amino acid residues whose side chains point toward the ligand for the amino acids with bulkier side chains, prevent JH binding by steric hindrance.

2. ***Binding of JH to the PAS-B domain likely results in a conformational change that disrupts the Met-Met complex and enables Met to form an active transcription factor with Taiman.***

   JH-dependent events, such as dissociation of Met homodimer and heterodimerization of Met with Taiman are prevented by mutations in the PAS-B domain of Met, disrupting the JH binding.

3. ***In the Met-Tai complex, Met is the ligand-specific sensor.***

   Met is responsible for the interaction with JH, Taiman does not contribute to the ligand binding. Thus, Met serves as the ligand-sensing unit in a fashion similar to that of nuclear receptors EcR or RAR that form dimers with Usp/RXR.

4. ***JH-dependent association of Met and Tai is mediated through the PAS-B domains.***

   The PAS-B domains of Met and Tai are necessary and sufficient for the JH-induced dimerization.

5. ***Met might play a JH-independent role in transcriptional regulation.***

   Similar to other members of the bHLH PAS protein family, Met interacts with the homolog of the universal dimerization partner Arnt, Tango. In the luciferase reporter assays, the Met-Tgo dimer activates transcription. Neither dimerization, nor transcriptional activation is affected by JH.

6. ***Met constitutes a novel type of intracellular hormone receptors – different from the nuclear receptor family.***

   Besides HIF-α and AhR, Met is another member of the bHLH PAS protein family that can sense external stimuli and transmit the signal to the nucleus to achieve activation of target genes. However, Met is the only known hormone receptor from this family of transcriptional regulators.

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