REVIEW

by Prof. Albena Jordanova - Sofia University "St. Kliment Ohridski", Faculty of Medicine, Department of Chemistry and Biochemistry, Physiology and Pathophysiology, member of the scientific jury, appointed by order No. 1035/01.12.2024 of the Director of the Institute of Molecular Biology "Acad. Rumen Tsanev" - BAS, Associate Professor Anastas Gospodinov, PhD

for a dissertation for awarding the educational and scientific PhD degree in the Field of higher education: 4. Natural Sciences, Mathematics and Informatics, Professional direction: 4.3. Biological Sciences, Scientific specialty: Molecular Biology

Author: *Teodora Krasimirova Dyankova-Danovska - full-time PhD student in the Laboratory of Genomic Stability, Institute of Molecular Biology "Acad. Rumen Tsanev" – Bulgarian Academy of Sciences*

Dissertation topic: *Kinetics of protein accumulation and removal from the replication fork during its stalling and restart*

Research supervisor: *Associate Professor Stoino Stoinov, PhD, Laboratory of Genomic Stability, Institute of Molecular Biology "Acad. Rumen Tsanev" - BAS*

1. General presentation of the procedure and the PhD student

The presented materials for reviewing the PhD study of Teodora Dyankova-Danovska are in full compliance with the Law on the Development of the Academic Staff in the Republic of Bulgaria, the Rules for the Implementation of the Law on the Development of the Academic Staff in the Republic of Bulgaria and the Rules for the Implementation of the Law on the Development of the Academic staff at the Institute of Molecular Biology "Acad. Rumen Tsanev", and includes the following documents:

- Regulations for the implementation of the law on the development of the academic staff at the Institute of Molecular Biology "Acad. Rumen Tsanev" at the Bulgarian Academy of Sciences;

- CV in pdf and doc format, including a complete list of publications and participation in scientific forums;
- Dissertation in pdf and doc format;
- Author's summary of dissertation in pdf and doc format;
- List of publications and 3 scientific articles on the topic of the dissertation, as well as separation protocols for the doctoral student's contributions to the conducted scientific research and analysis of the results;
- List of citations (145 citations) of publications on the topic of the dissertation (until 27.03.2024);
- Diploma for the acquired master's degree in Biotechnology, master's program Genetic and Cellular Engineering;
- Protocols of the passed mandatory exams of the PhD student;
- Order 363/30.06.2016 of the Director of IMB Prof. Iva Ugrinova for the enrollment of Teodora Dyankova in a full-time form of study for the acquisition of the educational and scientific PhD degree in the specialty "Molecular Biology", as well as order 482/21.06.2019 of the Director of IMB Prof. Iva Ugrinova for the withdrawal of the PhD student with the right to defense;
- Order No. 1035/01.12.2024 of the Director of IMB-BAS Assoc. Prof. Atanas Gospodinov for the appointment of a scientific jury for the defense of the dissertation work for the acquisition of the educational and scientific PhD degree;
- Certificates for participation in international scientific conferences and doctoral symposiums, etc.

The dissertation was presented and discussed at an extended seminar of the Laboratory of Genomic Stability at the Institute of Molecular Biology "Acad. Rumen Tsanev" - BAS on 19.07.2024.

2. Relevance of the topic

In the PhD Study of Teodora Dyankova-Danovska - a full-time doctoral student at the Laboratory of Genomic Stability, Institute of Molecular Biology "Acad. Rumen Tsanev" - BAS (dismissed in 2019 with the right to defend), a large-scale study of the observed dynamic processes in the accumulation and removal of key proteins involved in replication during stalling and restart of the replication fork was conducted through the application of modern highly informative molecular biological methods.

In this dissertation, experiments were performed with 3 double HeLa Kyoto cell lines expressing the proteins RPA-EGFP, POLD2-EGFP, PAXIP-EGFP and PCNA-mCherry and an approach is presented for the study of the dynamics of replisome components under conditions of replication stress and the subsequent recovery of the forks at a time resolution of 30 seconds. The removal of the clamp *Proliferating cell nuclear antigen* (PCNA), which acts as a processing factor for DNA polymerase δ in eukaryotic cells and is essential for the normal course of replication, as well as the accumulation of *Replication protein A* (RPA) - a major protein that binds to single-stranded DNA (ssDNA) regions in eukaryotic cells, upon hydroxyurea-induced depletion of the nucleotide reserve, was visualized, and the kinetics of both processes were precisely studied. PCNA repair and RPA removal during replication fork restart within the same replication foci have been identified and visualized. The influence of *ATM-related protein* (ATR) - a fundamental kinase that regulates replication origin initiation, replication fork stability and cell cycle progression; *Poly(ADP-ribose) polymerase 1* (PARP1), which rapidly detects damage to DNA structure and directs to repair mechanisms; *Ataxia telangiectasia mutated* (ATM) – the main protein kinase responsible for the phosphorylation of H2AX at sites of DNA damage, plays a role in the activation of cell cycle checkpoints, the repair of double-strand breaks and the regulation of the "guardian of the genome" p53 in response to DNA damage and *Meiotic recombination 11* (MRE11) – a protein involved in homologous recombination, maintenance of telomere length and repair of double-strand breaks in DNA on the dynamics of forks during their arrest and restart has been studied and analyzed in detail in the presented dissertation work.

Despite the mechanisms established so far in the processes of fork reversal, degradation and restart of the forks, the central role of BRCA1 and BRCA2 in a specific type of DNA damage *homologous recombination repair* (HRR) against nucleotide degradation of replication forks, as well as the role of PARP1 in the regulation of fork restart, there are still no definitive results regarding the replisome dynamics during the entire process of replication fork stalling, its reversal and restart at the single cell level. Therefore, the experiments conducted in the dissertation work, the obtained results and their in-depth interpretation are of great importance for clarifying the detailed mechanisms for the participation and kinetics of accumulation and removal of proteins from the replication fork during stabilization of a stalled replication fork and during its restart.

3. Knowing the problem

After getting acquainted with the dissertation work, the author's abstract and the scientific publications of Teodora Dyankova-Danovska, I can state that the PhD student is well acquainted with the analyzed scientific problem, creatively evaluates the scientific research of the cited authors and can accurately and competently interpret them. The goal that Teodora Dyankova-Danovska has set for herself is clearly and precisely formulated: to study the kinetics of accumulation and removal of key proteins involved in replication during fork arrest and restart. To achieve the set goal, 5 main tasks have been envisaged and implemented, which include the study of:

- the kinetics of accumulation and removal of PCNA and RPA upon stalling and restarting of the replication fork in the presence and absence of ATM and ATR kinase inhibitors;
- the kinetics of accumulation and removal of POLD2 upon stalling and restarting of the replication fork, both with active and inactivated ATR kinase;
- the influence of inhibition of MRE11-dependent resection on the dynamics of accumulation and removal of PCNA and RPA upon stalling and restarting of the replication fork.
- the influence of inhibition of PARP1-dependent paring on the kinetics of accumulation and removal of PCNA and RPA upon stalling and restarting of the replication fork.
- the influence of inhibition of the ATM replication checkpoint on the kinetics of PAXIP accumulation at sites of complex DNA damage.

4. Research methodology

To conduct experiments using *HeLa Kyoto* cell lines obtained by transfection with BAC (*Bacterial Artificial Chromosome*), which stably co-express the analyzed proteins: RPA-EGFP, POLD2-EGFP, PAXIP-EGFP and PCNA-mCherry. The selected research methods in the dissertation work: *cell cultivation and treatment; conducting and analyzing microscopic Time-lapse experiments using the computer program CellTool in its own and absence of various inhibitors (hydroxyurea, HU; HU and AZD6738 - ATR kinase inhibitor; HU and KU55933 - ATM inhibitor; as well as a combination of HU, AZD6738 and KU55933); conducting and analyzing experiments with microirradiation of living cells, Western blot analysis, etc*. are completely adequate, realistic and highly informative for achieving the set tasks and goals of the dissertation work.

5. Characterization and evaluation of the PhD work

The dissertation is excellently designed and presents in detail the research carried out by the PhD student. The dissertation covers 107 pages, contains 41 figures and 2 tables. The bibliography includes 234 literary sources from reputable and indexed scientific publications, a large number of which are from recent years.

The extremely detailed and up-to-date *Literature Review* for the dissertation includes modern ideas for determining the origin sites for the start of the replication process, the mechanisms of assembly of the replication fork, the unique processes of synthesis of DNA fragments in the leading and lagging strands, as well as the amazing processes observed during the termination of replication. Particular attention is paid to the factors that cause replication stress, as well as the role of cell cycle checkpoints, which are responsible for genomic stability and, in the event of DNA damage, stop cell cycle progression and provide an opportunity for DNA repair before the next stage of the cell cycle. My only remark on this section of the dissertation is that more figures could be included for a more informative and detailed illustration and presentation of the mechanisms and processes under consideration. Numerous scientific studies on the topic of the dissertation are cited and analyzed in the *Literature Review*, which makes an excellent impression. The goal of the dissertation is clearly formulated, and the tasks set for implementation (described above) summarize the guidelines for performing and analyzing the planned experiments.

The *Results* obtained by Teodora Dyankova-Danovska are described in detail and presented excellently with appropriate figures on 40 pages, with a subsequent *Discussion* section. Results were obtained in 6 main areas, and of fundamental importance for revealing the mechanisms studied in the dissertation work is the development of the highly informative computer platform *CellTool* for fast, easy and accurate analysis of microscopic images.

- The contribution of the PhD student in the development of the graphic interface design, image analysis protocols, writing of the documentation and testing of *CellTool* is described. With this computer program, single living cells were monitored in real time and the dynamics of PCNA and RPA1 were followed during the arrest and subsequent restart of the replication fork with hydroxyurea in the presence and absence of various inhibitors.
- The results obtained give with precision the times and rates of accumulation and removal of the studied proteins, and it was found that the inhibition of ATR dramatically increases

the amount and rate of accumulation of RPA1 at stalled replication forks. In addition, it was shown that ATR prevents the post-replication presence of single-stranded DNA after restart and subsequent mitotic catastrophe. Inhibition of ATM alone, a protein involved in the regulation of cellular responses to DNA damage, had no effect on the dynamics and amounts of PCNA and RPA upon stalling and restarting replication forks.

- When stalling the replication fork with hydroxyurea and inhibiting both kinases ATR and ATM with the inhibitors AZD6738 and KU55933, PCNA removal and slightly faster accumulation of single-stranded DNA were observed than in experiments with AZD6738 alone. The inhibition of ATM and ATR prevented the removal of RPA upon stalling of replication forks.
- When treating the studied cells with *mirin* (an inhibitor of the MRN complex, in which the nuclease MRE11 also participates) and an ATR inhibitor, no difference was observed in the dynamics of RPA and PCNA during fork arrest and restart.
- To establish the role of PARP1 during replication fork arrest and restart, talazoparib (BMN673) was used, as inhibition of PARP1/2 slowed the accumulation of RPA under conditions of nucleotide starvation induced by HU in the absence of ATR activity.
- The kinetics of accumulation and removal of RPA and PCNA proteins during replication fork arrest and restart were studied. It was found that the dynamics of PCNA and RPA are constant in the three studied cell lines in all experimental conditions, but differences in the accumulation and removal of RPA are observed.
- When the replication fork is stopped with hydroxyurea, the fluorescently labeled protein POLD2 (*DNA Polymerase Delta 2, Accessory Subunit*) is removed together with PCNA. When HU is removed, POLD2 is restored to the restarted fork together with PCNA. In the presence of the ATR inhibitor AZD6738, after stopping the replication fork and after treatment with hydroxyurea, PCNA foci decrease and POLD2 foci accumulate.
- The kinetics of accumulation and removal of PAXIP (a nuclear protein involved in the response to DNA damage and the regulation of gene expression) at the sites of complex DNA damage induced by UV laser in the presence and absence of the ATM inhibitor KU55933 were studied, and accumulation of PAXIP and PCNA at the damage sites was observed. In the presence of the ATM inhibitor, PAXIP does not accumulate, while PCNA levels remain the same, indicating that ATM inhibition is the reason for the lack of PAXIP at the sites of complex DNA damage.

- By Western blot it was measured that the levels of RPA-EGFP are 1:3, compared to the endogenous levels of RPA, while the levels of mPCNA-mCherry are approximately 1:4.2, compared to the endogenous levels of PCNA.

Based on the complex experiments conducted and analysis of the results obtained, 6 conclusions have been summarized and 2 scientific and scientific-applied contributions have been formulated.

6. Evaluation of the publications and personal contribution of the PhD student

The results of the research conducted in the dissertation work were published in the period 2018-2024 in 3 scientific articles with an impact factor (*International Journal of Molecular Sciences - Q1, IF=4.9/2024; International Journal of Molecular Sciences - Q1, IF=5.6/2023; Molecular Cell - Q1, IF = 14.714/2018*) and thus the legal requirements have been fulfilled for acquiring the scientific and educational PhD degree. In the last published scientific article (12.2024), the PhD student is the first author, which proves the leading role, the emphasized interest and commitment of Teodora Dyankova-Danovska to the researched issues. The obtained results have been reported at 10 International and National scientific forums. Over 140 citations of the scientific publications in which the doctoral student participated have been noted.

In conclusion, the presented dissertation work categorically proves that PhD candidate Teodora Krasimirova Dyankova-Danovska possesses serious and in-depth theoretical knowledge and professional skills in the scientific specialty of Molecular Biology, demonstrating exceptional qualities and skills for independently conducting, analyzing and presenting scientific research results.

7. Author's summary of dissertation

The presented author's summary of the dissertation work of Teodora Krasimirova Dyankova-Danovska is carefully prepared and in accordance with legal requirements. It fully corresponds to the content of the dissertation and provides comprehensive information about the experiments conducted, the results obtained, discussion and analysis of the research conducted.

CONCLUSION

The PhD study presented for defense is the result of a precisely conducted study, the results of which are of significant theoretical and applied significance. Based on all of the above, I can confidently state that the peer-reviewed dissertation on the topic "*Kinetics of accumulation and*

removal of proteins from the replication fork during its stopping and restart" represents an original scientific work. It meets all the conditions of the Law on the Development of the Academic Staff in the Republic of Bulgaria, the Regulations for its Application and the Regulations of the Institute of Molecular Biology "Acad. Rumen Tsanev" - BAS.

All of the above gives me reason to confidently express my positive assessment of the research carried out in the PhD study, the achieved results and contributions of a scientific and scientific-applied nature, and I propose to the honorable scientific jury to award the fully deserved educational and scientific PhD degree to Teodora Krasimirova Dyankova-Danovska in the field of higher education: 4. Natural sciences, mathematics and informatics, Professional direction: 4.3. Biological Sciences, Scientific specialty: Molecular Biology.

17/12/2024 **Author of the statement:**

(Prof. Albena Jordanova, PhD)