OPINION

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

on the doctoral dissertation "Study of the Dynamics of Processes in Living Cells Using Advanced Microscopic Approaches,"

submitted by **Alexander Sergeev Atemin** (Laboratory of Genome Stability, Institute of Molecular Biology, BAS) for the educational and scientific degree **"Doctor"**

The dissertation titled "Study of the Dynamics of Processes in Living Cells Using Advanced Microscopic Approaches," presented by Alexander Sergeev Atemin for the educational and scientific degree "Doctor," was conducted at the Laboratory of Genome Stability, Institute of Molecular Biology, BAS. Initially supervised by Assoc. Prof. Marina Nedelcheva-Veleva, and later in collaboration with Assoc. Prof. Dr. Stoyno Stoynov as the doctoral advisor.

The work of Alexander Atemin focuses on developing and applying innovative approaches to study highly dynamic processes at the cellular level, leveraging the advantages of confocal laser microscopy for real-time *in vivo* visualization. The combination of methods from molecular, cellular, and chemical biology (cloning and transfection to generate fluorescent marker lines, advanced light microscopy, treatments with chemical inhibitors) and programming (developing software algorithms for analyzing microscopic data) enabled new insights into endomembrane trafficking of viruses and the spatial-temporal distribution of specific nuclear proteins in human cells. Specifically, the dissertation focuses on two main directions: (1) studying the mechanisms of SARS-CoV-2 internalization in host cells and (2) the dynamics of key protein regulators of DNA replication during the cell cycle. The obtained results are significant, contributing to a better understanding of fundamental cellular processes and paving the way for potential innovative therapeutic strategies for combating diseases related to SARS-CoV-2 infection and irregular DNA replication and repair.

For the first research direction, the candidate collaborated with a partner laboratory from the University of Utah, which developed a protocol for obtaining, purifying, and fluorescently labeling SARS-CoV-2 virus-like particles with preserved conformation and stoichiometry of structural SARS-CoV-2 proteins, but with compromised replication. Using these particles, A. Atemin conducted detailed microscopic experiments to elucidate the sequence, duration, and coordination of the steps of viral entry into the host cell. After validating the preserved functionality of the virus-like particles, the candidate tracked their trajectories, movement speeds, and fluorescent signal strength using a custom algorithm developed at the Laboratory of Genome Stability, IMB-BAS, specifically for this study. Atemin played a significant role in testing and refining the software application. Consequently, it was determined that upon attachment to the cell surface, the particles slow down their movement, whereas their speed increases upon internalization into the cytoplasm. This entry occurs through dynamin-dependent endocytosis, where colocalization of the particles with dynamin foci and their acceleration (internalization) are

closely timed events, and inhibition of dynamin function prevents the internalization of the particles into the cell. Utilizing fluorescently labeled Rab5a, a marker for early endosomes, revealed a lack of colocalization with the internalized virus-like particles, which were instead detected in acidic compartments labeled with LysoTracker. Additionally, double-labeled particles with a protein pH sensor showed that the particles often reach acidic compartments before disintegrating, with acidification and increased movement speed occurring closely in time. It was demonstrated that the levels of the ACE2 receptor and TMPRSS2 protease in the host cell affect the number of internalized particles. Another important observation was that the speed and efficiency of virus-like particle internalization vary significantly depending on the target cell type. Particles containing a mutated Furin cleavage site in the S protein sequence were endocytosed similarly to non-mutated particles, indicating that this cleavage does not affect cell entry. Mutations in the Omicron variant also showed no impact on internalization dynamics. The candidate further analyzed the kinetics of nucleocapsid release relative to the stages of internalization. Using virus-like particles with fluorescently labeled N protein, it was established that nucleocapsid release and endosomal compartment acidification occur simultaneously. Overall, the precisely done measurements during the dissertation work revealed, for the first time, the dynamics and some regulatory mechanisms of SARS-CoV-2 endocytosis in host cells. The findings provide a foundation for developing new antiviral therapies targeting the internalization of viral particles in target cells.

For the second scientific direction, A. Atemin prioritized studying the dynamics of accumulation and distribution of five protein regulators of DNA replication in human cell lines throughout the cell cycle. These proteins are known to participate in the licensing and activation of replication origins, as well as replication control (RIF1, Claspin) and DNA damage repair (PCNA, RIF1). Using dual fluorescent marker lines, Atemin conducted measurements and data normalization from several individual cells to determine the average duration of the entire cell cycle and its phases. Quantitative analysis of the fluorescent signal and the size of PCNA foci, after applying a contrast-modulating filter to microscopic images, allowed the candidate to clearly distinguish three sub-phases of the S phase. Observing the studied protein regulators of DNA replication against the fluorescence background of PCNA-mCherry revealed that RIF1 and MCM6 peak in early G1 phase, followed by a gradual decline. ORC1 showed the highest levels in early S phase, while Claspin was characterized by maximal levels during mid and late S phases. The recorded dynamics of protein accumulation and localization within the nucleus correlated with their known functions in cell cycle processes.

A significant contribution by the candidate was his participation in the creation of two freely accessible databases, which systematize and visualize information generated during this and previous research at the Laboratory of Genome Stability.

The literature review and the list of 247 cited sources reflect the excellent knowledge of the candidate regarding existing research in the field of DNA replication mechanisms, as well as in an emerging field with a vast amount of scientific information related to SARS-CoV-2 and the disease COVID-19 caused by the virus. A. Atemin successfully summarizes the currently published data on the structure and life cycle of the virus in a synthesized form. Without diminishing the informational value, I believe that the strategies for treating COVID-19 are described in excessive detail, as they are not directly related to the experimental work of the

candidate. A review of the initial stages of replication and the key proteins involved in licensing replication origins and initiating the process is also included. Unlike the previous chapter of the review, here the candidate has not illustrated the text content with figures adapted from key publications on the discussed topic.

The aims and tasks of the dissertation are concisely and clearly presented. The experimental methods are exhaustively described, allowing for reproducibility. Minor inaccuracies were noted, such as descriptions of inhibitor and dye concentrations (p. 43). It is important to note that A. Atemin clearly distinguished his experimental contributions from those of other collaborators regarding both methodology and results.

The "Results" section constitutes nearly half of the dissertation's volume, excluding the bibliography, and includes 24 figures, most of which consist of several data panels. The experimental results and methods of calculation are clearly described and presented in a logical sequence, following the order outlined in the aims and tasks section. The applied approaches to study the trafficking of virus-like particles provide comprehensive information, allowing the candidate to draw well-supported conclusions. However, to definitively prove the movement of the particles along the microtubule network, I consider it necessary to conduct additional experiments in the future. The assumption that the increased speed of particle movement during internalization in the cell is likely due to active transport along microtubules is based on observed colocalization of fluorescent signals. These data could be further supported by other approaches, for instance treatment with photoswitchable inhibitors of microtubule dynamics, such as photostatins.

The "Discussion" section spans six pages, where A. Atemin successfully interprets the results he obtained in a concise and structured manner, aligning them with existing literature. Regarding the endocytosis of SARS-CoV-2, the discussion could further address the potential influence of the ACE2 receptor on the dynamics of viral internalization and nucleocapsid release. It is known that following SARS-CoV-2 recognition and binding to ACE2 via the S protein, the receptor enters the cell along with the virus. In the absence of a ligand, most receptor molecules undergo recycling (moving from the plasma membrane to endosomes and back), while only a small fraction is directed to lysosomes for degradation as part of the protein turnover. Many trafficking regulators participate in the recycling process. One such regulator (SNX27) has already been shown to potentially recognize a specific motif in the cytoplasmic domain of the S protein, in addition to ACE2. The binding of SNX27 to the viral particle suppresses ACE2 recycling, thereby reducing the receptor levels on the cell surface. The stage at which SARS-CoV-2 separates from ACE2 and how this process relates to the viral particle's entry remains unknown.

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 the publication activity of Alexander Atemin.

The candidate has published three articles on the dissertation topic in Q1-ranked international journals, where A. Atemin's individual contributions are clearly distinguished from those of other co-authors. In two of these articles, A. Atemin is the first author. The information provided by the candidate and the additional checks demonstrate A. Atemin's high scientific

activity and the quality of his research output. He is a co-author in five other scientific publications unrelated to the dissertation topic. To date, articles with his involvement have been cited over 115 times according to Scopus and over 175 times according to Google Scholar. Over an eight-year period, A. Atemin has participated in 11 scientific conferences in Bulgaria and abroad.

CONCLUSION:

The dissertation convincingly summarizes several years of fundamental research by Alexander Atemin. The candidate developed an innovative approach to studying cellular processes with high molecular dynamics, such as the entry of SARS-CoV-2 particles into host cells and the spatial-temporal distribution of protein regulators of replication during the cell cycle. The obtained results have potential implications for developing new approaches to treating socially significant diseases like COVID-19. The online platforms created with A. Atemin's participation, featuring processed data from experiments with advanced live-cell light microscopy, represent a valuable resource for other research groups working in the same field of research. A. Atemin's access to high-tech platforms at IMB has enabled him to develop into a highly qualified researcher in the fields of molecular and cell biology. The candidate also stands out for his significant publication activity, with high citation rates reflecting the relevance and importance of his findings. Based on the materials presented for review and additional revisions, **I confidently recommend that the esteemed academic jury award Alexander Sergeev Atemin the educational and scientific degree of "Doctor"** in the professional field 4.3. Biological Sciences, Scientific specialty "Molecular Biology."

15.11.2024

Prepared by:
(Kiril Mishev)