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Subject: Evaluation of Ph.D. thesis by Petra Sekyrová

"Functional analysis of the bZIP transcription factor dATF3 in *Drosophila* development"

It is my pleasure to write this letter of evaluation for the Ph.D. thesis of Petra Sekyrová, which describes the role of dATF3 (activating transcription factor 3), the single ortholog of human ATF3 and JDP2 bZIP proteins, during development of the fruit fly *Drosophila melanogaster*. Thus far ATF3 has been extensively studied in mammalian systems. Numerous studies in cultured cell lines have implicated its function in multiple cellular behaviors including cell proliferation, apoptosis, cell motility, or stress response. Even though animal models like *Atf3-/-* knock-out mice and rats or mice overexpressing human ATF3 have been developed, results were often contradictory and seem not to give coherent answer with regard to ATF3 function. Strikingly, *Drosophila* dATF3 has not been functionally characterized so far. *Drosophila* provides an excellent system and offers many genetic and molecular tools to study protein functions and interactions in the context of an intact organism and thus improves our general understanding of ATF3 function.

In her thesis Petra Sekyrová implicates dATF3 in *Drosophila* epithelial morphogenesis. Epithelial sheet spreading and fusion underlie important developmental processes. Well-characterized examples of such epithelial morphogenetic events have been provided by studies in *Drosophila* and the nematode *Caenorhabditis elegans*. During *Drosophila* development dramatic epithelial remodeling occurs, including embryonic dorsal closure, formation of the adult thorax, and wound healing. Surprisingly, much less is known about morphogenesis of the fly abdomen. Adult epithelia in *Drosophila* originate from imaginal cells, either imaginal discs or histoblasts, all of which become specified already during embryogenesis of the fly. At the onset of metamorphosis arrested diploid histoblasts rapidly proliferate and replace polyploid larval cells that had formed organs so far. In *Drosophila*, hormonal signaling controls the overall synchronicity of developmental events in distinct organs. Furthermore Jun N-terminal kinase signaling has been shown to play a role in most cases of epithelial remodeling.

Petra Sekyrová employes a molecular and genetic approach in combination with confocal laser microscopy to reveal cooperation of dATF3 with its predicted partner, the bZIP protein dJun and the first evidence for a developmental role of dATF3 *in vivo* is presented. My general impression of this thesis is that Petra has done an

outstanding job. All experiments, including controls are well documented and follow a logical order that can be easily reproduced. DIC, fluorescent and electron micrographs are of excellent quality and nicely grouped into collages with well written figure legends. Petra seems to be a very skilled experimentator, not running out of patience and working through a large numbers of experiments to finally make observations significant. The introduction and discussion demonstrate that Petra knows the literature very well and it must be a real challenge to discuss with her. Keeping in mind that English is not her native language, the style is really splendid. I would wish more of my students were blessed with these skills when released from the lab. Petra absolutely deserves her Ph.D. and according to the standard at our institution she would rank high on the exam.

COMMENTS AND QUESTIONS

The first part of Petra Sekyrová's Ph.D. thesis has been submitted as an research article, which now has been accepted for publication in "Development" a high profile journal in the field of "Developmental Biology". From the list of authors, I would assume that Petra was responsible for most, – if not all – experimental work.

The *Drosophila* ortholog of ATF3/JDP2 is an essential gene whose transcription is dynamically regulated during metamorphosis. To demonstrate direct binding of dATF3 with dJun Petra conducted co-immunoprecipitation experiments. In addition she performed a DNA mobility-shift assay with recombinant bZIP domains of dATF3, dJun and dFos to test for their DNA-binding properties. To test whether dATF3 and dJun interact *in vivo*, she conducted experiments in the *Drosophila* compound eye, whose precise structure of ommatidia sensitively reflects genetic interactions. In this system, the *datf3* misexpression phenotype could be completely suppressed by simultaneous RNAi against *djun* but not of *dfos*. In addition, RNAi-mediated depletion of dJun in animals that misexpressed *datf3* restored viability of adults (see also below). **Taken together**, these results clearly show that dATF3 cooperates with dJun. Given the capacity of both dATF3 and dFos to bind dJun, and based on the ability of dJun and dFos to enhance and suppress the dATF3 gain-of-function phenotype, respectively, it is suggested that dATF3 and dFos compete for their common partner dJun *in vivo*.

Overexpression of *datf3* in larval epithelial cells (LECs) but not in histoblasts disrupts abdominal metamorphosis and a dorsal cleft in the abdomen remained that could not be covered with the adult cuticle, and consequently most of the flies died inside the puparium. Temporally deregulated expression of dATF3 allowed initiation of LEC extrusion (constriction of the actomyosin cytoskeleton), but prevented its completion and probably apoptosis of LECs. RNAi silencing of RhoA produced a phenocopy of *datf3* misexpression, causing a dorsal abdominal cleft in 100% of adults. These results suggest a genetic interaction between Rho signaling and *datf3*, and support the idea that excess dATF3 prevents extrusion of LECs by stabilizing their cell-cell adhesion components. Supplying third-instar larvae with a dietary of 20-hydroxyecdysone suppresses the effect of ectopic dATF3 resulting in an increased number of enclosing adults. Since histoblasts proliferated normally, it is assumed that 20E counteracts the effect of *datf3* misexpression by promoting extrusion of LECs. The ability of histoblasts to proliferate, spread or differentiate was unaffected by the presence of ectopic dATF3. However, the adhering immortal LECs may present a barrier that the histoblasts could not overcome.

All together, these data demonstrate that the sustained expression of *datf3* prevents fusion of the adult abdominal epidermis by acting cell-autonomously in LECs, suggesting that the replacement of obsolete larval cells by adult histoblasts requires the developmental down-regulation of *datf3* expression.

The second part of this thesis presents data that not have been published so far First, a more detailed description how dATF3 affects components of cytoskeletal dynamics and may alter 20E response in LECs is given. Then, several *datf3* deletion mutants, two null alleles and tissue-specific RNAi lines were generated, phenotypically characterized and discussed with regard to abdominal morphogenesis and their function on the larval fat body. Finally, the observation that dATF3 is transcriptionally induced by ER stressors is consistent with results from human ATF3. **Taken together**, these experiments nicely complement the first part of Petra's thesis and hence give further detailed insights into the function of *Drosophila* dATF3.

Questions:

1) Do other invertebrate genomes contain a ATF3 homologs, and if yes is there anything known about their functions?

2) After overexpression of dATF3 in LECs Rho1 no longer localizes to adherens junctions but instead becomes dispersed throughout the cytoplasm. Excess of Rho1 can cause its mislocalization and thus perturb Rho1 activity. Does overexpression of dATF3 leads to a higher level of Rho1 in LECs?

3) dATF3 and dJun cooperate *in vivo* and gain-of-function phenotypes induced by overexpression of dATF3 rely on the availability of dJun. Is the level of dJun upregulated when dATF3 is overexpressed?

4) The dATF3 gain-of-function phenotype can be suppressed by stimulation ecdysone signaling, which is known to promote LEC extrusion and death. Does the accumulation of the catenin-cadherin complex not occur under these conditions or how is it dissolved?

5) Is the general organization of junctional complex along the lateral membrane domain similar in embryonic, larval and adult epithelia of *Drosophila*?

6) Is there a known function of the Crumbs-Stardust complex in larval and adult epithelia?

Yours respectfully,

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Dr. Hugo Stocker

Zürich, October 23rd 2009

Expertise on the PhD thesis by Petra Sekyrová

Functional analysis of the bZIP transcription factor dATF3 in *Drosophila* development

It is my honor to write an expertise on the PhD thesis of Petra Sekyrová presented to the Faculty of Science of the University of South Bohemia České Budejovice.

Petra Sekyrová set out to analyze the function of Activating transcription factor 3 (ATF3). ATF3 belongs to the family of basic region-leucine zipper (bZIP) transcription factors that are known to be involved in numerous stress-induced transcriptional responses. bZIP transcription factors act as homo- or heterodimers to activate (or to repress) target gene expression, and their functions have mainly been investigated in mammalian cell culture systems thus far.

Petra Sekyrová has chosen the fruit fly *Drosophila melanogaster* to explore the developmental roles of ATF3 (termed dATF3). The model system *Drosophila* offers a number of advantages that facilitate the analysis of dATF3's function. First, the bZIP family members dJun and dFos that together form the AP-1 complex have been well studied in *Drosophila*. They have been shown to be required in developmental processes such as embryonic dorsal closure and thorax closure during metamorphosis. Second, in contrast to the situation in mammals, there is a single locus encoding an ATF3 homolog in the fly genome. Third and most importantly, *Drosophila* genetics provides a rich toolkit allowing a rapid and detailed analysis of gene function in space and time.

In a first step, Petra Sekyrová had to generate tools for her genetic analysis of *datf3*, namely mutant alleles of *datf3* (by mobilizing a transposon insertion in the locus) and transgenic flies capable of activating *datf3* expression at wish (the so-called *UAS-datf3* flies that will drive *datf3* transcription in the presence of the transcription factor Gal4). Binding studies revealed that dATF3 heterodimerizes with dJun but not with dFos. The demonstration that *datf3* overexpression phenotypes depend on dJun function proved that dATF3 and dJun cooperate in vivo. In a very detailed phenotypic analysis of the effects caused by *datf3* overexpression during the morphogenesis of the adult abdominal epidermis, it is convincingly shown that sustained expression of *datf3* in the larval epidermal cells prevents their replacement by histoblasts and results in a failure of epithelial closure. The larval epidermal cells are not basally extruded from the epithelium because the cell-cell junctions among them are reinforced. As a consequence, the larval epidermal cells do not undergo apoptosis. In additional experiments, Petra Sekyrová also demonstrated that *datf3* genetically interacts with signaling by the small GTPase RhoA and with the Ecdysone signaling network. Those connections as well as the regulation of *datf3* expression need to be further explored in the future.

The results of the present study represent the first demonstration of a developmental function of ATF3. They also provide in vivo evidence for the existence of different dJun containing complexes that control distinct morphogenetic processes during *Drosophila* development. The conclusions of the thesis will stimulate further research on ATF3, especially with respect to tissue remodeling and wound healing in mammals.

To experimentally address the questions of interest, Petra Sekyrová has used appropriate methods that are state-of-the-art, and she presents a thorough analysis including the necessary control experiments. The experiments are carefully performed and clearly described, such that the reader is enabled to follow the rationale of the experimentation with ease. The thesis is written concisely, focusing on facts rather than exploring speculative avenues, and the precise language renders it a pleasure to read.

Taken together, the presented study is of high quality and clearly reaches the standards of PhD theses at my home institution, the ETH Zürich. I am happy to learn that Petra Sekyrová's work will be published in the prestigious journal *Development*.

Without any reservations, I recommend the thesis to be accepted by the Faculty of Science of the University of South Bohemia České Budejovice and to grant the PhD degree to Petra Sekyrová.

H. Stocker

Hugo Stocker

Oponentský posudek doktorské disertační práce Mgr. Petry Sekyrové „Functional analysis of the bZIP transcription factor dATF3 in *Drosophila* development"

Předkládaná disertační práce Mgr. Petry Sekyrové se zabývá rolí transkripčního faktoru ATF3 ve vývoji mouchy Drosophila melanogaster, zejména pak jeho funkci při odstranění larválních buněk z břišní stěny během metamorfózy (uzavření toraxu)a jejich nahrazení epitheliálními buňkami dospělého jedince.

Nosným pilířem předkládané práce je prvoautorský rukopis Petry Sekyrová (v recenzním řízení v časopisu Development) doplněný experimenty zaměřenými zejména na celkové či tkáňově specifické potlačení exprese dATF3. Disertační práce je sepsána velmi ujasněně a srozumitelně s přehledným úvodem, následovaným rukopisem výše zmíněné práce a posléze doposud nepublikovanými výsledky, stručným shrnutím presentovaných výsledků (bohužel chybí závěry z těch „nerukopisových") plus popisem experimentálních přístupů a citačními odkazy. Vedle nesporně zajímavých a vzhledem k jejich rozsahu i nelehce získaných výsledků oceňuji velmi dobrou angličtinu a to jak po formální, tak i obsahové stránce. Celou disertační prací se prolíná zodpovědný a pro poodhalení funkce dATF3 nesporně pozitivně zaujatý přístup autorky, čemuž i pak odpovídají získané a presentované výsledky. Celkově lze usuzovat (a tato úvaha je také zmíněna v diskuzní části), že cca. 70 kDa dATF3 v sobě spojuje vlastnosti svých savčích příbuzných ATF3 a JDP2 (oba jsou to cca. 20 kDa proteiny) a je kromě esenciální jak pro metobolismus lipidů (v tomto připomíná JDP2), tak pro migraci/apoptózu larválních epiteliálních buněk na břišní stěně Drosophily. Na rozdíl od některých jiných transkripčních faktorů z ATF rodiny – např. ATF2 nebo ATF4 je genetická či RNA zprostředkovaná inaktivace dATF3 letální již v larválním stádiu.

Stěžejním bodem této disertační práce je zevrubný popis a charakterizace většinově letálního fenotypu doprovázejícího nadprodukci dATF3 v larválních epiteliálních buňkách břišní stěny. Tyto buňky během metamorfózy ve stádiu kukly pak nemigrují z povrchu břišní stěny a jsou tak jen částečně nahrazeny epiteliálními z histoblastů odvozenými buňkami dospělého jedince. Potlačení exprese dATF3 či spíše funkce dATF3/dJun transkripčního faktoru je tedy nezbytné pro metamorfózu břišní stěny Drosophily. Důležitou roli v tomto procesu hraje dATF3 zprostředkovaná dislokalizace dRho1 a potlačení exprese eckdysonového receptoru. Celkově lze tedy jen konstatovat, že tato práce bezesporu přináší důležité poznatky nejen o funkci doposud nepopsaného transkripčního faktoru dATF3, ale zprostředkovaně i o evoluci transkripčních faktorů z rodiny ATF/CREB.

Několik dotazů a námětů:

1. Do úvodu by pro přehlednost mohlo být vhodné zařadit tabulku či obrázek porovnávající hmyzí a savčí faktory (alespoň některé) z rodiny ATF/Creb.
2. Na str. 23 není zmíněn jeden z recentně popsaných členů ATF/Creb rodiny - dATF-2 (Sano et a., MCB 2005).
3. Funkční interakce mezi dATF3 a dJun byla v předkládané práci prokázána několika různými přístupy – bylo by také možné, či zkoušeli jste imunoprecipitovat dATF3/dJun i z LEC buněk s nadprodukovaným dATF3.

4. Jaká může být vzhledem k masivnímu fenotypu nadprodukovaného dATF3 jeho role ve vývoji oka? Jaký fenotyp má potlačení exprese či inaktivace dATF3 v oku (tedy pokud jste tyto experimenty delali)?
5. Potlačení apoptózy v LEC se vyznačuje mnohem mírnějším genotypem než nadprodukce dATF3 a tudíž lze usuzovat, že dismigrace LEC je primární příčinou tohoto fenotypu. Bylo by ale možné např. pomocí hsFlp přístupu v těchto buňkách nadprodukovat např. Rip a ověřit si tak, zda by jejich (LEC) apoptóza mohla alespoň zčásti potlačit letální genotyp?
6. Jakým mechanismem může dATF3 potlačit expresi EcR? Analyzovali jste změny v transkripci jednotlivých komponent EcR v LEC s nadprodukovaným dATF3?

Celkově hodnotím předkládanou disertační práci velice kladně, autorce blahopřeji k velmi zajímavým výsledkům a vynikající budoucí publikaci a po úspěšné obhajobě disertační práce doporučuji udělení titulu PhD za jménem.

Praha, 20.10.2009

RNDr. Ladislav Anděra, CSc.