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Review of the doctoral thesis entitled **"REORGANIZATION OF *IN VITRO* RECONSTITUTED ACTIN-BASED NETWORKS"** by **Mgr. Ján Sabó**, Faculty of Science, Charles University

In his doctoral thesis the author focused on certain aspects of the actin cytoskeleton; while these had been previously studied in vivo the author attempted to reduce the complexity of the system by applying microscope-based in vitro reconstitution assays to reveal intrinsic activities of actin and actin-binding proteins.

In the first part the author studied the crosstalk between actin filaments and microtubules, specifically the role of the protein CKAP5 in this process. The experiments presented in the thesis revealed that CKAP5 bundles actin filaments and crosslinks them to the surface of a microtubule. Intriguingly, dynamic microtubules can pattern these actin bundles and subsequently, after catastrophe, can be guided in their extension by plus end-associated CKAP5 interacting with a persistent actin bundle. Hence, these experiments for the first time demonstrate that CKAP5 has intrinsic properties, which enable both patterning of the actin cytoskeleton by microtubules and of microtubules by the actin filaments. These activities are in accord with the previously described role of the protein in neuronal growth cones, a region of a prominent actin – microtubule crosstalk.

In the second part the author attempted to reconstitute in vitro actin networks, which are critical for establishment of the left-right asymmetry of embryos, to study the source of their chirality. By combining several constituents the author was indeed able to reconstitute the networks, and these were under specific conditions chiral. There was only a partial bias in chirality direction across a population of the networks. The developed experimental system will enable future studies of candidate factors for chirality induction.

In the final part the author participated in development of chips enabling more standardized and time efficient in vitro microscope-based assays. Roughness of the chip surface was identified as the critical factor and an optimized chip was developed.

The findings of the thesis are original, some of them rather unexpected, and they significantly contribute to understanding biology of actin and actin filament crosstalk with microtubules. Data on the activities of CKAP5 were recently published in Current Biology (the author of the thesis is the first author) and the development of chips for in vitro assays in Scientific Reports (co-author). The author managed to set up several complex experimental systems combining a number of purified proteins with actin filaments, microtubules, beads etc.; I find these prime examples of the level of complexity, which can be currently achieved in these in vitro reconstitution assays. At the same time availability of these experimental systems opens possibilities for further investigations of the questions addressed in the thesis.

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The thesis consists of typical parts. The introduction is appropriate in the scope and length. The only thing I missed was a brief introduction to the third part- development and testing of chips for the in vitro assays. However, a sufficient motivation was provided later in Results. Methods describe all used techniques and their modifications, though in some cases I would appreciate a bit more information, e.g. on composition of some of the buffers. Also I would find it useful to present gels of protein purifications (which could equally appear in Results). The Results chapter is clear, quantitative, imaging data are of high quality, particularly given the complexity of experiments. I would like to stress the clarity of this chapter- numerous modifications of assays were employed, but thanks to schematics used for each experiment and a concise description it is easy to navigate the chapter. Finally, the observations are discussed in the context of published knowledge. In general, the thesis is very well written. Typos are very rare and figures are neat. Clearly, a more than usual amount of attention was paid when putting the thesis together.

In conclusion, this work in my opinion fulfils criteria for a doctoral thesis as it demonstrates that the author is capable of addressing important scientific questions, designing experiments to test them, carefully performing complex experiments (at the limits of what is currently possible), analysing results, and summarizing findings in a format suitable for scientific publications. Therefore, **I fully recommend Mgr. Ján Sabó for further proceedings of the thesis defence**.

I do have several questions for the author of the thesis:

1/ Could the author explain what exactly does the coefficient of variation (COV) mean, how was it calculated, and how to interpret these measurements?

2/ In chapter 4.19 three classes of microtubule-recruited actin filament bundles were defined: (i) not prevailing in the image plane; (ii) prevailing in the image plane for a short time (5s-5min); and (iii) prevailing in the image plane for the entire length of observation (>5min (15 min total). Why was the cut-off of 5 min selected? How did the distribution of bundle duration times look like?

3/ In chapter 4.19 the following is stated: "The presence of methylcellulose constraints filaments like actin and microtubules close to the surface of the coverslip, which then enables their visualization by TIRF microscopy without physical tethering to the surface. As in the experiments described in Figure 4.10, microtubules were first introduced into the experimental chamber in the buffer containing Taxol. Then, actin filaments and CKAP5-mNG were introduced without Taxol presence (Figure

4.11A)" – I am wondering how was the introduction of actin filaments achieved without removing untethered microtubules?

4/ The following statement is found in discussion 4.1.12: "The different mechanisms might arise from CKAP5 bundling and visibly binding (even at 2 nM) to only thick actin bundles (32 ± 10, filaments, in vitro, intensity base estimate)82" - How do you picture this? How do thin bundles form in the first place, if CKAP5 only binds to bundles composed of a substantial number of filaments? How could you mechanistically explain the higher preference for the thick bundles?

5/ Discussion 4.2.6 states that: "The rigid rotational constraint of formins in MyoII-mDia1 aster in our in vitro reconstituted simplification might not be present"- could you think about an experimental way to address this?

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