Review of Ph.D. thesis of Rohit Joshi

„Study of protein-protein interactions of human E3 ubiquitin ligase Nedd4-2“

The main aim of the Rohit Joshi's thesis presented here is to shed more light on functional interplay between E3 ubiquitin ligase Nedd4-2 and regulatory 14-3-3 proteins in human cells. Impaired function of both interaction partners is directly related to serious human diseases, e.g. impairment of the Nedd4-2 -dependent ubiquitination of the epithelial sodium channel (ENaC) is manifested in a genetic disorder known as the Liddle's syndrome. The activity of the ligase is regulated also by interaction with 14-3-3 protein which interconnects different interaction networks. The student applies a broad spectrum of experimental techniques based in biochemistry, molecular cloning or biophysics for this aim. The molecular biology of the studied proteins is well described in the Theoretical background section, the experimental techniques used in this study are presented in the Methods and material section. The methods applied in this study are so abundant that it is difficult to characterize all of them efficiently with relation to the presented project. The student was successful in providing basic information, only it looks that the more the described technique is further from his primary expertise, the more he has a tendency to repeat the main characterization in effort for careful explanation, e.g. the Jablonski diagram is presented two times in the Chapter 4.2.6, although at the first time with more details, even more then it is generally necessary for the biological character of this study, maybe the relation between time-resolved fluorescence spectroscopy and time-resolved quenching should be more explained, the Bragg's law is repeated in the protein crystallography Chapter 4.2.9, where XRD abbreviation is used without further clarification.

Experimental sections of the dissertation provide interesting information about the Nedd4-2:14-3-3η complex. The student carried out enormous work on construction and preparation of all protein samples. From my own experience, to prepare all protein constructs in a quality sufficient for efficient and successful staining by fluorescent dyes it needs a lot of experimental work and expertise. Cysteine scanning was performed using site-directed mutagenesis. The purified Nedd4-2 variants were soluble and stable, which was confirmed by DSF analysis, and they formed complex with 14-3-3η protein in 1:2 stoichiometry. The variants containing single cysteine were labeled with 1,5-IAEDANS dye. The Nedd4-2:14-3-3η complex was characterized by time-resolved fluorescence spectroscopy, time-resolved fluorescence anisotropy and time-resolved fluorescence quenching techniques. All the fluorescence techniques showed

the hindrance of the WW3 and WW4 domains and the conformational change in the HECT domain. Chemical crosslinking coupled with mass spectrometry and limited proteolysis provided further data on the complex. Additionally, the student refined the protein structure of the Nedd4-2pS342:14-3-3γΔC complex. All this experimental work and results show applicant's qualification for scientific work. The experimental results presented in this thesis create a substantial part of two papers published in prestigious peer-reviewed journals, Biophysical Journal and Communications Biology. It is obvious that results and their analysis presented in the dissertation meet well the aims declared in the Chapter 3.

Questions for the defense:

1. I do not understand well the description below the eq. (9), could you please explain it more?
2. Could you please explain more the relation between time-resolved fluorescence spectroscopy and time-resolved quenching experiments in your thesis, i.e. the relation between eq. (2) and eq. (10)?
3. Could you please specify more whether the size exclusion chromatography with the Nedd4-$2^{186-975}$ variant was performed using Hiload column Superdex 200 or Superdex 75? I find different description in the Methods and material section than in the Results.
4. In the section 5.5 we can read: "Analysis of the XL-MS data from the full-length Nedd4-2:14-3-3 complex … ." Could you please explain whether the XL-MS experiments were done also with full-length Nedd4-2? Can you explain when and for what reason the DSG and DSS reagents are used for the cross-linking and when the DSA one?
5. For my orientation, could you please if possible compare molecular sizes of proteins and protein domains critical not only for this study: WW domains, HECT domain, Nedd4-2, 14-3-3 proteins (dimer) or tumor suppressor p53? When small molecules or short peptides are important for regulation of protein-protein interactions of the critical proteins as described in the Chapter 2.5.5, are there interaction regulatory networks based on even smaller molecules (small peptides) in cells?

To conclude my review, I consider the presented dissertation of Rohit Joshi to satisfy all requirements necessary for successful defense.

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