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Institut für Populationsgenetik

Vienna, 12 September 2012

TO THE SCHOOL OF DOCTORAL STUDIES IN BIOLOGICAL SCIENCES
UNIVERSITY OF SOUTH BOHEMIA IN CESKE BUDEJOVICE
FACULTY OF SCIENCE

Report by Dr. Thomas Flatt (Rapporteur) on the Doctoral Thesis of Jan Rynes

Ph.D. Thesis, by Jan Rynes , entitled "Role of the bZIP protein Atf3 in metabolic and immune homestasis and Molecular interactions of the insect juvenile hormone receptor Met", supervised by Prof. RNDr. Marek Jindra, CSc, Biology Centre of the Academy of Sciences of the Czech Republic, Institute of Entomology, Ceske Budejovice, 2012.

To whom it may concern:

I herewith provide a formal and structured evaluation of the Ph.D. thesis by Jan Rynes, entitled "Role of the bZIP protein Atf3 in metabolic and immune homestasis and Molecular interactions of the insect juvenile hormone receptor Met" (Thesis Advisor: Prof. Dr. Marek Jindra).

Overall structure of the thesis
The thesis consists of two main parts, which are already mentioned in the title: the first part deals with the role of the bZIP protein Atf3 in regulating metabolic and immune homeostasis, whereas the second part deals with the molecular interactions of the insect juvenile hormone receptor encoded by the methoprene tolerant locus, Met. Each of these two main parts consists of a peer-reviewed published paper and is framed by a statement of the research objectives, a general introduction, and a final section with conclusions. The structure of the thesis is extremely clear and logical; the research objectives at the beginning of each thesis main part are outlined very clearly, with each of the two parts addressing an important unresolved question in insect physiology. Similarly, the introductory parts as well as the conclusions, which frame the published research papers, are succinct and very well written. Of course, one could have tried, as an alternative, to write an overall introduction, which would have attempted to tie the two thesis parts together, however, I think this would have been difficult and somewhat artificial. Overall, therefore, I find the structure of the thesis excellent. I also especially appreciated the clear statement, at the beginning of the thesis, of what the candidate has directly contributed to each of the two publications.

Peer-reviewed, published research papers
The most important parts of the thesis are the two peer-reviewed, published research articles. The first paper, entitled "Activating Transcription Factor 3 regulates Immune and Metabolic Homeostasis", with the candidate as the first lead author, has been published in *Molecular and Cellular Biology*, which is a very respectable international journal. This very nice paper is the first to investigate in detail the functional role of Activating Transcription Factor 3 (Atf3) in *Drosophila*. Mammalian ATF3 is known to be a negative regulator of innate immunity, and its

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paralog JDP2 plays a role in lipid metabolism and obesity; however, not much is known about the detailed physiological functions of these genes/proteins. In a beautiful and very well-performed set of experiments in *Drosophila* the candidate and his collaborators now identify *Drosophila* Atf3 as the single ortholog of ATF3 and find that it integrates the functions of both mammalian ATF3 and JDP2. The results of these experiments in the fly are all highly novel, interesting and important, especially as they establish a major novel regulator of immune and lipid/energy homeostasis in the fly. This is particularly interesting in the light of the common but not yet well understood physiological links between fat metabolism and immune function. The candidate and has collaborator have used an impressive array of genetic, molecular, cellular, and physiological tools to investigate the function of Atf3 in the fly. As neatly laid out in the paper as well as in the conclusions section of part 1 of this Ph.D. thesis, these experiments have lead to 7 very clear-cut and important results: (1) Atf3 has an essential function in the growth of *Drosophila* larvae, with loss of atf3 function severely impairing larval viability; (2) atf3 mutant larvae suffer from chronic inflammation and stress, as evidenced by transcriptional changes; (3) atf3-deficient larvae exhibit an altered intestinal microflora; (4) atf3 mutants are obese yet at the same time seem to be experiencing starvation; (5) human ATF3 normalizes the fat content and gene expression in atf3 mutants, thus clearly demonstrating the functional role of Atf3 in regulating these processes; (6) when immunity is genetically attenuated by reducing the gene dose of Relish or FOXO in Atf3 mutants lipid metabolism as well as survival are improved, thus clearly establishing an important link between immunity and lipid metabolism; in conclusion, therefore, (7), it seems clear that *Drosophila* Atf3 is a novel, major regulator of both immune and metabolic homeostasis and that it integrates the distinct functions of the mammalian orthologs, ATF3 and JDP2. To my mind, these are highly interesting results that will be of major importance for the field. In addition to these beautiful results, I am impressed by the broad range of methods used in this paper for studying the functional role of Atf3 in the fly. Just to give one specific example, the rescue of fly Atf3 function by the human wildtype ATF3 protein represents a clever and very elegant rescue experiment that clearly proves the functional involvement of Aft3 in the processes discussed above. Overall, I find this to be an excellent research paper, and I am sure that it will be widely read and cited by people working in the field.

The second paper, which represents the main core of the second part of this thesis, is entitled "Ligand-binding properties of a juvenile hormone receptor, Methoprene-tolerant", published in the prestigious high-impact journal *Proceedings of the National Academy of Sciences USA* (*PNAS*), with the candidate as one of the co-authors. This paper is an important landmark contribution in our understanding of juvenile hormone (JH) function, a hormone whose receptor and detailed molecular function have remained mysterious for many decades. The Jindra group has recently made major discoveries that suggest that the protein encoded by the methoprene-tolerant locus, Met, migh actually really be the long-sought-after bona fide JH receptor. The current paper is therefore to be seen as part of a number of seminal contributions towards resolving the molecular details of JH signaling. Using an impressive array of biochemical, genetic and in-vivo experiments (e.g., ligand binding assays, protein structure modeling, protein interaction studies, RNAi and hormonal treatments in vivo, etc.) the candidate, in a collaboration with his colleagues, establishes at least 6 highly novel, major results: (1) Met ues the ligand binding pocket within its PAS-B domain to sense JH; (2) binding

of JH to this domain seems to cause a conformational change, disrupting the Met-Met homodimer and establishing the association of Met with the transcription factor taiman; (3) in this Met-Tai complex the Met part is the JH-ligand-specific sensor; (4) the Met-Tai association is mediated by the PAS-B domains of both Met and Taiman; (5) Met is also known to interact with Tango, however, although the Met-Tgo dimer activates transcription, JH does not affect Met-Tgo dimerization and transcriptional activity of this complex. In summary, these results provide extremely important additional evidence for the notion that the MET protein encodes a (or perhaps THE) functional JH receptor; together with previous papers they make MET the best candidate for the JH receptor. Importantly, the data clearly demonstrate that MET binds JHIII as well as synthetic analogs of JH, methoprene and pyriproxyfen, through its C-terminal PAS domain; this specific binding of JH to MET is functionally required for the ligand-dependent interaction between MET and its partner Taiman. In my opinion, this is beautiful and very important paper that will have a major impact on the field of insect endocrinology. It is clear (see below) that the candidate has made crucial contributions to this important paper.

Candidate's contribution to the two research papers

In the context of the first research paper the candidate performed colorimetric measurements of saccharides and triacylglycerols in *Drosophila* larvae, cultivated and identified the bacterial microflora, and performed respirometry, electron microscopy imaging as well luciferase reporter assays. The candidate also collaborated with others to analyze gene expression (involving RNA isolation, cDNA synthesis and qRT-PCR), to perform survival, rescue and starvation experiments using mutant *Drosophila* and participated in the analysis of the data. It is clear from these contributions that the candidate played the leading role in this project, a fact which is fairly reflected in his first-authorship on this paper. In general, I am impressed by the breath of molecular, cellular and genetics tools and methods the candidate has employed to answer the question at hand. Thus, in my opinion, the candidate's contribution to this research project is excellent. For the second research paper, the candidate has, as part of a collaborative team, made active and important contributions to the research by preparing plasmid constructs, expressing recombinant proteins in mammalian cell cultures and by testing JH-dependent protein-protein interactions using immunoprecipitation from transfected HEK293 cells. It is clear from my reading of this paper that the candidate's contributions to this project have been essential for the successful completion of this research and for the final publication in a high-profile, high-impact journal. Again, it is obvious from this paper that the candidate is an excellent experimentalist with a highly versatile experimental toolkit. Overall, I find the contributions of the candidate to both research papers excellent -- both projects clearly involved many non-trivial, rather complex and demanding laboratory experiments, both in vivo and in vitro, and with his excellent contributions the candidate has clearly established his ability to successfully apply a wide array of methods to solve such difficult experimental problems.

In conclusion, I find this Ph.D. thesis excellent: it is well written and presented; the methods are sound and experimental designs robust; the results novel, interesting, and innovative; and the thesis has resulted in two published papers in very good journals. I am very impressed by the high amount and quality of the scientific work produced by Jan Rynes in his dissertation work. I am therefore very happy to declare without hesitation that in my opinion the present

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Ph.D. thesis by Jan Rynes fulfills all the academic requirements for a successful Ph.D. thesis defense at your institution and that the candidate should be granted the Ph.D. degree.

Vienna, 12 September 2012

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OPONENTSKÝ POSUDEK NA DISERTAČNÍ PRÁCI

Autorka práce: Mgr. Jan Ryneš

Název práce: Role of the bZIP protein Atf3 in metabolic and immune homeostasis and Molecular interactions of the insect juvenile hormone receptor Met

Oponent: doc. RNDr. Jan Vondráček, Ph.D.

Předložená disertační práce představuje shrnutí dvou experimentálních studií, zabývajících se jednak úlohou transkripčního faktoru Atf3 v regulaci metabolismu a imunity, a molekulárními interakcemi PAS proteinu Met, jako kandidátního receptoru juvenilního hormonu. Obě tyto práce (v prvním případě se jedná o prvoautorskou publikaci) byly již publikovány ve vysoce kvalitních impaktovaných časopisech, s velmi náročným recenzním řízením, což dokládá, že se jedná o práce na velmi dobré vědecké úrovni, které představují kvalitní základ pro disertační práci. Práce je vhodně členěna na shrnutí teoretických předpokladů a současného stavu poznání, ze kterých autor vycházel, prezentaci vlastních výsledků ve formě publikovaných článků (včetně použité metodiky) a také dosud nepublikované výsledky, které jsou komentovány v závěrečné části obou experimentálních oddílů. Úloha autora a jeho podíl na jednotlivých studiích jsou jasně vymezeny, i když je pořadí z neznámých důvodů přehozené. Po formální stránce je předložená disertační práce velmi dobře zpracována, jen s minimem drobných chyb a nepřesností.

Konkrétní připomínky:

1) Část I.

Teoretická část stručně a přehledně shrnuje základní informace nezbytné pro interpretaci výsledků práce. Vlastní výsledky i experimentální přístupy autora jsou součástí prezentovaného článku. Jediná výtka, podobně jako v části II., se týká toho, že by bylo vhodné alespoň částečně komentovat data, která jsou součástí článku jako Supplementary Data. V rámci disertační práce by byl jistě prostor věnovat jim více pozornosti, než v rámci článku, kde je rozsah nutně omezený.

2) Část II.

Druhá část shrnuje výsledky studie, ke které autor významně přispěl především konstrukcí plazmidů, analýzou rekombinantních proteinů a proteinových interakcí proteinů ektopicky exprimovaných v buňkách HEK293.

3) Diskuse

V rámci výsledků a diskuse jsou prezentovány výsledky, které již prošly oponentním řízením v průběhu publikačního procesu. Proto nebudu podrobně rozebírat výsledky, ani komentáře k nim. Nicméně, vzhledem k tomu, že se jedná o velmi zajímavé poznatky, rád bych autorovi položil několik otázek, které vesměs vyplývají z prezentovaných výsledků:

1) Je známo, že savčí Atf3 je transkripční faktor, jehož aktivita je v řadě případů aktivována environmentálním stresem, ať již se jedná o genotoxiny, hypoxii, podvýživu apod. Podobně i z prezentovaných výsledků vyplývá, že např. hladovění zvyšuje hladinu atf3 mRNA. Přestože studie č. 1 byla primárně zaměřená na úlohu *atf3* v regulaci imunity a metabolismu, a ne na mechanismy regulující jeho transkripční aktivitu, dá se z výsledků usuzovat, že podobně jako u savců může být aktivován i různými toxiny a zprostředkovávat jejich dopad na imunitní systém hmyzu, funkce tukového tělesa apod.?

2) V rámci studie II jsou prezentována data dokladující, že Taiman funguje jako transkripční partner Met a aktivita Met-Tai dimeru je regulována přítomností ligandů Met. Zároveň bylo v poslední době publikováno i několik prací naznačujících, že Taiman je kofaktorem ekdysonového receptoru. V případě savčích PAS proteinů AhR a ARNT bylo popsáno, že mohou samostatně či společně vytvářet komplexy se steroidními receptory a různým způsobem modulovat transkripci genů primárně regulovaných např. estrogenním receptorem alfa. Dá se předpokládat, že komplex Met-Tai by mohl analogicky regulovat transkripci cílových genů EcR, nebo, že by mohlo docházet k přímé interakci mezi Met a EcR?

3) V poslední době se ukazuje, že jiný PAS protein, savčí Ah receptor, by se, vedle zprostředkování aktivity celé řady endogenních a exogenních ligandů, mohl podílet i na regulaci genů *CLOCK* a *PERIOD1*. Dá se předpokládat, že funkce Met receptoru je regulována cirkadiánními rytmy, nebo existuje možnost, že tento protein může přímo interagovat s dalšími členy PAS rodiny, které se podílejí na regulaci cirkadiánních rytmů?

Závěrem konstatuji, že předložená disertační práce představuje, po obsahové i formální stránce, vysoce kvalitní výstup postgraduálního studia. Autor prokázal své tvůrčí schopnosti a předložená práce zcela splňuje požadavky v daném oboru. Proto doporučuji, aby byla práce přijata k obhajobě a dalšímu řízení. Rovněž doporučuji, aby byl Mgr. Janu Rynešovi po úspěšné obhajobě práce přiznán vědecký titul Ph.D. dle § 47 Zákona o vysokých školách č. 111/98 Sb.

V Brně dne 11. 9. 2012

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