REVIEW

by Prof. Dr. Iva Ugrinova from the Institute of Molecular Biology, Bulgarian Academy of Sciences (БАН).

The review concerns Dr. Stefan Dimitrov's dissertation on obtaining the scientific degree "Doctor of Sciences." The dissertation topic is **"Chromatin: From Structure to Function."**

The review follows **Protocol No. 1 (dated 16.12.2024)** from the first meeting of the scientific jury, appointed by **Order No. 209-OB (dated 06.12.2024)** from the Director of IMB-BAS.

Documents and Procedure Validity:

The dissertation, its abstract in Bulgarian, and all required documents, including publications and a meeting protocol, comply with the current regulations governing this procedure.

Thematic Scope:

The research focuses on structural epigenetics, specifically understanding histones and chromatin fibers' structure and functional role. The study examines key topics such as histone H1 binding to the nucleosome, 3D organization of chromatin fibers, the influence of different histone variants on chromatin structure and functions

Scientific Field:

The research belongs to the field of chromatin biology and epigenetics, with a strong emphasis on Molecular biology, Biochemistry, and Structural biology

Methodological Approach:

The dissertation employs a multidisciplinary approach, utilizing advanced techniques such as Cryo-electron microscopy, Hydroxyl radical footprinting, and Biophysical analyses. This suggests that the research integrates structural and functional studies to advance understanding chromatin organization and its role in gene regulation.

Structure, sections, objectives, original results, and conclusions of the dissertation:

The dissertation consists of 309 standard typewritten pages. It is presented as a collection of scientific publications supplemented by a comprehensive literature review and a connecting discussion. The review covers the thematic scope of the dissertation, mainly focusing on chromatin and histone variants. It cites 178 bibliographic sources. The literature review Demonstrates exceptional knowledge of the research topic. It is readable and well-structured, easy to follow, and provides detailed information on the current state of research worldwide. This description suggests the dissertation is well-founded in existing literature while contributing original findings in chromatin biology and epigenetics. The dissertation sets out two clearly and logically formulated objectives, both related to the organization of chromatin in the nucleus. The first is to determine the crystal and cryo-EM structures of nucleosomes bound to H1 to shed light on their organization in solution. The second focuses on how the core histone variants macroH2A and H2A. Modulating the organization of

nucleosomes, Bbd, H2A.Z, and CENP-A allows the proper functioning of the nucleus. Thus, the formulated objectives solve two major scientific questions:

1. **How does linker histone H1 bind to the nucleosome?**
2. **How does the chromatin fiber fold into a denser chromatin structure?**

These questions have remained unresolved for more than forty years since the structure of DNA and the nucleosome particle was determined.

This dissertation presents an original, interdisciplinary approach that successfully addresses and resolves these two fundamental epigenetic problems. Regarding methodologies, the author employs hydroxyl radical footprinting, combined with EMSA (Electrophoretic Mobility Shift Assay) and cryo-electron microscopy, to map the contacts between linker histone H1 and DNA in mono-, di-, and tri-nucleosome complexes with single-base resolution (*Publication No.1: Syed et al. 2010, PNAS*). Using these data for DNA modeling, he develops a molecular model that illustrates how different domains of H1 interact with the nucleosome, predicting a specific H1-mediated "stem" structure in the linker DNA (*Publication No.2: Mayer et al. 2011, Nucleic Acids Res.*). This is followed by determining the crystal and cryo-EM structure of the nucleosome with H1 at molecular resolution (*Publication No.3: Bednar et al. 2017, Mol. Cell*). The study employs a 197-base-pair nucleosome complex containing symmetric 25-base-pair linker DNA arms in a complex with linker histone H1. The analyses reveal that H1 alters the nucleosome conformation by bringing the two linker segments closer and reducing their flexibility. The main conclusions the author draws are that the C-terminal domain of H1 localizes mainly on one linker, while the globular domain of H1 contacts the nucleosome dyad and both linker DNA strands, thereby H1 introduces asymmetry into the nucleosome, which likely influences the structure of higher chromatin levels. These structures are further validated through covalent protein crosslinking and hydroxyl radical footprinting.

To analyze the higher-order organization of chromatin, Dr. Dimitrov uses a hexanucleosome system bound to linker histone H1. In these experiments, crystallography at 9.7 Å resolution reveals the structure of this particle (*Publication No.4: Garcia-Saez et al. 2018, Mol. Cell*). The obtained double-helical structure demonstrates uniform nucleosome stacking but with lower density compared to the twisted 30-nanometer chromatin fiber. Data from cryo-EM and subsequent biophysical analyses confirm that small changes in ionic strength can lead to a compact, twisted form. Dr. Dimitrov's studies represent a breakthrough in structural epigenetics. Moreover, the determined 3D organization of the H1-bound nucleosome and the double-helical chromatin fiber structure have recently been confirmed *in vivo*. It is well known that chromatin is highly condensed in the cell nucleus, with the highest level of compaction occurring in mitotic chromosomes. By measuring the elasticity of mitotic chromosomes, Dr. Dimitrov proposes a model for their structure (*Publication No.5: Houchmandzadeh & Dimitrov, 1999, J. Cell Biol.*).

The cell relies on epigenetic mechanisms, such as chromatin remodeling, post-translational modifications of histones and DNA, and the incorporation of histone variants, to extract the necessary genetic information. **The second aspect** of the dissertation is related to histone variants and their role, covering 11 manuscripts (from No.6 to No.16), published in top journals such as *Nature, Molecular Cell, Nature Structural and Molecular Biology, The EMBO Journal, and Proceedings of the National Academy of Sciences, USA*. These articles

are pioneering and explore the interaction between different nucleosome conformations containing histone variants and their functional effects.

The first article, published in 2003 (*Angelov et al., Mol. Cell*), shows that macroH2A histone hinders the binding of transcription factors and the remodeling of nucleosomes by the SWI/SNF complex. MacroH2A is the most unusual histone variant, consisting of a histone H2A-like domain and a large non-histone region (NHR), with the specific properties of macroH2A nucleosomes primarily being due to the protein's NHR part. Taken together, the data suggest that macroH2A is involved in transcriptional repression.

Interestingly, H2A.Bbd nucleosome, which contains another histone variant, exhibits "opposite" properties (*articles No. 7–9*). The incorporation of H2A.Bbd results in lower nucleosome stability both in vitro and in vivo. Additionally, transcription activated by p300 and Gal4-VP16 appears more efficient for H2A.Bbd nucleosome arrays than for conventional H2A arrays.

Of particular interest are Dr. Dimitrov's contributions to elucidating the structure-functional relationships of the CENP-A nucleosome (*publications No.10 and 11*). CENP-A belongs to the H3 histone family and replaces H3 in centromeric chromatin. It is deposited in chromatin through a specific interaction between the CENP-A domain and its chaperone HJURP (*publication No.11*). Using cryo-electron microscopy, Dimitrov and his collaborators determine the dynamic properties of the CENP-A nucleosome in solution. Major biochemical, proteomic, and genetic analyses reveal that increased DNA-end flexibility reduces histone H1 binding to the CENP-A nucleosome. *In vivo*, replacing CENP-A with hybrid H3-CENP-A nucleosomes leads to H1 recruitment, mislocalization of kinetochore proteins, and significant mitotic and cytokinetic defects. The data show that the evolutionarily conserved flexibility of CENP-A nucleosome ends is crucial for ensuring proper mitotic progression. These scientific publications are key to understanding the role of centromeres in assembling active kinetochores in both normal and pathological conditions.

The histone variant H2A.Z, a mysterious protein with multiple functions, is also the subject of research by Dr. Dimitrov's team. Initially, the author and his collaborators identified the first chaperones of H2A.Z in mammals (*publications No.12, 13*). They demonstrate that ANP32E is a histone chaperone that removes H2A.Z from chromatin. YL1, on the other hand, is responsible for the specific deposition of H2A.Z and its replacement with conventional H2A. Through genomic studies in mice, combined with biochemical and omics approaches, the team shows that H2A.Z plays a role in premature aging and initiates DNA repair in double-strand breaks (DSB) in muscle fibers (*publications No.14, 15*). The depletion of H2A.Z in epidermal stem cells reveals a strong connection between innate immunity, epigenetics, and inflammation (*publication No.16*). These recent findings confirm that H2A.Z is a key factor in maintaining both genome integrity and nuclear homeostasis. Furthermore, they explain the pleiotropic functions of H2A.Z in fundamental nuclear processes—questions that have remained open since the discovery of H2A.Z as a histone variant.

The conclusions, as formulated in the dissertation, along with the described contributions, can be considered as a well-structured summary of the main results of the research program.

Publication Activity and Scientometric Characteristics

The original results are based on 15 publications published in the most prestigious international journals with a high impact factor, such as *Nature, Molecular Cell, Nature Structural and Molecular Biology, Nucleic Acids Research, The EMBO Journal, Proceedings of the National Academy of Sciences, USA,* and others.

- The total impact factor of these 15 publications is 219.
- The works have been cited 2,335 times.

This staggering scientometric data speaks for itself.

Abstract

The abstract appropriately compiles the structure and content of the dissertation.

Significance of the Research

This dissertation represents a significant breakthrough in structural epigenetics. Determining the structure of the H1-bound nucleosome and chromatin fiber at high resolution provides knowledge comparable in importance to the discovery of the DNA double helix and the nucleosome particle. Additionally, the functional studies on histone variants offer a comprehensive insight into their roles in transcription regulation, chromatin remodeling, genome stability, and nuclear homeostasis.

Recommendations:

1. I recommend the dissertation for the highest academic evaluation due to its revolutionary scientific contributions.
2. I recommend that the author further explore how these structural and epigenetic findings can be applied in therapeutic interventions for diseases associated with chromatin dysregulation, such as cancer and neurodegenerative disorders.

CONCLUSION:

Dr. Stefan Dimitrov's dissertation is an exceptional scientific study. It combines structural biology, biophysics, and functional genomics to answer fundamental questions in chromatin biology. The work is characterized by originality, methodological precision, and an interdisciplinary approach. The obtained results significantly expand our understanding of chromatin architecture and its regulatory mechanisms.

Beyond science, I have known Bat' Stefko for over 20 years as an excellent teacher, mentor, and friend. With his return to Bulgaria and the institute, he has also managed to convince colleagues of these incredible personal qualities.

Based on the above, I **positively evaluate** the dissertation and recommend that the honorable members of the scientific jury award the Doctor of Sciences degree to Dr. Stefan Ivanov Dimitrov.

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