REVIEWER'S REPORT OF THE DISSERTATION THESIS

Author: Pavel Grinkevich
Title: Structure and Function of the C-terminal Domain of the HsdR Subunit from the Type I Restriction-Modification System EcoR124
Supervisor: Prof. RNDr. Rüdiger Ettrich, Ph.D.
Reviewer: Assoc. Prof. Mgr. Radka Chaloupková, Ph.D.

The work entitled " Structure and Function of the C-terminal Domain of the HsdR Subunit from the Type I Restriction-Modification System EcoR124" presents an explanation of the possible role of the C-terminal domain of HsdR subunit of EcoR124 enzyme complex responsible for recognition, modification, and cleavage of foreign dsDNA in prokaryotic organisms. Based on the successful expression, purification, and crystallization experiments followed by X-ray structural analysis, the author was able to elucidate the structure of the C-terminal domain of HsdR subunit of EcoR124 complex, previously missing in the crystal structures of HsdR subunits. The author's results revealed a unique α-helical fold of the C-terminal domain of HsdR and allowed in-silico construction and refining of a complete structural model of HsdR subunit of the EcoR124 complex. Together with endonuclease assay, electrophoretic mobility shift assay and bacteriophage restriction assay performed with HsdR wild-type and its deletion mutants lacking C-terminal part followed by molecular dynamics simulations of complete HsdR structure, the authors uncovered that C-terminal domain is essential for appropriate formation of functional pentameric complex of EcoR124 where plays an important role in correct positioning of DNA thus facilitating its cleavage. The results presented in the dissertation thesis by Pavel Grinkevich thus add considerable novelty into the understanding of structure-function relationships of HsdR subunit in EcoR124 catalytic activity.

The whole thesis is divided into four chapters including introduction, materials and methods, results and discussion and conclusions. In the introduction, the author describes the role of restriction-modification enzymes in prokaryotic microorganisms. The main attention is paid to the description of current knowledge about the structure and function of individual subunits of pentameric enzyme complex EcoR124 belonging to the type I of restriction-modification enzymes. This chapter provides a nice summary of the field, the only problem I have with this chapter is that it is quite difficult to read due to a large number of used abbreviations. For example, the abbreviations of individual domains of EcoR124 subunits used in the text only a few times could be replaced by their full names, which would facilitate readability of the introductory part. Also, I missed the numbering of individual steps of all EcoR124 enzymatic activities summarized in Figure 8 and described in the text. While in the text the individual steps of three possible scenarios of EcoR124 catalytic behavior are described together with the numbering of the steps, in Figure 8 the steps numbering is missing.

The second chapter of the dissertation consists of a description of the materials and methods employed to achieve the results described in the following results chapter of the thesis. Since the results part of the thesis is based on the three publications, each of which contains its own "Material and Methods" section, that describe very similar information as chapter 2, it is a question of why the author presents the methods as a separate chapter of the thesis and does not include the experiments description directly into the methodological parts of individual publications.

The third chapter of the thesis consists of two published articles and one manuscript. The first article aimed at expression, purification, crystallization, and X-ray diffraction data analysis of C-terminal domain of HsdR subunit of the EcoR124 complex. The second publication is focused on X-ray structural analysis of the C-terminal domain of HsdR subunit of EcoR124 complex, building of the full-length structural model of HsdR subunit and biochemical analysis of HsdR wild-type and its deletion mutants lacking the C-terminal part. The third manuscript presents molecular dynamic simulations of the whole HsdR subunit containing the newly solved C-terminal domain. Two of the three described articles have already been published in international journals and have therefore successfully passed demanding review process that confirms the novelty and high scientific level of the presented results. The only problem I've found in this chapter is numbering the figures and tables in this chapter that do not follow the chronological order of all figures and tables presented throughout the whole thesis.

I have a few additional questions which should be discussed:

- Three fusion constructs of the C-terminal domain of HsdR with pHluorin were constructed. Could the author describe how these constructs were designed? Why was pHfluorin selected as a fusion partner for the C-terminal domain of HsdR, and no some other fusion protein (such as thioredoxin, GST, SUMO or ubiquitin) often used to improve solubility and expression of the target protein?
- MD simulations of HsdR subunit of EcoR124 was performed at three different temperatures 280 K, 300 K, and 340 K. How are these temperatures relevant to the functionality of EcoR124 enzyme complex? What is the optimal temperature at which the HsdR subunit associates with MTase to form the functional EcoR124 complex? What is the optimal temperature for modification and restriction activity of EcoR124? What was the temperature at which in vitro and in vivo biochemical experiments were performed with HsdR wild-type and its deletion mutants lacking C-terminal domain?

To sum up, the candidate for Ph.D. degree has performed a large amount of insightful research and obtained new original results which broaden our understanding of the role of HsdR subunit in EcoR124 catalytic activity. The dissertation work has been performed at a high

scientific level. Most of the important results presented in the dissertation thesis are published in peer-review journals. In my opinion, the reviewed thesis meets all requirements posed on the dissertation theses. I recommend the work for defence and I believe the author should be awarded the Ph.D. degree.

In Brno, December 2, 2018

Assoc. Prof. Mgr. Radka Chaloupková, Ph.D.

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BIOMEDICÍNSKE CENTRUM
Slovenská akadémia vied

OPONENTSKÝ POSUDOK
doktorskej dizertačnej práce

(ďalej v anglickom jazyku)

Title:	**Structure and Function of the C-terminal Domain of the HsdR Subunit from the Type I Restriction Modification System EcoR124**
Author (PhD defender):	Pavel Grinkevich, MSc.
Supervisor:	Rudi H. Ettrich, RNDr., MSc., Ph.D.
Place:	University of South Bohemia, Faculty of Science, České Budějovice
Date of defense:	December 10, 2018
Opponent:	Ivana Nemčovičová, MSc., Ph.D.

The thesis of Pavel Grinkevich entitled *'Structure and Function of the C-terminal Domain of the HsdR Subunit from the Type I Restriction Modification System EcoR124'* is carrying out a detailed analysis of the C-terminal domain of the HsdR subunit from the Type I restriction-modification enzyme EcoR124 using methods of structural and molecular biology, biochemistry, bioinformatics and molecular dynamics simulations. Results are based on three papers (2 published and 1 in preparation) that are put in results chapter in chronological order as well as some additional experiments that were not present in publications.

Even though the first crystal structure of HsdR was solved almost ten years ago, a large part of the C-terminus (about 150 residues) was not resolved neither in five subsequent structures. Therefore, the main research goal of this work was to solve the missing part of HsdR structure and elaborate on the possible function of the C-terminal within the HsdR subunit and the whole EcoR124 complex.

Pavel's efforts were focused on preparation of MTase, HsdR and its C-terminal deletion mutant, and pHluorin-HsdR fusion proteins and these were successfully prepared within the study. The production of recombinant proteins seems to be a critical step in structural and biochemical characterization; and includes, but is not limited to vector design, cloning, overexpression and various purification stages. Therefore, I really appreciate Pavel's work on developing the strategy to express and purify the C-domain; to perform and optimize the crystallization experiments; to collect and analyze the X-ray diffraction data and to solve the C-domain structure.

Moreover, Pavel's work on the preparation of relevant model of the full-length HsdR subunit by combining the previous solved wild-type and the C-terminal domain structures clearly demonstrates a role of this domain by means of X-ray diffraction crystallography and explore its function using various methods. That was probed experimentally (both *in vivo* and *in vitro*) by biochemical and microbiological experiments, and nicely confronted with computational predictions, bioinformatical and structural evaluation.

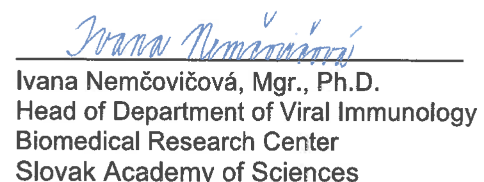
The thesis is based on 3 scientific papers, currently 2 records in PubMed. The Acta Crystallographica F Structural Biology Communications paper (IF2017=0.989) that Pavel authored as a first author, therefore contributed the most to both experimental and writing tasks. The Journal of Biological Chemistry paper (IF2017=4.010) that is also his first authored paper where he participated in majority on performing experiments and data analysis as well as writing. The third paper was also presented in the thesis, but not published yet. The contribution to this paper is also major. In addition, two experimental data sets were deposited in PDB database; C-terminal domain (PDB ID 5J3N) and full-length crystal structure of HsdR (PDB ID 6H2J). Those 3 papers together with 2 crystal structures, I therefore consider as a core of his PhD work. There is a reasonable potential that papers will be cited well based on previous work published from the research group as well as good journal selection. Therefore, the topics of this PhD thesis is obviously actual with international recognition.
The thesis has all together 119 pages divided into main 5 chapters and the supplementary material. The individual chapters are well distributed. However, the chapter *‚Results and Discussion'* could be organized better. For example, it was a bit confusing to read standard text, then text based on paper where it gets the reader out of flow. It would be better to attach whole papers as they are, not fraction those in chapters.
Overall, Pavel obviously contributed extensively to the experimental as well as theoretical part. He got exposed to various experimental techniques by his own hands and during his PhD study he also conducted a couple of internships (with Dr.Mesters and Dr.Weiserova). I would like to also appreciate Pavel's contribution in writing of the manuscripts. All those are good attributes to become an independent scientist. Therefore, the thesis as a whole constitutes a substantive original contribution to the knowledge of restriction modification enzymes.

Questions for the defense that should be addressed by the candidate:

1. The pHluoring-fusion expression; pros and cons in protein expression, purification and X-ray crystallography.
2. The principle of EMSA and discuss the usage within the thesis.

Finally, I can clearly state that the candidate fulfills all criteria for being awarded a PhD degree. Therefore, **I can fully recommend Pavel Grinkevich's thesis for the PhD defense.**
(Záver: Pavel Grinkevich vo svojej práci jasne preukázal svoje tvorivé schopnosti i vedomosti a preto táto práca bezpochyby spĺňa požiadavky kladené na dizertačné práce v tomto odbore. ***Odporúčam prácu k obhajobe****.)*

Bratislava, 22.11.2018



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Pavel: Grinkevich: Thesis Defense

Title: Structure and Function of C-terminal Domain of HsdR Subunit from the type I restriction Modification System EcoR124

Over all Comments: Mr. Pavel Grinkevich (Dr. to be) had worked on a protein system that is fundamentally very important. One of the complicated, yet beautiful system, to use to study protein/structure function. Pavel along with guidance from Dr. Rudi Ettrich, and from various others, have completed the project necessary for completing the Ph.D. requirements. This project involves areas like Molecular Biology (Cloning, Expression..), Biochemistry (Protein Purification, Characterization by Restriction Assay and Electrophoretic Mobility Shift Assay (EMSA), Microbiology (Restriction/Cleavage Complementation and Bacterial-Phage Plaque Assay), X-Ray Crystallography, Bioinformatics (Sequence Comparison and Identifying Motif's), and Molecular Dynamics (MD) Simulations and Analysis. In all of this Pavel Grinkevich, with guidance and with his own independence especially with molecular biology, biochemistry and microbiology, has shown overall excellence. His Molecular Biology area stands out and appears as his expert area amongst plethora of area's that had been covered to prove the importance and significance of the C-terminal part/domain of the HsdR from Type I EcoR124 system. Pavel's molecular biology techniques, understanding and application of it, to prove the role of the C-terminal domain in HsdR structure/function is the highlight of the thesis. He has done a great job of it. The area that probably could have been better defined: is the clear distinction between helical domain and C- terminal domain (the basis of protein fold difference?, a distinct functional difference?. Although, the interaction of helical domain with the rest of the protein is less, compared to the "C-terminal domain" that clearly influence ATP dependent translocation/Endonuclease activity, more justification might be required to separate the domains?. In other words, can we call helical and C-terminal domain as one large functional domain? Nonetheless, the elucidation of the C-terminal structure and its function is very important and it had to be completed, so that one can look at the overall protein structure/function of all three (HsdR, HsdM and HsdS) as one giant complete pentameric multiprotein system. Can you elaborate more on the tetrameric versus pentameric (in other words explain/hypothesize why tetrameric has translocase activity but no cleavage activity? Is translocase/ATP-hydrolysis and endonuclease activity coupled? If so what is the time scale?, the complete enzyme (pentameric) has all activities a) Recognition b) Methylation c) Translocation and d) Cleavage). Compare the tetrameric and pentameric at the amino acid residue level. Create a figure of your own to compare tetrameric and pentameric and discuss structure/function. This would tell you why the homo dimeric (R2) part of the pentameric subunits are interacting better as oppose to monomeric (R1) of tetrameric is less interactive with the deficiency of last activity?

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Helicase I and 2: It seems only helicase 2 binds ATP and not 1 (or it has it not been discussed? Or not clear?). Can you discuss/hypothesize the rationale behind the helicase 2 influence, over 1 is what is really a matter of the problem/concern? In other words why only one ATP/two helicase domains?

Though your focus is on C-terminal part can you discuss why there is no "classical ATP binding motif e.g. GxxGxxK or GxxGK or similar versions in this case (i.e. helicase 2)?. Clearly helicases split 1 ATP/1 bp movement. Or is this process dependent of C-terminal residues that along with helicase 1 and perhaps 2 forms the complex that could mediate 1ATP hydrolysis/1bp movement? Also, just as a curiosity the movement is random (i.e. nick made could be anywhere) is there consensus? As to how far it had to move/translocate before making a cut/nick? Wouldn't it be cost saving in terms of ATP if it were to move short distance and make a cut? or multiple cuts? Is the ATP in anti or syn orientation, when it binds to helicase 2?

You do suggest the importance of the C-terminal motif (^{1016}KKXXXXXK^{1023}) and various motifs, and you plan to do site directed mutagenesis of the K's. Do you think you could actually make one triple mutant (AAXXXXXA) to test this hypothesis?

Being a biochemical/molecular biology advocate can you not go to IUBMB to change the EcoR124 to StyR124? Especially since you have the complete structure now.

You have painfully used many different techniques to establish "the role of C-terminus" which are all pointing out to one single concept "C-terminus" is very important, an idea that has been around, which you have completed/proved, and it is very important for the scientific world. The challenging part of the whole project is of course is to crystallize the C-terminal part. Using novel pHluorin vectors, fusion protein strategy etc.. you have managed to complete the overall structure elucidation. This is the strength of your thesis and experimentally you have learnt many nuances of Molecular biology. Kudos.

Can you compare bacterial 3N/6N? Adenine methylation versus mammalian Cytosine 5 methylation? Any hypothesis/thoughts as to why nature chose one versus the other? What advantage does it have structurally to methylate adenine?

To think of Hsd system as the early immune response system, is fascinating. Very sophisticated, in its own right, at the very molecular level. Indeed fascinating system that you have chosen to work with. "Early Defense Mechanism".

Overall the thesis has been put together extremely well and congratulations. Feel free to hypothesize/discuss bit more at the end of each section. This what you would have to be doing as a Ph.D.

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Specific Comments/Edits

Page I: Annotation: Line 4: Follow the exact order of the biological events: a) Recognition b) Methylation c) Translocation and d) Cleavage. Use this order through out the manuscript in describing whereever you are describing it all (a-d) at once.

Page 7: line 10 (...enzyme [illegible] available...)

Page 9: paragraph 1: [illegible]

Table 2: [illegible]. Can you also comment on evolutionarily advanced nature of type I or is it?

Table 2: Under type III: Nonpalindromic, bipartite (instead of ?) required for...

Can you possibly insert both (bipartite) consensus sequences (the actual sequences) for both Type I and Type III.

Page 11: line 1: insert: rings in [illegible] presence....

Page 13: line 2: delete "[illegible] (i.e. the sentence ends with "precipitate".)

Page 13: Last Paragraph: Can you discuss little bit about survival/speciation (feel free to use your own thoughts/hypothesis). I feel you should talk about this interesting evolutionary concept. Evolutionary Hierarchy?/parallel evolution?

Page 14: 3rd para: line 1: genetics of (instead of genetical aspects

of) Page 14: 3rd para: line 6: genetic not genetical

Page 16: Figure 3 legend: to be consistent "HsdR not [illegible].

Page 16: Section 1.3.2: line 3: its own in [illegible] case of (insert [illegible] between in and case)

Page 17: Figure 4C: You have used linear depiction of the HsdS. Very nice. Can you do the same for HsdM and HsdR (i.e. do the corresponding linear depiction of domains in their respective figures)? It would make it very visual for people of one dimensional viewers.

Page 19: Last paragraph is SAM anti or syn?

Page 21: Section 1.3.4: Paragraph 2: line3: Is it E220A? and Not [illegible] ?

Page 22: Figure 6: can you label the very end "N and C" so that we know endonuclease is the amino terminus and helical is the C-terminus.

Page 23: What is the rate of ATP hydrolysis (Kcat/Km)? It doesn't have typical Walker or HxxH motif?, what is the role of Lys 313? Would it not perform charge repulsion? Explain better.

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Page24: line 1: EcoKI is produced, (insert is and , after produced,)

Page 24: Has anybody done mixing and matching of type I/II/III/IV subunits? And asked the question for function?

Page 24: paragraph 3: Line 2: Insert the abbreviation at the first time ([illegible])

Page 24: can you elaborate more on the role of C-terminus on "cleavage" as opposed to more of anything else?

Page 28: Line 2: Is it possible to show the double strand?

Page 28: paragraph 1, 2 and 3: [illegible] .towards the end of 1st paragraph talk about bipartite and the spacer of 5-6 nt (it might be repetitive, but it would be good to bring it in) I only see two G in your sequence. You say 3 G in paragraph 2, 5th line: where is the 3rd G is it in the complementary strand?

Also is it 2 A's on sense strand and 1 A on antisense

strand? Base flipping: could it be "anti to syn" as well?

Page 29: Paragrapg3: Line 3: is it antisense

strand? Overall I loved your pages 28 and 29.

Page 33: 2.1.1: line 5: can you say " [illegible] " instead of "[illegible]" also is it 2X YT or 2xYT?

Page 33: do we need a comma for [illegible] instead of [illegible] g ?

Page34: Table 3: Column 2 (title): instead of saying "[illegible]" "[illegible]"

Page 34: Can you explain why 320 nm? Also why not subtract the 260 nM for potential contaminant of NA that could absorb at 280 nm as well?

Page 35: 2.1.2: line 6: [illegible] LB instead of [illegible] LB?

Page 35: 2.1.2: paragraph 2: line 1: is it [illegible] or Tris-Acetate? Instead of just

[illegible]? Page 36: Last para: line 4: deletion [illegible] instead of deletion present

Page 37: 2.2: line 6: This [illegible] instead of This [illegible]

Page 39: 2.3: expand EMSA (being the first encounter of the abbreviation EMSA)

Page 39: 2.3.1: Line 1: "activity of [illegible] mutant" instead of "activity of

mutant" Page 39: Also what is the approximate in vivo translocation length?

Page 40: 2.3.2: Tris-HCl not Tris

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Page 40: 2.4: instead of cultivated? What is LA? Liquid Agar? Expand.

Page 43: 3.1: Line 1: to instead of carryout?

Page 44: Para 2: last line: not latter?

Page 44: 3.4: line5: simulation instead of simulation length?

Page 45: Figure 9 Legend: and insert are ligated

Page 46: Figure 10 legend (A): is it linear plasmid?

Page 47: 3.2.1: line: 3: not

Page 57: Figure 11 legend: instead of

Page 58: 3.3.2: line before last (penultimate line): clones instead of clones

Page 58: 3.3.3: Both WT and mutant instead of "mutant"

Page 60: Figure 13 legend: Can you expand for abbreviation, also in the previous paragraph.

Page 69: line 1: delete " " and say " "

Page 70: Conceptually what is your basis for calling domain 5 instead of saying one long helical domain? Discuss it bit more on your helix in red interaction versus helix in green interaction. Structurally both contains alpha-helix and coils.

Page 70: Figure 4 legend: What is the DNA concentration? Is it 10 nM? Lane 8 has more free DNA? Loading inaccuracy? To form pentamer do you need more than 100-120 nM MTase?

Page 74: Line 4/5: instead of " " say " "

Page 74: 5th line from the bottom: instead of .

Page 77: Rationale or Reason or basis, behind why only helicase 2 has bound ATP and not helicase 1? If truly helicase 1 doesn't bind ATP explain/hypothesize why?

Page 95: 3rd line from bottom: where which... delete which

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Page 105: I assume it is plasmid DNA and not genomic. Has it ever been genomic? In other words, can speciation form through evolution of bacterial genomic DNA? I know it is off topic. But a line or two on that?

Page 106: Middle para: deletion mutant instead of mutant.

Overall Comment: Very Good Thesis.

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12/5/2018

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