MICHIGAN STATE
UNIVERSITY

May 1, 2024

Dear Ms. Maria Pega,

Please find attached my review of the dissertation of Katarzyna Dziubek, titled “Dissecting the mechanism and functional landscape of cancer PD1 signaling in osteosarcoma”. I thank you and my colleagues from the International Centre for Cancer Vaccine Science University of Gdańsk, Poland for trusting me with this responsibility. I am honored.

Please let me know if any further information is required.

Kind regards,

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From: Prof. Dr. Vilma Yuzbasiyan-Gurkan, Michigan State University
To: Colleagues at University of Gdansk and Maria Pega
Date: May 1, 2024

I am pleased to provide this report of the thesis submitted by Katarzyna Dziubek, from the Intercollegiate Faculty of Biotechnology, University of Gdansk and Medical University of Gdansk, titled "Dissecting the mechanism and functional landscape of cancer PD1 signaling in osteosarcoma" submitted for completion of her PhD.

The studies included in this work address an important aspect of cancer biology, focusing on characterization and dissections of the molecular interactions of a key immune checkpoint molecule PD1, program death protein 1, in osteosarcoma. Osteosarcoma is a challenging cancer for which advances in outcomes for patients have been lacking for over three decades.

The work utilizes the human osteosarcoma cell line, U2OS, widely used in studies of osteosarcoma. Of note, the study reveals that PD1 is expressed by this cell line robustly intracellularly, as determined both by Western blot analysis as well as immunofluorescence microscopy, whereas membrane expression appears to be limited to a few percent of the cells, as determined by flow cytometry. Studies using siRNA probe the effects of suppression of PD-1 expression by three different siRNA constructs, two targeting different regions of exon 2, and one the 3'UTR, of the gene *PDCD1* which encodes PD1. Functional studies measuring migration and wound healing in scratch assays and cell viability demonstrate variability in the effect of the three siRNAs and the author goes through careful review of potential factors that may be at play, and reasons that an off-target effect associated with siPD#2 is likely the explanation of viability. Studies of the changes in the cellular proteome following the perturbation of PD1 expression with the siPD1s are particularly novel. Interestingly, the siPD treatment induces enrichment of MAPK signaling. I would like to note here that the presentation of this data is comparing the control to the siPD1 treatment (Figure 20), and the double negatives involved in processing and describing the data (page 62) is quite confusing and would suggest that this be more clearly stated or the graph revised to make the comparison based on siPD1 vs control, so that processes that are enriched by siPD1 treatment are reflected in positive values. Nevertheless, this finding is important as it indicates that PD1 may play a similar role in osteosarcoma as it does in T cells. Other processes also enriched included cell adhesion, migration, and motility. Further studies of the functional enrichment of proteins across all the siPD1 treatments also provided support for MAPK signaling and mTORC1 mediated signaling as being significant results of the perturbation of the PD1 equilibrium. This also points to the similarity of the role of PD1 in osteosarcoma cells as in T cells, with PD1 repressing mTOR signaling. Other major proteins upregulated by siPD1 treatment included cell adhesion molecule ALCAM, MUC18, Talin-1 and CSK, indicting a potential role of PD1 signaling in cancer metastasis, which requires further confirmatory studies. Silencing of PD1 was also found to downregulate the proteins, SERC (also called PSAT-1, phosphoserine aminotransferase), sulfiredoxin-1 and pirin, the latter two being related to oxidative stress. These are interesting observations and can form the basis for future studies.

Identification of proteins interacting with PD-1 in the osteosarcoma cell line constitute another set of important contributions of this doctoral thesis. The approach of overexpressing tagged recombinant PD1 protein in U2OS cells (used at bait) followed by pull-down experiments and the study of the proteins (the prey) thus isolated and analyzed after tryptic digestion with LC-MS/MS was used. Use of both N terminal and C-terminally tagged PD-1 was useful and revealed that the C-terminal tag resulted in appropriate post-translational modification and expected bands on Western blot analysis- and was chosen for further studies as the bait. In addition, phosphor tyrosine mutations (Y223F and Y248F) mutations were introduced to study how protein-protein interactions may be influenced by PD phosphorylation. With this approach, overexpression of PD-1 in both the membrane and the cytoplasm was achieved and the pull-down experiments revealed 52 interacting protein candidates with WT PD1 and 79 proteins for each

of the mutants, with 38 being shared across all PD1 forms and 8 being unique to the WT PD1. Of note, the known PD1 interacting partners, such as PDL1, PTEN and SHP2 were not identified in the analysis. However, there are many limitations of this approach which were well articulated in the thesis, including the preferential detection of highly abundant proteins, and the constraints inherent to the structure of the peptides generated. The list of 36 proteins identified as potential PD1 interaction candidates will likely provide much fertile ground for future studies. Included among these 36 proteins, the receptor tyrosine kinase AXL (originally named UFO, for its unidentified function and now recognized as an important player in immune-evasion and cancer growth) was noted as a highly promising target and was further studied.

The interaction between PD1 and AXL was confirmed by LC/MS as well as proximity ligation assays and followed by molecular modeling and molecular dynamic simulations. As full crystal structures of neither PD1 intracellular domain nor AXL were available, the sequences of extracellular domain (ECD) and intracellular domains (ICD) of AXL and intracellular domain of PD1 each were obtained and modeled using RoseTTFold. The crystal structure of the extracellular domain of PD1 was available. The modeling and docking results for the AXL-PD1-ECD revealed a strong binding affinity, and a yet stronger affinity for the AXL-PD1-ICD complex with Pro811 in AXL forming a hydrogen bond with Glu247 in PD1, as did Ser685 in AXL and Trp230 in PD1. Similar studies were carried out with the tyrosine mutants with the AXL-PD1-ICD (Y248F) showing the highest binding affinity. Conformational analysis by long-scale molecular dynamic simulations were carried out to study the dynamic behavior of the AXL receptor and PD1 ECD and ICDs. As anticipated, the ICDs were shown to be able to interact only when embedded in within the same membrane. Significantly, the thermodynamic studies point to conformational stability of the AXL-ICD and PD1-ICD interactions. In total, this set of findings point to the possibility for AXL-PD1 interactions and the possibility of non-canonical activation through heterodimerization with each other, and lead to the hypothesis that combined inhibition of PD1 and AXL may provide clinical benefit in osteosarcoma and other cancers.

The thesis is well written, with an excellent review of the literature and description and discussion of the findings. The studies are well documented, and the data presented in an organized fashion and followed by thoughtful interpretation including limitations. The findings are novel and constitute a significant contribution to our understanding of key molecules important in cancer biology.

I am pleased to confirm that the doctoral dissertation meets the requirements set for doctoral dissertations by The Higher Education and Science Act dated 20 July 2018 (Polish Journal of Laws of 2018 item 1668, as amended. Therefore, I am applying to the Council of the Biotechnology Discipline for admission of Katarzyna Dziubek to further stages of the doctoral procedure.

Dr. Vilma Yuzbasiyan-Gurkan

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