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Opinion on doctoral dissertation of mgr. Antona Slyvki entitled "Specific recognition and processing of modified cytosine bases in DNA" prepared under the supervision of Prof. Matthias Bochtler carried out in the Institute of Biochemistry and Biophysics at the Polish Academy of Sciences in Warsaw and International Institute of Molecular and Cell Biology, Laboratory of Structural Biology in Warsaw.

The methylation of DNA based on the addition of methyl groups to nucleotides of DNA broadens a genetic code for additional canonical four bases with consequences in the genetic and epigenetic roles.
5 - methyl cytosine (5mC) is present in the DNA of all organisms and in some viruses playing an important role in the regulation of gene expression. The presence of 5-hydroxymethylcytosine (5mhC) in bacteria and bacteriophages has been known for over 50 years, however, their presence in mammalian genomes is relatively new. The discovery that the presence of 5hmC is not a result of spontaneous damage of DNA but the product of enzymatic oxidation of 5-methylctosine opened the road to studies of the biological role of this nucleotide. Both 5mC and its oxidized form 5-hydroxymethylcytosine (5hmC) have to be recognized by proteins to convey their epigenetic

function. Nevertheless, the molecular details of such recognition remain poorly understood. The main aims of the presented dissertation are concentrated on two aspects of the molecular activity of the epigenetic role of 5Mc and 5hmC.

The first one concerns the exact mechanism of recognition of methylated cytosine residues by proteins that convey their epigenetic messages. As a model for studies of this problem the author used interaction of the *Escherichia coli* EcoKMcrA enzyme that recognizes and cleaves the DNA containing 5mC or 5hmC. Understanding the action mechanism of this enzyme and the mechanism of its interaction with DNA will help for a better understanding of the epigenetic role of 5mC and 5hmC. Based on genetic experiments and molecular modeling, it was suggested that EcoKMcrA senses modified cytosine with its N-terminal domain, and cleaves DNA with the HNH domain.

The second one concerns the process of active DNA demethylation based on the process of 5mC oxidation and removal of oxidized derivatives. In eukaryotes, 5hmC is a product enzymatic oxidation of 5mC by TET dehydrogenases and removing the product from DNA by further oxidation to 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC), excision from DNA by TDG glycosylase and replacing by unmodified cytosine. The precise DNA demethylation is absolutely required in the process of mammalian development.

The aim of this doctoral research was to understand the mechanism that underlies the specific recognition of modified cytosine bases in DNA and to get more information on reactions catalyzed by the enzymes involved in active DNA demethylation. Those two main aims of the presented doctoral thesis are in the front of contemporary studies in molecular biology and studies on the epigenetic role of DNA methylation.

The presented doctoral dissertation does not have a traditional scheme for such work. It includes a short introductory summary allowing for fast understanding of general ideas of undertaken studies and a much longer (71 pages) "Introduction". In this part of the thesis, the author presents up to date, based upon 310 positions of literature, information on DNA modifications concentrating mainly on C5 and 5hmC modification in prokaryotes and eukaryotes.

A large part of the Introduction is devoted to the biogenesis of 5mC and 5hmC, regulatory and defensive roles of 5C methylation in prokaryotes, restriction-modification systems and phage anti-R-M mechanisms. In deep details, the author describes the mechanisms of the recognition of modified bases by proteins, including recognition with flipping and without flipping and a detailed description of all known types of recognition domains. The presentation of this information is very precise showing a profound knowledge of the presented materials.

The main parts corresponding to a traditional Dissertation: **Results**, **Materials and Methods and Discussion** showing the scientific achievements are presented in this dissertation in the form of four articles published between 2018 and 2020. In two of these articles, mgr. Anton Slyvka is the first author and in others signed by two to eight other co-authors working within the team supervised by dr. Böchtler from IIMCB. All of these articles were published in the most acclaimed scientific journals such as Nucleic Acid Research, J. Mol. Biol. and Scientific Reports. The papers published in this class of scientific journals (Impact Factor; 10,162 NAR, 4.122 SR and 4.760) guarantee their highest scientific value and that they were carried out with use of the most contemporary techniques.

As with all papers signed by several authors belonging to a research team it is difficult to get an exact estimation of the extent and importance

of the participation of particular members. Based on the declarations of mgr. Anton Slyvka and co-authors of the publications included in his dissertation I can conclude that mgr. Anton Slyvka contributed substantially not only in the execution of the experimental parts of the research but also in the developing general ideas of research, planning and designing of the experiments as well as in the interpretations and discussion of the results and manuscript writing which resulted in the scientific values of these publications. His participation was mostly connected with the expression of genes encoding of EcoKMcrA and NEIL1 and TDG glycosylases, their purification, examining biochemical properties formation of crystals as well as participation in editing the manuscripts.

The first two papers contributing to **Results,** concern the activity, structure and crystal structure of the N-terminal domain of EcoKMcrA. The specific in vitro activity of this enzyme toward methylated cytosine residues of DNA was shown for the first time in these papers. In contrast to strong *in vivo* activity, the *in vitro* activity was relatively weak but enough to determine biochemical properties including the determination of the crystal structure of the enzyme without DNA. In this paper, mgr. Anton Slyvka took part mainly in the determination of the vivo activity of the enzyme. His ability to carry out more technically advanced experiments was demonstrated in the second paper dealing with the EcoKMcr enzyme where he expressed, purified and co-crystallized the N-domain of the enzyme (NEco) with DNA duplexes. These studies show that the N-terminal domain of the EcoKMcrA is not phylogenetically related to other domains responsible for the recognition of methylated cytosine residues and has a unique protein fold recognizing 5mC and 5hmC in a novel way without flipping.

The next two papers describe experimental works with the enzymes participating in the excision of the product of oxidation of 5mC. The author has cloned, expressed and purified the recombinant human glycosylase and through a series of different types of biochemical experiments showed that NEL-1 can directly excise 5caC but not 5fC from DNA and cleave the DNA phosphodiester backbone by its β-δ lyase activity and stimulation of glycosylase activity of TDG on 5fC and 5caC. In next paper the author produced NEI1 and TDG glycosylases and their catalytic mutants as well as carried out some biochemical experiments that provided evidence for understanding the mechanism of their activity.

In summary, I can fully agree with the author, that the data presented in the dissertation significantly expanded our current understanding of the interaction of modified cytosine residues in DNA with specific proteins. The data presented in these papers unrevealed mechanism recognition of 5mC and 5hmC and cleavage of DNA by EcoKMcrA and showed uniqueness in the N-end domain of enzymes among analogous domains.

The results of this work contributed strongly to a better understanding of the reaction catalyzed by NEL1, TEG and TET dioxygenases in the process of active demethylation.

The dissertation does not have a separate part entitled "Discussion". The discussion of the results is included in a particularly original publication and in another part of the dissertation where the author confronts the results obtained during his experimental work with similar works in published papers. In my opinion, considering scientific values, novelty and editorial diligence, this dissertation is fully acceptable as a doctoral one and should be rewarded appropriately.

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