Opinion

on the doctoral dissertation of Teodora Krasimirova Dyankova-Danovska

"Kinetics of Protein Accumulation and Removal at the Replication Fork During Stalling and Restart"

submitted for the award of the academic degree of "Doctor" in the scientific field of "Molecular Biology."

Reviewer: Assoc. Prof. Dr. Anastas Gospodinov

1. General Overview

Teodora Dyankova-Danovska conducted her doctoral research at the Institute of Molecular Biology "Acad. Roumen Tsanev" of the Bulgarian Academy of Sciences in the Laboratory of Genome Stability under the supervision of Assoc. Prof. Dr. Stoyno Stoynov. The dissertation is 108 standard pages long, includes 41 figures, and cites over 240 references. The work has been presented and approved at a meeting of the extended scientific seminar of the Institute.

2. Relevance of the Topic

The dissertation presents an innovative study of the dynamic processes that occur during the stalling and restart of the replication fork. The applied original approach allowed for quantitative real-time tracking of changes in the levels of key replication factors and single-stranded DNA (ssDNA)—a critical signaling structure in replication stress—with second-level resolution. This is achieved for the first time. The significance of the obtained results and the research approach stems from the fact that replication stress is a major factor in the etiology of cancer. Furthermore, replication stress results from the action of most anticancer agents.

3. Knowledge of the Field

The literature review in Teodora Dyankova-Danovska's dissertation thoroughly addresses key aspects of DNA replication, replication stress, and mechanisms for coping with it. It explores the main stages of replication—initiation, elongation, and termination—while focusing on the function of the CMG complex, the roles of replication proteins such as PCNA and RPA, and the dynamics of the leading and lagging strands. Replication stress is described as a primary factor compromising genome stability, with detailed discussion of physical barriers to replication forks, including DNA damage and hard-to-replicate genomic regions, as well as metabolic factors such as nucleotide pool depletion. The review highlights the central role of ATR kinase in ssDNA detection and response and its interactions with other proteins involved in genome stability maintenance. The dissertation's literature review is well-prepared, demonstrating high competence in the field, and provides an excellent basis for understanding the scientific significance and innovative aspects of the study.

4. Methodology

The main experiments were conducted using HeLa Kyoto cells modified via BAC recombineering to express fluorescently labeled PCNA and RPA1 proteins. This technique ensures protein expression at near-endogenous levels, providing physiological relevance to the results.

The primary method used was live-cell microscopy with a temporal resolution of 30 seconds. A combination of Airyscan and spinning-disk microscopy was employed to monitor PCNA and RPA1 dynamics in real time. Replication stress was induced with hydroxyurea (HU), and the roles of ATR and ATM kinases were studied using specific inhibitors.

Microscopic image data were analyzed using custom-developed algorithms and software, which enabled measurement of PCNA and RPA1 signal intensities in individual replication foci and quantitative determination of bound and free protein fractions. Western blot analysis was used to validate protein expression levels and compare them with their endogenous counterparts.

The results demonstrate that during replication stress, PCNA is rapidly removed from the forks, while RPA gradually accumulates on ssDNA. Restoring the nucleotide pool after HU treatment allows rapid fork restart and RPA removal. ATR inhibition significantly accelerates RPA accumulation, leading to RPA depletion and residual ssDNA, while ATR and ATM co-inhibition results in mitotic catastrophe. The study also highlights the role of RAD18 in PCNA removal and fork stabilization, whereas MRE11 inhibition has no significant effect.

The methods applied enable detailed real-time tracking of protein dynamics and provide new insights into the cellular response to replication stress. This study represents significant progress in understanding these processes and opens new avenues for the development of anticancer therapies targeting ATR and ATM signaling pathways.

5. Results Achieved

The dissertation presents an innovative study of replication fork dynamics during stalling and restart. Under replication stress induced by hydroxyurea, two main processes were observed: the rapid removal of PCNA, reflecting a decrease in DNA synthesis, and the gradual accumulation of RPA on ssDNA, reaching up to 2400 nucleotides per fork, even with an active intra-S checkpoint.

ATR inhibition accelerated RPA accumulation ninefold, depleting cellular RPA reserves within 20 minutes. During fork restart, ATR inhibition caused residual ssDNA (up to 600 nucleotides per fork). While ATR inhibition did not affect PCNA re-accumulation or RPA removal kinetics during restart, it resulted in a residual fraction of RPA1 remaining at replication foci. Co-inhibition of ATR and ATM led to even greater ssDNA accumulation and mitotic catastrophe. On the other hand, restoring the nucleotide pool after HU treatment allowed rapid fork restart without residual ssDNA, ensuring smooth cell cycle progression.

Additional experiments revealed that MRE11 inhibition does not affect PCNA and RPA dynamics, while E3 ligase RAD18 accumulates at stalled forks alongside PCNA removal. Using quantitative analysis and mathematical modeling, the kinetics and precise amounts of proteins involved in these processes were determined. These findings not only expand understanding of the cellular response to replication stress but also provide a foundation for developing new anticancer therapies targeting ATR and ATM signaling pathways.

6. Conclusion

Teodora Dyankova-Danovska’s dissertation is a high-quality scientific study that provides original results elucidating the cellular response to replication stress, with potential applications in biomedical sciences. The author demonstrates excellent competence in the field and has successfully applied state-of-the-art experimental methods developed in the Laboratory of Genome Stability.

The dissertation is supported by three related publications, one of which has been submitted for publication (with DOI). The total impact factor of the publications exceeds 20, and they have been cited over 140 times. Based on this, I conclude that Teodora Dyankova-Danovska is a highly qualified young researcher who fully meets the requirements for the academic degree “Doctor.”

I recommend that the members of the esteemed scientific jury award Teodora Dyankova-Danovska the academic degree of “Doctor.”

Assoc. Prof. Dr. Anastas Gospodinov
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