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Review of the doctoral dissertation of Mr. Thiliban Manivarma

The doctoral dissertation of Mr. Thiliban Manivarma submitted for evaluation to the Faculty of Physics, Astronomy and Informatics of the Nicolaus Copernicus University in Toruń is titled *"Regulation of Ferroptosis through 15LOX-1/PEBP1 Complex – Computational Modeling at Molecular Level"*. The thesis was supervised by Dr. hab. Karolina Karolina Mikulska-Rumińska, prof. NCU and prof. dr hab. Wiesław Nowak, both from the Department of Physics, Institute of Physics, Nicolaus Copernicus University.

The research topic of Mr. Manivarma's dissertation concerns ferroptosis, one of the mechanisms of programmed cell death, discovered only over a decade ago. Since cell death is a crucial process occurring either under physiological or stress conditions, the processes that lead to controlled cell death could undergo artificial regulation and the molecules involved could be targets for inhibitory drugs. The topic covered by the thesis is therefore up-to-date and related to potential therapeutics.

Ferroptosis is a regulated form of cell death during which lipid peroxides accumulate. The lipid peroxides form enzymatically with the help of lipoxygenases (LOXs, iron-containing enzymes), which catalyze the incorporation of oxygen into polyunsaturated fatty acids. Mr. Manivarma investigates how ferroptosis regulation is triggered by the complex of a 15-lipoxygenase-1 (15LOX-1) protein and phosphatidylethanolamine (PE)-binding protein 1 (PEBP1) at an atomistic level of detail.

The aims of the thesis listed on page 33 of the dissertation are clearly presented. The objectives were well-defined, fully accomplished, and are consistent with the presented results. This doctoral thesis aimed to elucidate the molecular mechanisms underlying ferroptotic lipid peroxidation mediated by the 15LOX-1/PEBP1 protein-protein complex, which is taking part in the ferroptosis process. The investigation of this complex included (i) explaining the dynamics of this complex alone and in the presence of a membrane and the stearoyl/arachidonoyl-PE (SAPE) substrate, (ii) explaining how the structural changes influence substrate entry and binding, and (iii) investigating the effects of mutations at the protein-protein interface to explain how critical interaction sites affect complex formation and catalytic function. The complex and mutations were investigated, focusing on their

role in directing substrate specificity, oxygen diffusion pathways, the conserved residues, and water molecules in the 15LOX-1 catalytic pocket.

The dissertation comprises two published articles in prestigious scientific journals and one article deposited in an archive. Both published articles already underwent external peer review by experts in the field. The manuscript deposited in the archive is probably still under review or revision as it has not appeared in a journal.

The articles are preceded by an Introduction written in English, approximately 30 pages in length. The Introduction covers regulated cell death mechanisms, the process of ferroptosis, explains the simulated systems, and the employed methods. Following the Introduction is a short description of the publications that constitute the thesis, Mr. Manivarma's contribution statement, and the aims of the PhD project. Subsequently, the author included about 150 references to scientific papers and software cited in the Introduction, along with reprints of the two published articles and one preprint. The Supporting Information files of these papers were not included in the thesis, likely due to their large size. Next, co-author contributions are attached in the form of their signed statements, followed by the Summary. An extensive Appendix explains how the author applied *WatFinder* to determine water bridges/clusters and the protocol to identify hotspots at the interface of the 15LOX-1/PEBP1 complex. The organization of the thesis is commendable and adheres to academic standards. I especially appreciate the figures in the publications, as the studied systems and results are well presented and labelled.

The first article was published in 2023 in *Free Radical Biology and Medicine* with the title "*Membrane regulation of 15LOX-1/PEBP1 complex prompts the generation of ferroptotic signals, oxygenated PEs*". The impact factor (IF) of this journal is 7.1. In this paper, Mr. Manivarma is listed as the first author out of 11 authors, which is understandable because this publication presents both experimental and computational research. This is a highly ranked journal in the field of redox biology.

This publication describes the molecular mechanism by which the 15LOX-1 complex with PEBP1 generates pro-ferroptotic lipid signals. Through molecular dynamics simulations and mass spectrometry experiments, the authors discovered that membrane association triggers a conformational change in the 15LOX-1/PEBP1 complex that creates an opening between the proteins, facilitating the access of the SAPE substrate to the catalytic site. The binding of SAPE induces further conformational changes that stabilize the complex in a closed form, which is optimal for the oxidation reaction. Both computational and experimental approaches confirmed the critical role of the P112E mutation of PEBP1 disrupting the complex and preventing the generation of ferroptotic death signals.

Author's contribution to *Article A* was in the computational approaches, which included molecular docking (using HDOCK and Smina), selecting the models, preparing the complex (including the mutants) and membrane system for simulations (using CHARMM-GUI), molecular dynamics simulations, and trajectory analyses (angles, channels, electrostatics). Molecular dynamics simulations were performed at the atomistic scale using the NAMD software with the CHARMM force field. Mr. Manivarma also wrote a Tcl script for VMD to compute the specific angles as a function of the simulation time. Overall, the computational part of *Article A* revealed how membrane

association triggers conformational changes that open a pathway for the SAPE substrate to access the catalytic site, and how substrate binding locks the complex in a catalytic closed conformation.

The second article, *Article B*, titled "*WatFinder: a ProDy tool for protein–water interactions*" was published in 2024 in *Bioinformatics*. This is a high-quality journal, with an impact factor of 4.4, publishing articles related to bioinformatics and computational biology. The work is a collaborative effort with seven authors, and Mr. Manivarma is listed as the fifth author.

The *Bioinformatics* paper introduces *WatFinder*, a tool (integrated into the ProDy Python API) for analyzing and visualizing protein-water interactions, including direct water bridges, water-mediated clusters, water channels and networks. The tool can analyze ensembles of structures, which is useful for trajectory analysis. Many capabilities of *WatFinder* were demonstrated through case studies included in the Supplementary Material. *WatFinder* provides the user with sites that could be susceptible to interactions with water molecules, which could help identify spots for inhibitor design by water replacement or blocking specific water channels. The tool conveniently generates plots and heat maps showing water-mediated interactions.

According to Mr. Manivarma's contribution statement, for *Article B*, he tested all functions and verified various parameters to assess water clusters using PDB and trajectory files.

In *Article C*, available as a preprint in *SSRN* (submitted to *Redox Biology*), titled "*The presence of substrate warrants oxygen access tunnels toward the catalytic site of lipoxygenases*", Mr. Manivarma is listed as the first author out of seven authors altogether. This work describes the molecular mechanisms by which oxygen molecules access the catalytic site of 15LOX-1 lipoxygenase during ferroptotic lipid peroxidation. Through molecular dynamics simulations (20 μs total) and redox lipidomics analysis, the authors identified two oxygen tunnels in this enzyme, a primary (*Entrance A*) and secondary (*Entrance B*) pathway, used in about 80% and 20% of cases, respectively. The study also reveals that SAPE substrate binding induces a conformational change that opens *Entrance A*, facilitating oxygen access to the catalytic site. The membrane was found to direct oxygen molecules more efficiently toward the tunnels. Furthermore, the study identified conserved sequence motifs and structurally important residues across the LOX family that maintain these oxygen pathways, highlighting the evolutionary importance of this mechanism for ferroptotic lipid peroxidation.

Mr. Manivarma's contributions to this work were the following. He performed molecular dynamics simulations of the 15LOX-1/PEBP1 complex, both without and with the SAPE substrate and the membrane, and under various oxygen concentrations. Mr. Manivarma also performed sequence alignments, electrostatic potential, and perturbation response scanning analyses. He applied the *WatFinder* tool to identify oxygen and water clusters in molecular dynamics trajectories, showing functional water networks. He created most of the panels in Figures 1–5 of the preprint.

Overall, I believe that Mr. Manivarma had a dominant contribution towards generating the computational results for *Articles A* and *C*, and an important contribution to testing the tool in *Article B*. Therefore, his contribution to results of this thesis warrants accepting him to the next stages of the doctoral procedure, namely the thesis defense.

According to my role as a Reviewer, I have a few comments regarding the thesis. Some are out of curiosity, and I hope the author can discuss these during the defense. Others are minor comments regarding the presentation of the thesis topic in the Introduction.

page 16: The author writes that "...ferroptosis is characterized by increased iron loading...". I was wondering in what situations the increased iron loading into cells occurs? When would this iron over-increase happen and how does the cell know it must turn on ferroptosis?

page 17: There is a minor typographical error on this page with the phrase "particularly oxidized" written twice.

page 18: The author describes numerous inhibition mechanisms, which makes the story difficult to follow if one is not an expert in the variety of proteins related to ferroptosis. A scheme would be useful to better understand the many inhibition mechanisms.

page 18: The sentence on "...preventing ferroptosis through multiple mechanisms." raises a question: Why would a cell want to prevent ferroptosis if this programmed cell death is so desirable?

page 19: The last sentence of section 1.2. This sentence is crucial for the thesis and is only at the end of this section, looking somewhat hidden.

page 22: Figure 5 could be more informative, at least secondary structure elements could be colored or combined with Figure 4. The figures in the Introduction stand out in terms of lower quality as compared to the great figures presented in the *Articles*.

page 24: It would be nice if Figure 5 showed how the iron cation interacts with the binding site.

page 27: "...analyses of both biological molecules and small molecules." Small molecules can also be biological, so I do not understand what the difference is.

page 27: Many water models are listed without any explanations or citations provided.

page 27: The sentence "Energy minimization aims to identify the system's global minimum energy configuration..." is not necessarily true. The author even writes in the next sentences that methods such as steepest descent guide the system towards the nearest energy minimum.

page 27: Last sentence. "Additionally, correcting the protonation states of titratable residues..." The author probably meant "assigning" not "correcting".

page 28. In the Introduction and also *Article A*, there is only a brief explanation regarding the force field parameters for iron, ("CHARMM force field parameters for bonded iron were obtained using Gaussian [131] (DFT B3LYP/6–31(d,p) method)." However, what does "bonded iron" mean? Since the author studied an iron-binding protein, I expected more information as to how iron is coordinated and discussion of the limitations of classical force fields for modeling iron-containing systems. Are specialized force fields not more appropriate for the catalytic iron? A similar comment pertains to *Article C*.

Article C. The 20 μsec cumulative trajectories are an impressive time scale. However, does the author believe that 200 ns trajectories for each system are enough to draw meaningful conclusions? Was there any statistical analysis performed to confirm the observed phenomena, particularly the opening of *Entrance A*?

I would like to understand how the homology model was validated in *Article A*. Was it through metrics like Ramachandran plots, QMEAN scores, or comparison with other available structural data?

I was wondering why this particular membrane composition (50% DOPC, 30% DOPE, 20% SAPE) was used. I could not find a justification for this specific composition.

Article B. I would like to know how the results of water behavior might depend on the water model used in molecular dynamics simulations and if this was tested.

Article B. I did not find a discussion on the efficiency or scalability of *WatFinder* and how this tool performs with large systems or extensive trajectory data, including memory requirements.

Article C. The Supplementary Figures and Tables were not included in the thesis and are also not available via the preprint link.

Overall, regardless of the above minor comments and questions, I believe that Mr. Manivarma's dissertation has made a significant contribution to the field of ferroptosis, which has not been fully understood. Mr. Manivarma is undoubtedly an expert in performing and analyzing molecular dynamics simulations, and also possesses knowledge in predicting protein-protein complexes, their interaction surfaces, and hotspot residues that make these interactions stable. He has mastered a range of molecular modeling and simulation techniques, from molecular docking, through sequence alignments, to molecular dynamics simulations and extensive analyses of trajectories. The protein-protein system he studied was difficult from many perspectives, due to the size, iron binding, peripheral interactions of proteins with membranes, and their low-frequency membrane-dependent internal dynamics.

In summary, I conclude that Mr. Thiliban Manivarma's doctoral dissertation, submitted as a collection of three thematically related scientific articles, shows that Mr. Thiliban Manivarma had a distinct and dominant contribution to computational work, as confirmed by his and co-author statements. The dissertation presents an original solution to a scientific problem, confirms Mr. Thiliban Manivarma's theoretical knowledge in the field of exact and natural sciences in the discipline of physical sciences, and demonstrates his ability to conduct independent scientific research. I conclude that the doctoral dissertation submitted for my review meets the conditions specified in Article 187 of the Act of July 20, 2018, Law on Higher Education and Science (Journal of Laws 2018, item 1668, as amended). Therefore, I propose to admit Mr. Thiliban Manivarma to further stages of the doctoral procedure.

J. Trylska

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