OPINION

by Assoc. Prof. Dr. Kiril Mihaylov Mishev (IPPG-BAS)

on the dissertation "Mechanisms of spreading of γH2AX and MDC1 beyond the DNA damage site",

submitted by **Georgi Todorov Danovski** (Laboratory of Genome Stability, IMB-BAS) for the award of the educational and scientific degree "**Doctor**"

The dissertation titled "Mechanisms of spreading of γH2AX and MDC1 beyond the DNA damage site", submitted by Georgi Todorov Danovski for the award of the educational and scientific degree "Doctor", was developed under the supervision of Assoc. Prof. Dr. Stoyno Stoynov from the Laboratory of Genome Stability at IMB-BAS.

Georgi Danovski's work is dedicated to the investigation of the mechanisms of repair of complex DNA damage in human cells, specifically the kinetics of accumulation and subsequent removal of repair proteins during the DNA repair process. As a result of the impact of chemical or physical agents, several different types of damage can be induced simultaneously within the same DNA region. Each of these damages triggers a specific DNA repair mechanism, associated with the activation of repair proteins and their placement in a confined space around the DNA lesion in a strictly defined sequence. These processes are subjected to multifaceted post-translational regulation to ensure their precise coordination in space and time. However, the high dynamics of the individual steps of the repair process significantly complicates its study using conventional approaches. Therefore, the doctoral candidate applies an interdisciplinary strategy based on the combination of methods from molecular and cell biology, programming, and mathematical modeling.

A major focus of Georgi Danovski's work is the creation and optimization of software for analyzing microscopic images, which allows for the study of the kinetics of DNA damage repair. Among the key advantages of the computer program CellTool are the capabilities for high-throughput processing of a large number of files from time-lapse experiments, including automated segmentation and tracking of DNA repair foci. The algorithms embedded in the program for visualizing the results and mathematical modeling of the data have recently been successfully used to establish the dynamics of association and dissociation of approximately 70 repair proteins in human cells *in vivo*. In the present dissertation, Georgi Danovski uses the algorithms he developed to study the early stages of the repair of complex DNA damage. Using cell lines expressing fluorescently labeled protein markers, the doctoral candidate tracks the kinetics of the spreading of phosphorylated H2AX within and beyond the damage site. Precise measurements of the accumulation times of ATM kinase and the mediator protein MDC1 after DNA damage induction reveal an unexpectedly high rate of activated ATM exchange. This high rate is not consistent with a model published by another scientific team for the spreading of phosphorylated H2AX, based on chromatin loop extrusion. The validity of the aforementioned model is also not confirmed by additional experiments, in which the doctoral candidate does not establish a direct role for cohesin-associated proteins in the spreading of phosphorylated H2AX. Based on the experimental data,

Georgi Danovski formulates a working hypothesis for the existence of freely diffusing activated ATM, which, before inactivation, phosphorylates H2AX in chromatin regions distant from the damage site. To validate this hypothesis, a mathematical model was created, which describes the obtained experimental data with high accuracy and proves that the kinetics of γH2AX spreading and the associated MDC1 spreading are based on the diffusion of activated ATM kinase. Overall, the experimental and theoretical studies described in the dissertation are of fundamental nature and significantly contribute to the understanding of the dynamics of molecular processes in the early stages of DNA repair. Hence, the significance of the obtained results in the context of a large number of human diseases caused by genome instability.

The literature review and the list of references (a total of 274 sources) demonstrate the doctoral candidate's excellent knowledge of the existing research in the field of genome stability. The main types of DNA damage are discussed, as well as the physical and chemical agents most commonly associated with DNA damage induction. A review of DNA repair mechanisms is provided depending on the type of damage, with the sequence of events and the involved proteins comprehensively illustrated with figures adapted from key review publications on the topic.

The aims and objectives of the dissertation are presented clearly and in a concise manner. The methods used by the doctoral candidate to achieve the set objectives include approaches for inducing DNA damage through UV laser micro-irradiation, modern light microscopy techniques (immunofluorescence, confocal microscopy analysis of the localization of fluorescently labeled proteins in live cells, FRAP), mathematical tools for solving partial differential equations, and regression analysis of microscopic data with mathematical modeling. The description of the methods in this part of the dissertation is comprehensive and allows for the replication of experiments if necessary.

The "Results" section covers 29 pages (about one-third of the dissertation excluding the bibliography) and contains 1 table and 16 figures, most of which are composed of multiple panels of data. The experimental results and mathematical modeling are described clearly and logically, following the sequence set in the section with the aims and objectives. At the beginning, the doctoral candidate presents the main tools and protocols embedded in the CellTool software he developed. The main advantages of the program in analyzing time-lapse experiments are automated segmentation and tracking of DNA repair foci, embedded protocols for FRAP analysis, graphical representation of processing results, and modeling of the experimentally obtained kinetics of protein accumulation and removal from the studied focus with an embedded or user-defined mathematical apparatus. After experimentally validating the analysis protocols, the doctoral candidate uses CellTool to study the dynamics of ATM, MDC1, and cohesin accumulation following the induction of complex DNA damage by micro-irradiation. Unlike MDC1, which is characterized by lower mobility due to its binding to chromatin, ATM exhibits rapid exchange at the DNA lesion site. Georgi Danovski finds that the spreading of γH2AX and MDC1 precedes the accumulation of cohesin, thus questioning the validity of a published model that links the distribution of nucleosomes phosphorylated by H2AX with cohesin-dependent chromatin loop extrusion. Based on the recorded kinetic constants, the doctoral candidate formulates an alternative hypothesis for the spreading of γH2AX and MDC1, attributed to the free diffusion of activated ATM kinase. To prove this hypothesis, Georgi Danovski develops spatiotemporal mathematical models of MDC1 spreading that account for the diffusion of activated ATM. The models are tested with experimentally measured diffusion coefficients of ATM and MDC1.

The "Discussion" section covers 5 pages, in which Georgi Danovski concisely summarizes the advantages of the software application he developed. Here, the doctoral candidate could better highlight the contributions of his work. The high processivity provided by his program for semi-automated image analysis could be compared with a sample protocol for manual processing of time-lapse movies using a combination of multiple separate applications. Furthermore, the doctoral candidate discusses the obtained results in light of published research by other authors, emphasizing the high agreement of his mathematical model with experimental data. A hypothesis is formulated linking the approach used for inducing DNA damage with the identified differences in the mechanism of γH2AX and MDC1 spreading compared to previously published models.

The conclusions and contributions at the end of the dissertation are clearly formulated and fully reflect the presented experimental results. The submitted abstract accurately summarizes the dissertation and Georgi Danovski's publication activity.

The doctoral candidate's articles on the dissertation topic total three. Two of them are published in international journals with a high impact factor, with the total number of citations exceeding 150! In these articles, Georgi Danovski's individual contributions are clearly distinguished from those of the other co-authors. The third publication is under revision, with the manuscript publicly available as a preprint in a repository. The additional information provided about publications not included in the dissertation and participations in scientific conferences testifies to Georgi Danovski's high scientific activity.

CONCLUSION:

The dissertation convincingly summarizes several years of basic research by Georgi Danovski. The doctoral candidate applies an interdisciplinary approach that successfully overcomes the existing methodological limitations in studying a process with high molecular dynamics, such as the repair of complex DNA damage. The experiments were conducted and analyzed meticulously using modern microscopy methods with high sensitivity and resolution. Georgi Danovski's access to high-tech platforms at IMB has enabled him to develop into a highly qualified researcher in the field of molecular and cellular biology. At the same time, the doctoral candidate has gained impressive experience in regression analysis and mathematical modeling, which he successfully applies to study the early stages of the recognition and marking of damaged DNA regions. In this context, original scientific results have been obtained that contribute to a more comprehensive understanding of the processes involved in maintaining genome stability in human cells. A significant contribution from Georgi Danovski's work is the creation and optimization of a computer program for the analysis of microscopic images of DNA damage sites where repair processes occur. In addition to innovative algorithms for segmentation, tracking, analysis, and modeling of data, the software application stands out for its open access and published code, which paves the way for increased future interest of the scientific community working in this field. The high citation rate of the presented scientific publications on the dissertation topic attests to the relevance and significance of the obtained results. Based on the materials presented for review, I consider that the regulatory requirements have been fully met, and **I confidently recommend that the esteemed scientific jury award Georgi Todorov Danovski the educational and scientific degree of "Doctor."**

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Signature:
(Kiril Mishev)