OPINION

By **Prof. Dimitrinka Yordanova Atanasova-Dimitrova, PhD**

Member of the scientific jury, approved by Order No. P-109-222/28.04.2025

of the Rector of the Medical University "Prof. Dr. Paraskev Stoyanov" - Varna

of the dissertation work

of **Iskren Boyanov Velikov**, MD

on the topic

"ROLE OF THE TRANSCRIPTIONAL FACTOR ZBTB20 IN CEREBELLUM DEVELOPMENT"

For awarding the educational and scientific degree "Doctor of Philosophy"

In the scientific specialty "Anatomy, Histology and Cytology"

In professional direction 7.1 Medicine,

An area of higher education 7. Health care and sports

Scientific supervisor of the PhD student: Prof. Dr. Irina Stoyanova – Van Der Laan, MD, PhD

This opinion has been prepared with a view to the public defense before a scientific jury of the dissertation presented by the doctoral student Iskren Boyanov Velikov for the acquisition of the educational and scientific degree "Doctor of Philosophy" in the scientific specialty "Anatomy, Histology and Cytology".

I. Brief biographical data about the doctoral student

Iskren Boyanov Velikov, MD, was born on March 1, 1983, in the town of Razgrad, Bulgaria. He graduated with honours from the High School of Natural Sciences and Mathematics "Acad. Nikola Obreshkov" in his hometown, specializing in "Biology with English". In 2007, he earned a Master's degree in Dental Medicine with distinction from the Medical University of Plovdiv. After a brief clinical internship, he began working as an assistant professor in 2009 at the Medical University "Prof. Dr. Paraskev Stoyanov" – Varna, where he has been conducting practical classes in anatomy, histology, and embryology for students in medicine, dental medicine, and pharmacy.

In 2013, he obtained a specialty in "Anatomy, Histology, and Cytology". In 2019, he began full-time doctoral studies at the Department of Anatomy and Cell Biology at the same university. His dissertation focuses on the molecular regulation of cerebellar development, with an emphasis on the

transcription factor Zbtb20. Iskren Velikov, has a B2 level of English proficiency, certified by an international qualification.

Currently, Dr. Iskren Velikov is a member of the Bulgarian Anatomical Society, the European Federation for Experimental Morphology (EFEM), and the International Federation of Associations of Anatomists (IFAA).

II. General impressions and relevance of the topic

The dissertation work of Iskren Velikov makes a strong impression due to its scientific **maturity, experimental precision, and conceptual soundness**. The study is the result of systematic work that combines modern methods of immunofluorescence, quantitative morphometric analysis, and critical interpretation of the results in the context of contemporary neuroscience.

The topic of the role of the transcription factor **Zbtb20 in cerebellum development is scientifically relevant and original**. Although Zbtb20 is the subject of growing interest in other brain structures (e.g. hippocampus, neocortex), its function in cerebellar morphogenesis and cell differentiation is poorly understood. Velikov's work fills this gap, revealing the selective glial expression of Zbtb20 and its role in modulating foliation, Purkinje cell populations, and oligodendrocyte proliferation.

The combination of morphological and molecular approaches, along with the analysis of age- and region-specific effects, lends the study high scientific value and an innovative character. This makes it not only relevant within the framework of fundamental neuroscience but also potentially relevant for clinical neuropathology, where Zbtb20 is associated with diseases from the spectrum of neurodevelopmental disorders.

III. Structure and clarity of the exposition

The dissertation on the topic "Role of the transcription factor Zbtb20 in the development of the cerebellum" is written 142 pages and is divided into sections as follows: *Table of Contents* – 3 pages, *Abbreviations used* – 1 page, *Introduction* – 2 pages, *Literature review* – 30 pages, *Aim and objectives* – 2 pages, *Material and methods* – 3 pages, *Results* – 56 pages, *Discussion* – 16 pages, *Conclusions* – 2 pages, *Contributions of the dissertation work* – 1 page, *Publications and reports related to the dissertation work* – 1 page, *Bibliography* – 24 pages including 254 literary sources, all in Latin, *Acknowledgements* – 1 page. The work is illustrated with 97 figures.

The **introduction** to Iskren Velikov's dissertation effectively fulfils its purpose of presenting the topic, its scientific significance and relevance, as well as the primary motivation for conducting the

research. It is structured logically, starting with a general overview of the development of the central nervous system and gradually focusing on the cerebellum and its morphogenetic features. A brief summary of the role of transcription factors is presented, with an emphasis on Zbtb20, which smoothly leads to an outline of the scientific problem.

The style of presentation is scientific, precise and clear, without deviations from the topic. The introduction demonstrates a good understanding of the problem and prepares the reader for the specific goals and objectives of the dissertation, which follow immediately thereafter. The doctoral student successfully justifies the need for his research by highlighting the insufficient knowledge of the role of Zbtb20 in cerebellum development – a topic with a clearly expressed innovative and unexplored aspect.

The **literature review** in the dissertation is highly comprehensive and systematic. It provides a thorough overview of the key aspects related to cerebellar development, neurogenesis, transcription factors (especially Zbtb20), as well as the cellular and molecular markers used in this context. The structure of the review is logically organized and well-aligned with the objectives of the study.

Anatomical and functional basis (items 2.1–2.2) The sections on morphology, histology and embryonic development of the cerebellum are comprehensive, with clear anatomical and histogenetic explanations. Modern sources are used, and phylogenetic and species comparisons are presented, which enriches the context.

Neurogenesis (item 2.3) is comprehensively described, with an emphasis on both embryonic and postnatal neurogenesis. The place of the rhombic lip and the ventricular zone as sources of different cell lineages is clarified. Cell migrations, the influence of factors such as Shh and the roles of radial and Bergmann glia are well-traced.

Transcription factors (section 2.4)- The review on Zbtb20 is the strongest part of the literature review, as it presents structural characteristics, functional roles in different organs and tissues, effects of deficiency, involvement in pathologies, and development. Own analyses of niches in the existing literature are included, which justify the need for the present study. The section on Pax2/Pax6 and other factors, such as Tbr1/Tbr2, complements the understanding of brain architecture regulation and provides a basis for experimental hypotheses.

Neuronal and glial markers (section 2.5) Excellent systematization of the most commonly used markers: BrdU, Calbindin, NeuN, GFAP, DCX, etc. Basic properties and applications are deduced, including limitations and a lack of data for some markers in the cerebellum of Zbtb20-/- mice, which contributes to the scientific justification of the dissertation.

The "**Goal and Objectives**" section is structured clearly and precisely. The doctoral student first formulates the primary scientific goal and then lists nine specific tasks aimed at realising this goal. The formulations are specific, realistic, and entirely consistent with the intended research direction.

The "**Materials and Methods**" section is methodologically sound, technically correct and suitable for reproduction. Modern histological, molecular, and statistical approaches were employed, fully compatible with the dissertation's goals. The section is detailed, consistently presented and logically justified. It includes all key subcomponents: 1) type and origin of the animals (Zbtb20+/+, Zbtb20-/-); 2) age periods of analysis (E16, P4, P8, P12, P30); 3) histological and immunofluorescence techniques; 4) list of antibodies used (primary and secondary); 5) preparative and analytical protocols (fixation, cryosectioning, microscopy); 6) Software and statistics.

The *Zbtb20-knockout mouse model* used is adequate and fully meets the study's purpose. The author used homozygous mutants, which allows for complete blockade of the Zbtb20 function. An ethical protocol (LAVES, Germany) is included, which demonstrates compliance with European requirements.

Recommendation: There is no information on the number of animals used at ages E16 and P30, while for the others (P4–P12), n=6 per group is clearly defined.

The **Results** section is presented at an exceptionally high scientific level. The results of a series of experiments, including morphological, cellular and molecular studies performed on the cerebellum of wild-type and Zbtb20-/- mutant mice, at several key postnatal age stages (P4, P8, P12), as well as at embryonic day E16 and adulthood (P30), are systematically presented. Each section includes an introduction to the specific experiment, a description of the observations, quantitative analysis, visualization by photomicrographs, and statistical processing (t-test, p-values).

In the dissertation work of Iskren Velikov, the expression and functional role of the transcription factor Zbtb20 in the development of the cerebellum in a mouse model was systematically followed. The doctoral student demonstrated that the normal expression of Zbtb20 is limited to germinal and glial zones – outer and inner rhombic lips, outer granular layer and Purkinje layer – with a lack of expression in postmitotic neurons, including NeuN+, Calbindin+, Calretinin+ and Tbr2+ cells. Immunohistochemical and quantitative analyses showed that Zbtb20 is expressed in neuronal precursors and Bergmann glia, which clearly defines its role as a regulator of early differentiation and glial maintenance.

Morphological analysis in Zbtb20-/- mutants revealed abnormalities in cerebellar foliation (additional clefts and subfoliations in areas III, IV–V, VI, IX, X), with preserved overall volume,

indicating the involvement of Zbtb20 in the topographic organisation of the cerebral cortex. Analysis of layer morphology (EGL, ML, IGL, Purkinje cell layer) revealed focal but statistically significant differences in thickness and cellular composition, especially at P12, including the retention of EGL and altered numbers of Calbindin+ cells, suggesting impaired migration, differentiation, or survival. Granule neurons (Pax6+) did not exhibit a global reduction; however, regional disorganisation was observed in Zbtb20-/-mice. Proliferative activity (Ki-67+) was also unchanged globally but increased locally in specific folia at a later postnatal stage. Interneuronal (Tbr2+) and brush-type (Calretinin+) cells were not quantitatively affected, highlighting the cell selectivity of Zbtb20. Zbtb20 was also found to be involved in the oligodendrocyte lineage (Olig2+) without any indication of pathological glial proliferation. In summary, the work demonstrates that Zbtb20 plays a vital role as a cell-specific and topographically restricted regulator of glial differentiation, architectural patterning, and cellular stability in the developing cerebellum.

Recommendations: 1) multivariate analysis: I recommend using ANOVA for group comparisons to exclude random differences; 2) Correlations between parameters: There are no analyses linking foliation to cellular parameters – for example: foliations with increased proliferation and additional folds. 3) tables: I think it would be useful to summarize the results by cell type, age and effect in a table.

The Results section presents a systematic and detailed study with a multi-layered approach, combining anatomical, cellular and molecular analysis. Velikov demonstrates a high level of analytical thinking, the ability to integrate histological and statistical data, and the capacity to draw original scientific conclusions. The results show both new findings and confirmation of known hypotheses, emphasizing the importance of Zbtb20 as a regulator of local morphogenetic and cellular processes in the developing cerebellum.

The **Discussion** section represents a comprehensive and in-depth component of the dissertation, successfully integrating the experimental results within the context of contemporary neuroscientific literature. The doctoral student demonstrates a high level of scientific culture by tracing the connections between Zbtb20 expression and the regulation of glial and precursor neuronal populations in the developing cerebellum. Through a systematic analysis, it is argued that Zbtb20 primarily functions in the glial niche, including Bergmann glia, astrocytes, and oligodendrocytes, and thus indirectly influences neuronal differentiation, migration, and the organisation of cortical plasticity. The discussion of disorders in the foliation and dynamics of Purkinje cells is scientifically convincing and timely placed in the context of the spatial regulation of cerebellar development. The

PhD student objectively reports both the observed effects and the lack thereof on specific cell populations (e.g., interneurons, brush-like cells), emphasising the specificity of Zbtb20's action. The argument is substantiated with references; however, I recommend including molecular mechanisms or model schemes of interaction. Overall, the section serves as an example of scientific maturity and a well-constructed conceptual framework.

The **"Conclusions"** section contains 14 clearly formulated and logically structured conclusions that reflect the main results of the dissertation research. Velikov consistently presents the key effects of Zbtb20 on various cell populations in the cerebellum, emphasising its selective expression in glial cells and its role in cerebellar foliation and cellular organisation. The conclusions are objective and entirely consistent with the results obtained, demonstrating full coverage of the set goals and objectives.

The PhD student formulated both original and confirmatory **contributions**. Among the original ones, the quantitative and qualitative analysis of neuronal and glial populations in the cerebellum of Zbtb20-/- mice and the proposal for a new concept for glial-mediated regulation of brain development by Zbtb20 stand out. The contributions are significant, well-argued and clearly defined, attesting to the originality of the scientific research and its value for neuroscience.

The **conclusion** presents a semantic and conceptual summary of the dissertation work, placing it in a broader biomedical context. The author not only emphasises the significance of his findings but also demonstrates scientific maturity by highlighting unresolved questions and providing directions for future research.

The **"Publications and Scientific Communications"** section effectively presents the scientific commitment and activity of the doctoral student throughout the entire dissertation preparation period. The inclusion of a scientific publication in a peer-reviewed journal with content closely related to the dissertation topic demonstrates the author's ability to formulate and publish significant scientific results. Participation in reports and posters at six scientific forums, including international conferences and national congresses related to anatomy, neuroscience, and development, is particularly impressive. These appearances show that the doctoral student not only works in depth on his topic but also actively presents it to the scientific community, demonstrating confidence, consistency and the ability to defend his results

The doctoral student's publication activity is adequate at the time of the defence, but it has significant potential for expansion and international recognition. It is recommended that the dissertation research results be developed into at least two or three thematically focused publications

in international, specialised journals with a higher impact factor. This will increase the visibility and citation of the work, and the contributions will receive wider recognition in the scientific community.

The abstract of Iskren Velikov's dissertation clearly and comprehensively presents the main highlights of the dissertation work, synthesising in a concise form the scientific justification, objectives, methods used, main results, and contributions. The abstract is logically structured, with well-formulated sections that provide a comprehensive overview of the course and research content. The scientific terminology is used correctly, and the style is concise and clear without straying from the scientific focus. The presented results are synthesised to emphasise the most significant observations and original aspects of the work. The conclusions and contributions are consistent with what was actually achieved and convincingly formulate the value of the research.

IV. Conclusion

The dissertation work of Iskren Velikov represents a methodologically precise, scientifically sound, and original study on the role of the transcription factor Zbtb20 in cerebellum development. The topic is timely and of high importance for fundamental neuroscience, as the work successfully fills an existing gap in knowledge about the functions of Zbtb20 outside of the classically studied structures, such as the hippocampus and cortex. The experimental approach is modern and combines various histological, immunofluorescent, and quantitative methods applied to a well-characterised animal model. The PhD student has formulated clear goals that have been fully achieved, and the results have been comprehensively analysed and objectively discussed in the context of the international scientific literature. The conclusions reached not only confirm existing concepts but also introduce new perspectives on glial mediation in neuronal organisation. The scientific contributions are clearly defined, and the publication activity has the potential to expand.

I confidently declare that Iskren Boyanov Velikov possesses the qualities of a researcher, and I confidently express a positive opinion about the developed dissertation work; as a member of the Scientific Jury for the procedure, I cast my positive vote for awarding the educational and scientific degree "Doctor of Philosophy " to Iskren Boyanov Velikov.

Prepared the opi

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06.06.2025г.

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