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**Review of the PhD thesis entitled
“Structural characterization and conformational changes of Retinol Binding Protein 3”
by Luca Gessa.**

The PhD thesis under review was prepared at the Institute of Physical Chemistry, PAS at the International Centre for Translational Eye Research in Warsaw under the supervision of Prof. Maciej Wojtkowski and Dr. Humberto Fernandes. The dissertation is based on an original research study devoted to the structural and functional characterization of retinol-binding protein 3 (RBP3), a key component of the interphotoreceptor matrix involved in retinoid transport and the visual cycle.

The subject of the dissertation addresses an important and scientifically challenging problem situated at the interface of structural biology, biophysics, and vision research. Despite the fundamental role of RBP3 in retinal physiology and its association with several retinal pathologies, structural information on the full-length native protein has remained very limited. Therefore, the topic of the thesis fills a significant gap in our understanding of the molecular mechanisms underlying retinoid transport in vertebrate vision.

The most important achievements and original findings described in this thesis, which substantially advance the current state of knowledge regarding RBP3 structure and function, include:

- (i) determination of a 3.67 Å resolution cryo-EM structure of full-length native porcine RBP3 without the use of antibody fragments;
- (ii) identification of rigid and flexible regions within the four-module architecture of the protein;
- (iii) experimental demonstration that binding of retinoids, but not fatty acids, induces measurable conformational changes of RBP3 in solution;
- (iv) identification and comparative analysis of putative ligand-binding cavities and inter-domain clefts, providing a structural framework for understanding retinoid transport and exchange.

In the Introduction, the PhD candidate presents a comprehensive and well-structured overview of the visual cycle, the biological role of the interphotoreceptor matrix, and the current structural and functional knowledge of RBP3. The literature is discussed competently and critically, allowing the reader to clearly understand the context of the undertaken research and

the specific questions posed in the thesis. This part of the dissertation demonstrates the candidate's excellent command of the subject matter and an ability to synthesize complex information.

The Materials and Methods chapter describes the tools used to achieve the research goals. Although the list of techniques is relatively detailed, numerous inconsistencies are noticeable. These include, for example, inconsistent notation of buffer concentrations (alternating use of “x”, e.g., 1x TAE buffer, and “X”, e.g., 1X Taq buffer), as well as inconsistent spacing between numerical values and unit abbreviations. Units are decoded in the List of Abbreviations and in some cases at first use in the text, although this is not done uniformly. For instance, the abbreviation “ng”, first used to specify template concentration in the PCR reaction described in subsection 2.1.1.1, is only explained later in subsection 2.1.1.3, concerning restriction enzyme digestion. Additionally, restriction enzymes should be denoted using Roman numerals rather than Arabic ones (e.g., BamHI instead of BamH1).

I also noted occasional missing spaces before parentheses. In subsection 2.1.2, it is not specified whether “0.01 mM” refers to the final IPTG concentration or that of the working solution. Moreover, this value appears inconsistent with subsection 3.1.1, which states that 50 μ M IPTG was used for induction and that, among several concentrations tested, 0.1 mM was ultimately selected. In subsection 2.1.2.1, it is not clarified whether glycerol concentration is given as m/v, v/v, or another ratio. A similar ambiguity applies, for example, to the ethanol concentration in the all-trans retinol solution in subsection 2.1.2.6.

In summary, this chapter contains an almost complete list of the experimental techniques used by the PhD student. For an experienced researcher, the above-mentioned nuances and the comparatively concise procedural descriptions should not pose major difficulties in reproducing the experiments. Importantly, MSc. Luca Gessa consistently provides at least brief explanations of the underlying principles of the applied methods, which significantly improves the overall comprehensibility of the work. Nevertheless, the thesis should be treated as an integral whole, because in some cases, particularly in the Results section, information about the expected outcomes of experiments and the implications of negative results is missing. For example, in subsection 3.1.3, it is not emphasized that the lack of all-trans retinol binding by the *Bos taurus* RBP3 M4 module, demonstrated by fluorescence analysis, effectively disqualified this preparation from co-crystallization trials with the ligand. This conclusion only appears later, at the beginning of subsection 3.2.1.

The Results section constitutes the core of the dissertation and documents an extensive experimental effort combining cryo-EM, small-angle X-ray scattering (SAXS), biochemical purification and binding assays, and molecular docking analyses. The presented results can be divided into two main parts. In the first, MSc. Luca Gessa focuses on attempts to obtain soluble fragments of *Bos taurus* RBP3 using a bacterial expression system. Individual modules (M1-M4), as well as tandem constructs (M1M2, M2M3, and M3M4), were produced as independent recombinant proteins with the aim of probing retinoid binding via X-ray crystallography. Because full-length RBP3 is glycosylated in most higher eukaryotes, and glycosylation likely

contributes to protein solubility, it is not surprising that most overexpressed modules produced in bacteria accumulated in inclusion bodies. The GST-bRBP3M4 fusion protein was partially soluble, and considerable effort was invested in obtaining sufficient material for crystallization. However, the resulting preparation proved nonfunctional, as it did not bind all-trans retinol, for reasons discussed further in subsection 4.1 of the Discussion. This raises the question of why no attempts were made, prior to isolating full-length RBP3 from bovine retinas, to express individual modules or the full-length protein in eukaryotic heterologous systems, such as yeast, insect, or mammalian cells.

In the second part of the Results section, the PhD student describes an ambitious strategy aimed at studying full-length, functional RBP3 isolated from natural sources using cryo-EM and SAXS. Initial attempts involved *Bos taurus* RBP3 complexed with various ligands (all-trans retinol, 11-cis retinal, and DHA). When these efforts failed due to the inability to prepare grids suitable for single-particle analysis, analogous experiments were conducted with the *taur* orthologue. However, the quality of 2D class averages for ligand-bound complexes precluded reliable 3D reconstruction, which the author attributes to increased local flexibility induced by ligand binding. At this point, it would be useful to know whether ligand-to-protein ratios other than 1:2 were tested, and whether excess unbound ligand was removed prior to grid preparation. The structural data obtained for *Sus scrofa* RBP3 are described in considerable detail, which deserves commendation. The extensive commentary on the 3D structure allows the reader to identify all major conformational features and puts them into homologous context through comparative analysis using the DALI algorithm. The resulting model exhibits higher resolution in the central region (from domain B of module 2 to domain A of module 4) than near the N- and C-termini. This higher-resolution core was used for docking simulations with 11-cis retinal, all-trans retinol, DHA, and oleic acid. The docking analyses revealed up to 20 potential binding sites, including several previously unreported ones, each of which is discussed in detail.

The Discussion section closely mirrors the structure of the Results section, which facilitates navigation and allows for easy cross-referencing between experimental observations and their interpretation.

General comments regarding the Results and Discussion include the following:

1. Table legends are placed below tables rather than above them. In the case of Table 2, this leads to clarification of the table contents only after turning the page (pp. 61-62) and causes an unnecessary text break, with the preceding text ending on page 63 instead of 61.
2. Some figure legends lack essential information, for example:
 - a) In Figures 3.5, 3.7, 3.12, 3.18, and 3.19, fractions analyzed by PAGE are not clearly designated, nor is it specified which fractions were used in subsequent steps.
 - b) In Figure 3.15 (p. 56), the type of SEC column used should be indicated (presumably Superdex 200).
 - c) In Figure 3.21 (p. 60), panel C shows scatter plots without error bars; error

estimates should be provided for fitted curves.

- d) In Figures 3.23 and 3.25, indicating the order of modules 1-4 or at least marking the N- and C-termini would make it easier to follow.
 - e) Labels in Figures 3.28 - 3.30 and ligand representations in Figures 3.32A and B are difficult to read; alternative rendering styles may improve clarity.
 - f) Figure 3.33 (p. 79) lacks a Kratky plot for RBP3 titration with oleic acid.
 - g) In Table 8 (p. 88), the unit of the reported values should be given as percent (%).
3. In some cases, a brief explanation of the purpose of a particular procedure or a clear reference to another section would be beneficial. For example, subsection 3.2.2 begins on page 54, but the rationale for purifying the mAb5 antibody does not become clear until page 56.
 4. There are occasional labeling errors or inconsistencies, such as Asn1114 versus Asn1014 on page 59, and inconsistent use of three-letter amino acid codes (e.g., p. 71).
 5. In Table 3, the first-column header is misleading and should refer to selected binding sites rather than ligands.

Despite these issues, the candidate demonstrates a high level of expertise in modern structural biology techniques, particularly in cryo-EM data processing and interpretation, as well as in integrating SAXS data with high-resolution structural models. The conclusions are formulated with appropriate caution, especially regarding docking-derived ligand-binding sites, and the limitations of these approaches are clearly acknowledged.

As a topic for scientific discussion during the public defense, it would be interesting for the candidate to elaborate further on the functional relevance of the observed ligand-induced conformational changes. Specifically, to what extent might the partial opening of the RBP3 structure observed by SAXS be sufficient for regulated retinoid loading and unloading *in vivo*, and how might this process be influenced by local protein-protein interactions within the interphotoreceptor matrix? Furthermore, could the observed conformational flexibility contribute differently to physiological versus pathological roles of RBP3? How does the candidate assess the relative functional importance of inter-domain clefts compared with deep domain B cavities, particularly considering partially overlapping docking predictions? Finally, how would future experiments be prioritized to experimentally validate the proposed binding sites?

The comments presented above primarily concern the formal and editorial aspects of the dissertation. While several areas could benefit from improved editing and systematization, there is no doubt that the obtained results, especially the improved-resolution structure of full-length RBP3 constitute a significant contribution to our understanding of the visual cycle in higher eukaryotes, including humans. This is due to the close evolutionary relationship between human RBP3 and the *Bos taurus* and *Sus scrofa* orthologues that were studied here. A particularly valuable element of the thesis is the discussion of disease-associated mutations in

the context of the newly determined structure. The structural interpretation of mutations linked to retinitis pigmentosa and other retinal disorders is convincing and provides a solid basis for future functional and biomedical research. The technical complexity of the project also deserves recognition, as obtaining cryo-EM data for a dynamic protein such as RBP3, especially in complex with ligands, is highly challenging. The candidate's reflections on potential methodological improvements further demonstrate critical thinking. The high scientific value of the dissertation is further confirmed by the publication of its core results in the reputable international journal *Open Biology*, in which the candidate is a shared first author.

The dissertation under review fulfills the requirements set forth in the Act of July 20, 2018, the Law on Higher Education and Science (Journal of Laws 2018, item 1668, as amended). Therefore, I recommend that the Scientific Board of the Institute of Physical Chemistry of the Polish Academy of Sciences admit the dissertation to the subsequent stages of the PhD defense procedure.

Sincerely,



Elżbieta Nowak