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REVIEW

of the doctoral dissertation of Teresa De Cicco, M.Sc.
entitled: "The role of the actin-remodeling proteins Cap2 and Cttn in the development of the neuromuscular synapse."
made under the supervision of Dr. Hab. Tomasz J. Prószyński
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The doctoral dissertation presented for review concerns basic research on the role of two actin-binding proteins, i.e., cortactin (Cttn) and cyclase-associated protein 2 (CAP2), in the development of neuromuscular junctions (NMJs). Studying the development and organization of NMJ is essential as there are some diseases connected to malfunctioning NMJs such as some muscular dystrophies, peripheral neuropathies, or Myasthenia gravis. Thus, the conducted studies conducted by M.Sc. Teresa De Cicco are important.

The doctoral dissertation, covering 124 pages, has a typical layout for this type of work. It is divided into the following chapters: **Introduction**, **Hypothesis**, **Materials and Methods**, **Results**, **Discussion**, and **References**. The dissertation also includes **Abstracts** in Polish and English and a list of **Abbreviations**.

The list of **Abbreviations** has been adequately prepared. Some abbreviations in the list are missing, e.g., BSA for bovine serum albumin, IF for immunostaining, Pi for phosphate group, PBS for phosphate-buffered saline, or WB for Western blotting. One-page **Abstracts** in Polish and English have been appropriately edited and contain all the most critical elements of the dissertation. However, in the Polish version, the order of presented information does not precisely correspond to the English version. Nevertheless, it is just a minor flaw.

The introduction gives us extensive information about the development and composition of NMJ. We also get acquainted with three significant groups of cytoskeleton structures. In fact, apart from microfilaments, microtubules, and intermediate filaments, the cytoskeleton includes septins, which have recently turned out to be the fourth type of cell skeleton structure. We also get information about the CAP2 and Cttn. Only two pages are devoted to Cttn, while in the case of CAP2, the situation is slightly better as CAP2 is described on four pages. Subchapters on CAP2 and Cttn should contain more information about these two proteins as the dissertation focuses on them. This could have been done on the cost of redundant subchapters about the usefulness of cultured myotubes in studying NMJs because the M.Sc. De Cicco did not use such cultures. Additionally, the subchapter with general information about the nervous system could be shorter. I would like to know, for instance, whether there is some data on Cttn's involvement in embryonal development, how *CTTN* expression is regulated, and whether Cttn's level varies among tissues. Figure 1.14 shows that it can be phosphorylated by Pak, Erk, and Src kinases. Could the PhD Student elaborate more on that topic during the defense, as it is not addressed in the dissertation? I would like to know also about the modulation of Cttn activity by HDAC6. Moreover, information about the regulation of CAP2 activity would be appreciated. Finally, I got really intrigued by the statement that "Agrin-activated MuSK induces cortical actin polymerization" (p. 31). Is anything known about which actin polymerization proteins are involved in that process and which actin paralogue (isoform) is involved in that?

As a person interested in actin cytoskeleton, I have a few comments on giving the readers information about actin cytoskeleton. On p. 26, it is written "[…], and TM binds Ca^{2+}, […]" – here it is meant Tropomyosin. In Fig. 1.6, it is shown that Troponin (Tn) binds the calcium ion. Could this discrepancy be addressed during the defense? I think presenting two chains of a helix of a microfilament in different colors is misleading. For instance, from the analysis of Fig. 1.16, one could deduce that there are six types of actin monomers incorporated into the microfilament, which is invalid. In the caption to this figure, we have the following sentence: "ATP-charged actin monomers are incorporated at the barbed end of the filamentous actin by ATP-hydrolysis." It is not valid. As shown in Figure 1.16, ATP-actin monomers are present in the microfilament in the region close to the barbed end. I would also avoid using the expression "inactive ADP-actin monomers." What does it mean?

Active regarding what? Finally, I am curious what was meant by writing that γ-actin is positively charged/basic (p. 39). I know that both β and γ-actin N-termini, which differentiate these two non-muscle actins, are somewhat acidic. Could the "α-, β- and γ-" nomenclature for actin paralogues be explained during the defense? Writing that the balance between G- and F-actin is maintained by treadmilling (p. 39) is not precise. *In cellula*, there are several actin-binding proteins out of which Rho GTPases tightly regulate the key players. Their action influences the ratio of F:G actin in a cell. In Fig. 1.13, N-WASP-VCA is shown to be still attached to the branching site while elongation of a new filament occurs. After binding Cttn to the Arp2/3 complex bound to N-WASP-VCA, the elongation of filaments starts after the dissociation of N-WASP-VCA. There are other minor oversimplifications or unprecise formulations concerning actin. However, I will not mention them here.

In the next chapter of the dissertation - **Hypothesis**, the Ph.D. Student clearly outlines what goals guided her work. The main goal of Ms. De Cicco was to decipher the role of CAP2 and Cttn in the organization of NMJs in mice. The small thing here is that I'm afraid I have to disagree that CAP2 and Cttn are novel actin-organizing proteins. These proteins have been known for many years.

In the **Materials and Methods** chapter, the materials used in the work are listed, and the protocols of individual experiments are described in detail. This part of the dissertation was prepared appropriately, though I have some comments on this part. Maps of all generated by the Ph.D. Student plasmids should be shown in the dissertation. It needs to be explained why, instead of using pBiFC-aDB-VN173 plasmid, it was necessary to prepare the pcDNA3.1(+)-aDB-VN173 plasmid. Could it be addressed during the defense? Could we also get some details about the AAV2/9 CAP2-GFP adenovirus? In the dissertation, it is written solely that it was received from the Nencki Institute (p. 66). Does the expression of this vector lead to the production of CAP2 tagged with GFP, or are both proteins produced separately, with GFP serving as a marker of transduced tissue? This information is essential to correctly interpreting the experiment presented in subchapter 4.1.6. A reference to "standard preparation for acrylamide gels" (p. 69) is missing. In Table 3.3, indications of fluorescent dyes for α-Bungarotoxin and phalloidin are missing.

The next chapter of the dissertation describes the **Results**. M.Sc. De Cicco obtained several interesting results. Based on the presented outcomes, we can draw a conclusion that the abnormalities in morphology of NMJs caused by the depletion of CAP2 are coming from changes on the "neuronal side" of the NMJs and not on the "muscle side" of it. I found it intriguing that in animals with CAP2-KO, the fraction of medium-sized NMJs was much smaller than in control animals, leading to a higher percentage of small and enlarged NMJs. Regarding the Cttn part of the dissertation as the most interesting result, I consider that Cttn is apparently more important for slow-twitching muscles than

for fast-twitching muscles. Additionally, it was proven that Cttn indeed interacts with αDB1. I miss controls for some experiments. For instance, in the case of electroporation of muscles with GFP-CAP2- or Cttn-GFP-coding construct (subchapter 4.1.5 or 4.2.1, respectively) showing a parallel experiment, where a GFP-coding construct would be used is lacking. Apropos accumulation of GFP-CAP2 in muscle tissue, does the characteristic pattern within a sarcomere correspond to the M-line to which the pointed ends of microfilaments extend (subchapter 4.1.5)? And how is it about the Cttn-GFP accumulation within sarcomeres (subchapter 4.2.1)? On p. 90, it is written that the negative controls for the BiFC experiment were performed but have not been shown. Could M.Sc. De Cicco show this controls during the defense. Based on the information presented in the **Materials and Methods** section, I understood that the tagged POIs were expressed under the CMV promoter, which is a very strong promoter. Usually, for experiments focusing on studying the interaction between proteins, weak promoters are chosen not to obtain false positive results. Finally, having antibodies recognizing either CAP2 or Cttn, the lack of studied proteins in analyzed tissues should be shown at least by Western blotting. In **Materials,** an antibody for CAP2 is listed. However, in the **Results**, no single experiment shows this antibody's usage. Using these antibodies could show whether CAP2 is localizing within NMJs. Or maybe these antibodies can be used only for Western blotting? Could the Ph.D. Student explain the issue of not showing any results using these antibodies in the dissertation? I believe an analysis of the distribution of NMJs regarding their sizes should be conducted after using adenoviral vectors (subchapter 4.7). Figures 4.9D and 4.19B were either not referred to or described in the text. Pictures of MN at NMJs for WT animals should be shown in Fig. 4.9. Information about stained structures is missing for Fig. 4.3, 4.4, and 4.14 (red signal).

In **Discussion** M.Sc. De Cicco critically refers to the obtained results and compares them with the data described in the available literature. This part of the dissertation is very well written. Including a subchapter, “Conclusions,” in the Discussions was a very good idea. This systematizes the most significant achievements of the PhD Student. I have a question here. Have the phenotype of mice with systemic and muscle-specific KO been described by other groups? If yes, what were their observations? Maybe I missed this information in the dissertation; if so, I apologize for that.

Moving on to the assessment of the dissertation from the formal and editorial point of view, it should be noted that the thesis was written correctly in language and carefully edited. There were only a dozen typos and minor grammatical and punctuation errors. For me, it was disturbing that some of the figures were placed in subchapters to which they did not belong and, thus, were not referred. For instance, Fig. 1.4 should be in subchapter 1.2.1.4.1 and not 1.2.1.4.2, though it should also be referred to in the latter one. I noted the misplacement of the following figures: Fig. 1.8 or 1.13. Additionally,

in subchapter 1.3.5, Fig. 1.13 should be referred to. Some figures, e.g., 1.2, 1.4, 1.7, or 1.15, contain details that should have been described in the main text or caption. In the captions to Fig. 1.8, 1.11, and 2.2, information about the staining of postsynaptic machinery should have been given. Figure 3.4 is not referred to in the text. Some tables in the Materials and Methods need to be found. Sometimes proteins are given with the first capital letter and sometimes with the first small letter (e.g., Agrin and agrin, p.22). Minor shortcomings are listed outside the review.

The PhD student lists 184 items in the **Reference** section. Only half of the cited positions (less than 90) were published in the last ten years. This proportion could be better. Internet sources given on pages 40 and 71 should be listed in the **Reference** list together with the dates of access. Regarding Fig. 3.6, a reference needs to be included. Writing "adopted from the Internet" is insufficient. Could the Ph.D. Student give a reference to "The sliding filaments theory" (a caption to Fig. 1.6). Nevertheless, the cited positions have been correctly selected, which proves the proper theoretical preparation of the Ph.D. student.

The listed errors and shortcomings do not affect the substantive value of the work. The results obtained by the PhD Student will certainly be used to plan further research. Looking at the organization of a growth cone of MN could be one of the possible ways to understand how CAP2 deficiency could lead to malformations of NMJ. I am sure that the M.Sc. De Cicco put much effort into both projects. The expectations concerning the designed experiments were presumably higher, and obtained results showing, e.g., the lack of substantial influence of Cttn-depletion on the formation of NMJ could be disappointing. But this happens rather often than seldom in science. Negative verification of several hypotheses leads eventually to important discoveries. Nevertheless, the formulated goals of this dissertation were fully achieved. Ms. De Cicco is the coauthor of two papers published in journals from the JCR list. One of these publications concerns the influence of CAP2-KO on muscle morphology. Thus, experiments described in part of the evaluated dissertation were a natural continuation of previously published studies.

The doctoral dissertation meets the conditions specified in Article 187 section 1-4 of the Law on Higher Education and Science (Journal of Laws of 2018, item 1668, as amended). To sum up, I present to the High Scientific Council of the Hirszfeld Institute of Immunology and Experimental Therapy Polish Academy of Sciences, an application to admit Teresa De Cicco, M.Sc. to further stages of the procedure for awarding a doctoral degree in the field of natural sciences in the discipline of biological sciences. [Rozprawa doktorska spełnia warunki określone w art. 187 ust. 1-4 Ustawy Prawo o szkolnictwie wyższym i nauce (Dz.U. 2018 poz. 1668 z późn. zm.). Reasumując, przedstawiam Wysokiej Radzie Naukowej Instytutu Immunologii i Terapii Doświadczalnej im. Ludwika Hirszfelda Polskiej Akademii Nauk wniosek o dopuszczenie M.Sc.

Teresy De Cicco do dalszych etapów postępowania o nadanie stopnia doktora w dziedzinie nauk ścisłych i przyrodniczych w dyscyplinie nauki biologiczne].

Signed by / Podpisano przez:

Antonina Joanna Mazur
Uniwersytet Wrocławski

Date / Data:
2023-09-13 12:01

Examples of stylistic and editorial errors, etc.:

p. 18 “[…] and these types of cells are called cells, […]” – there is “Schwann” missing;

p. 43 “In vertebrates and also in mouse […]” – mouse belongs to vertebrates;

p. 46, p. 95, and in other places, “falloidin” – it should be“phalloidin”;

p. 57, there is no information in what PFA was dissolved;

p. 58 “OCT” abbreviation is not explained;

p. 58, it is not written for how long the isolated fibers were incubated at RT on a moving shaker;

for concentrations expressed in %, there is no information whether it is a w/v, w/w or v/v concentration;

the Ph.D. Student expresses the rotation speed in “g” while the correct notation is “x *g*”; after all, it is not about weight; sometimes, she expresses centrifugation speed in rpm.