REVIEW

on the dissertation work of Georgi Todorov Danovski "Mechanisms of the spread of γH2AX and MDC1 beyond the DNA damage site" presented for the award of the educational and scientific degree "Doctor" in the field of higher education 4. "Natural sciences, mathematics and informatics", professional direction 4.3. Biological sciences, doctoral program: "Molecular Biology".

Reviewer: Assoc. Prof. Dr. Anastas Georgiev Gospodinov

1. General Part

Georgi Todorov Danovski developed his doctoral dissertation at the Institute of Molecular Biology "Acad. Rumen Tsanev" (IMB) of BAS, in the Genomic Stability Laboratory, under the doctoral program "Molecular Biology" under the scientific supervision of Assoc. Prof. Dr. Stoyno Stoynov. The dissertation work covers 108 standard pages and is illustrated with 23 figures, citing more than 270 literary sources. The work was reported at a meeting of the extended scientific seminar of the IMB and was unanimously approved for defense. A review of the documents related to the defense shows that the procedure for enrollment, training, and discharge of the doctoral student has been followed, and the documentation has been prepared in accordance with the requirements of the Law on Academic Staff Development in the Republic of Bulgaria (LASDRB) and its implementation rules.

2. Biographical Information

Georgi Todorov Danovski was born on February 1, 1990, in Sofia. He graduated from the National High School of Mathematics and Natural Sciences "Acad. L. Chakalov" in 2009. In 2013, he completed a bachelor's degree in "Molecular

Biology" at Sofia University "St. Kliment Ohridski", and in 2015, a master's degree in "Biochemistry" at the same university.

From September 2014 to March 2015, he was an intern under the "Erasmus +" program at the Nuclear Oncology Research Team at the University of Nantes, France.

In 2016, he was enrolled as a doctoral student in the specialty "Molecular Biology" at IMB "Acad. Rumen Tsanev". Georgi Danovski is the author of 8 scientific articles (with a total impact factor over 33.7), of which 3 are directly related to his dissertation. These have been cited around 200 times. He has participated in 18 scientific conferences and courses and has been involved in numerous national biology olympiads and in preparing students for the national and international rounds of the biology olympiad.

3. Relevance of the Developed Topic

Georgi Danovski's dissertation is dedicated to studying the mechanisms of spreading γH2AX and MDC1 beyond the DNA damage site.

H2AX is a substrate of kinases ATM (Bakkenist & Kastan 2003) or DNA-PK (Bakkenist & Kastan 2003; Jackson 2002) in response to double-strand breaks and ATR in signaling replication stress (Cimprich & Cortez 2008; Zou & Elledge 2003). In mammalian cells, phosphorylated H2AX encompasses large chromatin domains and forms nuclear foci that can be easily visualized by immunostaining (Rogakou et al. 1998). The spread of gamma-H2AX in nuclear regions of megabase size around the double-strand break is attributed to an amplification mechanism involving the MDC1 protein. The gamma-H2AX-bound MDC1 binds Nbs1 and stabilizes the MRN complex at the double-strand break (Stewart et al. 2003), leading to further ATM binding (Lou et al. 2006; Stucki et al. 2005). This creates a positive feedback loop for the spread of H2AX phosphorylation (Savic et al. 2009). A similar interaction of MDC1 with TopBP1-bound ATR links the ATR kinase to chromatin under conditions

that favor the generation of single-stranded DNA (Lee et al. 2010). Despite these results, the mechanisms of gamma-H2AX spread remain unclear. For example, Gaelle Legube's group highlights the role of cohesin-mediated loop extrusion in the spread of gamma-H2AX in double-strand breaks. This process involves the binding of cohesin at the double-strand break sites, allowing the extrusion of chromatin loops and leading to H2AX phosphorylation on either side of the double-strand break and focus formation (Arnould et al. 2021).

The topic is of great significance as DNA damage and its repair are fundamental processes for maintaining genome stability. Numerous studies in this field demonstrate the relevance of the problem.

4. Knowledge of the Problem

The literature review in Georgi Danovski's dissertation includes an extensive overview of existing studies and theoretical models related to DNA damage and repair mechanisms. The main sections of the review are:

- Types of DNA Damage: Various types of DNA damage, including single-strand and double-strand breaks, oxidative damage, and damage caused by external agents like ultraviolet light and chemicals, are discussed.
- DNA Repair Mechanisms: The main mechanisms of DNA repair, including direct repair, base and nucleotide excision repair mechanisms, different double-strand break repair pathways, DNA crosslink repair, and mismatch repair, are described. The role of histone H2AX and associated repair factors is outlined. The function of MDC1 as a mediator in the DNA damage signal, facilitating the recruitment of other repair proteins to the damage, is discussed. Molecular interactions between γH2AX and MDC1 and their role in spreading the DNA damage signal beyond the damage site are described. Existing mathematical models describing the kinetics and dynamics of repair protein spread in the nucleus are reviewed.

5. Achieved Results:

The main goal of the dissertation work is to study the process of spreading γH2AX and MDC1 beyond the damage zone during the repair of complex DNA damage as a consequence of ATM kinase activity and to propose a mathematical model describing its mechanism. To achieve this goal, four tasks were formulated:

- Development of software for analyzing microscopic experiments with UV laser micro-irradiation and FRAP;
- Tracking the kinetics of accumulation and removal of the studied proteins at sites of complex DNA damage;
- Development of a mathematical apparatus and software for calculating biological models of reaction-diffusion and modeling the spread of MDC1 and ATM in the nuclei of cells with complex DNA damage.

Various methods from different scientific fields were applied for their execution: live cell microscopy in which the studied proteins are fluorescently labeled, micro-irradiation to induce complex DNA damage, and FRAP (fluorescent recovery after photobleaching) of the studied cells, immunofluorescence microscopy, complex mathematical apparatus for biophysical modeling of repair processes, development of complex software products for image analysis and calculation of mathematical models.

As a result, Georgi Danovski's dissertation presents significant results that can be divided into two major groups:

- Development of CellTool software: Original software for analyzing microscopic images was developed and validated. The software was used to measure the

kinetics of accumulation and removal of MDC1 and ATM proteins in live cells and the ongoing exchange processes at damage sites.

- Modeling the spread of γH2AX and MDC1: Several mathematical models describing the spatial and temporal spread of γH2AX and MDC1 were developed. The data support the hypothesis that the diffusion of activated ATM can explain the observed spread of γH2AX and MDC1 beyond the DNA damage site. The theoretical models are confirmed through various experimental techniques such as fluorescence microscopy and FRAP, proving that the spread of γH2AX and MDC1 does not require processes like chromatin loop extrusion.

The core of the work is the developed software CellTool, which allows quantitative processing of a large volume of data from the conducted microscopic studies in the Genomic Stability Laboratory. The program combines all necessary tools for analyzing the kinetics of proteins involved in DNA repair processes and for analyzing FRAP experiments. The program offers a user-friendly graphical interface, providing quick, easy, and accurate image analysis. The software has an integrated file manager, tools for image preparation (cropping specific fragments), algorithms for filtering and segmenting images, object tracking, visualization of measured results, and data analysis - regression analysis using predefined models for FRAP and micro-irradiation, as well as the ability to introduce new models. The value of this freely available software for genomic stability research is clearly confirmed by its application in several publications.

As a user of the software, I would like to note that besides high functionality, CellTool offers convenience for work with a well-thought-out interface made with the researcher's needs in mind. This is extremely rare among the many software applications for solving various tasks.

In the execution of the experimental tasks of the dissertation, it was established that:

1. MDC1 quickly accumulates at the damage site with a half-time of 55 seconds, reaches maximum levels around 900 seconds, and then spreads beyond the damage site;
2. ATM accumulates faster than MDC1 (half-time of 40 seconds) but does not spread beyond the damage site. FRAP experiments show that ATM exchanges quickly at the damage site;
3. The NIPBL factor loading cohesin and the cohesin subunit RAD21 accumulate at the damage site significantly later than MDC1, suggesting that loop extrusion is not necessary for the initial spread of γH2AX/MDC1.

As a result of applying a complex mathematical apparatus, 3 models for describing the spread were created:

1. Standard model - The model assumes that both the bound and free activated ATM (αATM) phosphorylate H2AX. It describes both the accumulation and spread of MDC1 and the dynamics of ATM. The model assumes that the effective diffusion coefficient of the activated ATM (αATM) is much smaller than that of the inactive ATM, likely due to multiple binding and release events.

2. Minimal model: This model assumes the immediate release of activated ATM without the need for other intermediate interactions. The model provides good agreement with experimental data, suggesting that the bound intermediate states are not necessary to explain the spread of MDC1.

3. Restricted model: This model assumes that ATM is only active when bound to the damage site, without the diffusion of αATM, restricting phosphorylation to the immediate vicinity of the damage site. This model failed to predict the early stages of MDC1 accumulation and its spread beyond the damage site, indicating that the diffusion of activated ATM is critical.

It is concluded that the diffusion of activated ATM is a sufficient mechanism to explain the spread of γH2AX and MDC1 beyond the DNA damage site, rejecting the proposed mechanism which suggests that loop extrusion is necessary for the spread of H2AX and MDC1 phosphorylation. Thus, through experimental data and mathematical modeling, a comprehensive understanding of the spatiotemporal dynamics of key DNA repair processes is achieved.

6. Conclusion

According to Nobel laureate Sydney Brenner, progress in science depends on new tools, new discoveries, and ideas, "probably in that order of decreasing importance." This is fully justified in Georgi Danovski's dissertation. By creating a new effective and convenient tool, Georgi Danovski not only solves the tasks of his dissertation but also enables others in the field to work successfully with minimal effort, thus making others' research endeavors possible. In this sense, the value of the reviewed work far exceeds the usual for such a type of work. With the help of the created software and the application of many other state-of-the-art cell biology techniques, the dissertation achieves significant results in understanding a key process for maintaining genomic stability. Given the above, it is not surprising for the reader that the dissertation is written precisely, with attention to detail, and a deep understanding of the issues. It clearly shows that Georgi Danovski is a versatile researcher capable of working at the intersection of several scientific fields, applying the most appropriate approaches to solving scientific tasks, regardless of their complexity. Wishing him similar and even greater success in his future professional path, I firmly recommend that the esteemed scientific jury award Georgi Danovski the well-deserved educational and scientific degree of "Doctor."

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