# Review report on doctoral thesis Rohit Ashok Joshi : Study of protein-protein interactions of human E3 ubiquitin ligase Nedd 4-2

This doctoral thesis is devoted to studies of protein Nedd 4-2 complex with its negative regulator 14-3-3η. The interaction is important in control of ubiquitination of different target proteins as its dysregulation could result in serious health problems including epilepsy, respiratory distress and Liddle syndrom.

Work is written in the full form in English and is accompanied by two publications of author. The text of the thesis has a standard structure, it is 181 pages long including supplements and the list of references contains 279 items.

I found only a minimal number of typos and mistakes in the text, which I have already passed to the author. Figures and graphs are in professional quality.

The literary overview summarizes contemporary view on the structure and function of Nedd4-2 proteins and especially on its interaction with 14-3-3 proteins.

The first part of the Methods consists of a detailed description of the preparation, isolation, modification, labelling and purification of protein constructs, which have been further studied by a series of advanced methods described in the second part of the methods. Experimental techniques to further characterize the interactions of the molecules under study include mass spectrometry, differential scanning fluorimetry, several methods of fluorescence spectroscopy, chemical crosslinking and protein crystallography. Special attention is paid to the fluorescence spectroscopy methods covering anisotropy and time-resolved fluorescence.

Different variants of Nedd 4-2 and 14-3-3 proteins were expressed in bacteria E.coli, isolated and purified. The aim of preparing different variants of Nedd 4-2 protein was to obtain molecules containing only a single cysteine residue localised in different important epitopes suitable for subsequent labelling with 1,5-IAEDANS. Fluorescent probe lifetime and time resolved anisotropy were measured in Nedd 4-2 alone and Nedd 4-2 together with 14-3-3η protein. Signals were compared for molecules labelled in different selected regions to detect localisation of changes induced by 14-3-3η binding. Complementary results were obtained by measurement of quenching of 1,5-AEDANS fluorescence by acrylamide showing differences and changes in quencher accessibility to different protein regions.

The relative position and sites of interaction were studied also by chemical crosslinking combined with mass spectrometry and by limited proteolysis of complex by trypsine. Structure of Nedd 4-2 -14-3-3 complex at atomic level has been proven by co-crystallization of Nedd 4-2 fragment which carries phosphorylation site S342 together with 14-3-3γΔC protein followed by Xray analysis.

The results obtained by the different methods were discussed in detail and compared with each other properly. Discussion of the results is appropriate and the stated goals of the work have been met.

Valuable original results include precise localization of intermolecular interaction sites between Nedd 4-2 and 14-3-3.

There is no doubt about the quality, experimental level and timeliness of the achieved results given that two publications that contain most of the results of the thesis have successfully passed the peer review process in international scientific journals. The author has demonstrated the ability of independent creative scientific work. Therefore, I recommend the thesis to be defended and recommend that the candidate be awarded a PhD.

Prague, March 19th 2024

RNDr. Jan Krůšek, CSc.

I have following questions:

1) It is known that 14-3-3 proteins frequently form dimers and also interact in the form of dimers with their binding partners .

You used shortened ΔC variant of 14-3-3γ protein in X-ray analysis. Are these shortened variants forming dimers in solution as natural 14-3-3 protein do? Are they interacting with Nedd4-2 and its shortened variants and fragments as monomers or as dimers?

2) You used chemical cross-linking coupled to mass spectrometry between different points of the Nedd 4-2 protein as a tool to identify the sites of interaction with the 14-3-3η proteins. In Figure 5.10 you show the crosslink sites within the Nedd 4-2 protein and crosslink sites between Nedd 4-2 and 14-3-3η. Have you also observed the crosslinks

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