OPINION

by Assoc. Prof. Kiril Mihaylov Mishev, Ph.D. (Institute of Plant Physiology and Genetics, Bulgarian Academy of Sciences)

on the doctoral dissertation titled *"Kinetics of Protein Accumulation and Removal from the Replication Fork During Its Stalling and Restart"*,

submitted by **Teodora Krasimirova Dyankova-Danovska** (Laboratory of Genome Stability, Institute of Molecular Biology, BAS) for the acquisition of the educational and scientific degree "**Doctor**"

The dissertation titled "Kinetics of Protein Accumulation and Removal from the Replication Fork During Its Stalling and Restart", presented by Teodora Krasimirova Dyankova-Danovska for the acquisition of the educational and scientific degree "Doctor", was conducted under the supervision of Assoc. Prof. Stoyno Stoynov, Ph.D., from the Laboratory of Genome Stability at the Institute of Molecular Biology, BAS.

Teodora Dyankova-Danovska's work focuses on the study of mechanisms for overcoming replication stress in human cells. The doctoral candidate developed a novel approach to tracking the dynamics of key replication and DNA repair protein factors at replication forks at single foci during the stalling and subsequent resumption of DNA synthesis. To achieve this, T. Dyankova-Danovska generated fluorescent marker lines and applied live-cell confocal microscopy with high temporal resolution in the presence of specific inhibitors of the studied proteins. For the quantitative analysis of fluorescence intensity in the monitored replication foci during the cell cycle, a computational algorithm was created as part of the CellTool software package developed in the Laboratory of Genome Stability. This allowed the doctoral candidate to conduct precise measurements of the kinetics of accumulation and removal of the studied proteins at the replication forks. These studies are fundamental, but also have potential implications for identifying and characterizing novel pharmacological targets as part of future strategies for treating replication stress-related diseases.

The experimental work of T. Dyankova-Danovska is based on the use of a fast and highly efficient approach for reversible stalling and restarting of replication forks using the ribonucleotide reductase inhibitor hydroxyurea (HU). When HU is added to the cell culture medium, DNA synthesis is halted due to the depletion of deoxyribonucleotide content, and the process resumes shortly after the inhibitor is removed. Using this approach, the doctoral candidate tracked the dynamics of accumulation and removal of fluorescently labeled PCNA (a key component of the replication fork) and RPA1, a marker for the spread of single-stranded DNA regions in the replication focus. The time intervals for PCNA focus disappearance were measured as a function of suppressed *de novo* accumulation in the presence of HU. The re-binding of this protein clamp to the replication fork region upon recovery of the nucleotide pool was also followed. The precise analysis of the fluorescence signal intensity, combined with previously published data from other groups on the absolute number of PCNA molecules in HeLa cells, allowed the doctoral candidate to determine with high accuracy the number of these complexes during each phase of stalling and restart of a single replication fork. Similar calculations were performed for the RPA1 protein,

which progressively accumulates under conditions of halted DNA synthesis and ongoing unwinding of the two polynucleotide strands, and is rapidly depleted from the fork region after HU washout. The full recovery of the replication process in each examined single cell ensures the subsequent unimpeded progression of the remaining phases of the cell cycle.

This experimental setup was utilized by T. Dyankova-Danovska to analyze the regulatory mechanisms that ensure overcoming of the replication stress effects. Key regulatory proteins identified include ATR and ATM kinases, which have numerous proven protein substrates involved in replication and DNA repair processes. One of ATR's functions is associated with coordinating polymerase and helicase activity. Accordingly, during replication fork stalling, the doctoral candidate observed significantly intensified generation of single-stranded DNA and accumulation of RPA1 under conditions of suppressed ATR activity. Restarting the fork in the presence of an ATR inhibitor did not lead to the complete removal of single-stranded DNA coated with RPA1, subsequently resulting in impaired mitosis. Unlike ATR, suppressing ATM activity did not affect the dynamics of PCNA and RPA1 accumulation and removal during fork stalling and restart. However, through combined inhibition of ATM and ATR, T. Dyankova-Danovska highlighted the significance of ATM in reducing single-stranded DNA regions, whose presence impedes progression to subsequent phases of the cell cycle. Using specific inhibitors, the doctoral candidate also examined the roles of other DNA repair proteins in replication stress induction, such as MRE11 and PARP1. The universal nature of the findings regarding the trends in PCNA and RPA1 accumulation changes during replication fork stalling and restart was confirmed via identical experiments conducted with two additional human cell lines. The approach for inducing replication stress through HU treatment was also applied by T. Dyankova-Danovska to test the interaction between PCNA and subunit 2 of the DNA polymerase δ complex regarding their dynamics during the interruption and resumption of DNA synthesis. The data from all the aforementioned microscopy experiments were processed using the CellTool software package, which enables high-throughput processing of numerous time-lapse experiment files, including automated segmentation, tracking of fluorescent foci, result visualization algorithms, and mathematical data modeling. T. Dyankova-Danovska played a significant role in the development and optimization of this program within the Laboratory of Genome Stability at IMB-BAS.

The literature review and the list of references (a total of 234 sources) successfully synthesize the currently available information regarding the individual stages of the replication process, the main participating protein complexes, regulatory mechanisms, and sources of replication stress. This part of the dissertation is comprehensively illustrated with figures and diagrams adapted from key review publications on the discussed topic. A drawback is the absence of introductory information about the structure and functions of the DNA repair proteins MRE11 and PAXIP1, which are subject to experimental work in the dissertation.

The objectives and tasks of the dissertation are precisely formulated. However, the task related to tracking the kinetics of PAXIP1 accumulation at sites of complex DNA damage under conditions of suppressed ATM kinase activity is not appropriately integrated into the dissertation. The doctoral candidate has not convincingly justified the necessity of these studies, and it is unclear how the obtained results with PAXIP1 contribute to achieving the stated aim of the dissertation. The materials used and the methods and approaches applied are described in detail, ensuring reproducibility of the experiments if validation is required.

The "Results" section spans 40 pages (just under half of the dissertation's total volume, excluding the bibliography) and contains 29 figures, most of which consist of multiple panels with data. The doctoral candidate has made a precise analysis of the obtained results, based on which well-founded conclusions have been drawn. In the "Discussion" section, T. Dyankova-Danovska discusses the model she has formulated for the dynamics of PCNA and RPA1 during and after replication stress in light of previously published results. The advantages provided by the experimental system used, compared to previous approaches, are emphasized, namely its high temporal resolution and large throughput in processing the microscopy images, ensured by CellTool. The conclusions and contributions at the end of the dissertation are clearly formulated and fully reflect the experimental results presented. The abstract correctly summarizes the dissertation of T. Dyankova-Danovska.

T. Dyankova-Danovska has published three articles on the subject of her dissertation, two of which are published in international journals with a Q1 quartile and high impact factor, while the third is under revision, with the manuscript publicly available as a preprint in a deposit database. In two of the publications, the doctoral candidate is the first author (either alone or co-authored with Georgi Danovski). The clear distinction of individual contributions from those of the other co-authors makes an excellent impression, as T. Dyankova-Danovska provides a contributor statement for two of the articles. She is also a co-author in another scientific publication unrelated to the dissertation topic, and the total number of citations of articles with her involvement exceeds 100. During her work on the dissertation, T. Dyankova-Danovska participated in 10 scientific conferences in Bulgaria and abroad and was part of the scientific team in 4 projects funded by the Ministry of Education and Science and the National Science Fund. These participations demonstrate the doctoral candidate's high scientific activity.

CONCLUSION

The presented dissertation by T. Dyankova-Danovska summarizes several years of research aimed at elucidating the regulatory mechanisms for overcoming replication stress in human cells. The application of modern microscopy approaches with high temporal resolution, combined with newly developed algorithms for high-throughput image processing and mathematical data modeling, enabled the doctoral candidate to conduct precise kinetic measurements at single replication foci. The obtained information has substantial contributory value and enhances our understanding of the plasticity of the replication fork which determines its stability. In the course of this work, valuable genetic constructs were generated and characterized, which can be used in future research dealing with stress factors that affect fork dynamics. The experimental results have been published in reputable international journals, and some of them have already been widely cited. Based on the materials provided and additional explorations, I believe that all normative requirements have been fully met, and **I strongly recommend to the respected scientific jury to award Teodora Krasimirova Dyankova-Danovska the educational and scientific degree "Doctor"** in the professional field 4.3. Biological Sciences, scientific specialty "Molecular Biology".

16.12.2024 Prepared by:
(Kiril Mishev)