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Review of the PhD thesis of mgr Piotr Cywoniuk

presented in order to obtain a PhD degree from the Faculty of Biology at the Adam Mickiewicz University in Poznań.

Thesis title: **Determination of functional RNA binding sites for MBNL proteins using antisense oligonucleotides**

The research presented in the thesis was conducted at the Department of Gene Expression, Institute of Molecular Biology and Biotechnology, Faculty of Biology, the Adam Mickiewicz University in Poznań, under the supervision of Professor Krzysztof Sobczak.
The work aimed at better understanding of the role of the *Muscleblind*-like (MBNL) protein family interacting with primary transcripts as a group of alternative splicing regulators essential during the development of particular tissues and involved in the pathomechanism of myotonic dystrophy (DM) disorder. This topic has been attacked by Prof. Sobczak' team in recent years with large success. The thesis documents the original contribution of mgr Piotr Cywoniuk to the development of research carried out at Prof. Sobczak's laboratory.

The thesis consists of three original scientific articles which are preceded by 9 pages of Preface with 63 citations. In the article published in *Scientific Reports* in December 2017 mgr Piotr Cywoniuk is the first among four co-authors. In the article published in *Nucleic Acids Research* in October 2016, he is the fourth among nine co-authors. Finally, in the article published in *Nucleic Acids Research* in June 2018 mgr Piotr Cywoniuk is the third among six co-authors. Each article is accompanied by statements of all the co-authors specifying their contributions to the study. It has to be underlined that the statements are very detailed, thus the contribution of mgr Piotr Cywoniuk to each article could have been clearly evaluated.

In the Preface, in the Introduction section, the Author briefly describes members of the MBNL protein family, their role in the regulation of alternative splicing and engagement of these proteins in the pathomechanism of myotonic dystrophy. The interactions of MBNL proteins with toxic RNAs bearing uncontrolled expansions of short nucleotide repeats are briefly reviewed. The Author also describes the principle of application of the antisense oligonucleotides strategy to the studies of protein-RNA interactions. Next, in the Aims section, specific goals of the thesis are formulated. Finally, in the Results section, the results of each of the three co-authored articles are briefly described with special emphasis on mgr Piotr Cywoniuk's contribution to the studies (the illustration materials which he prepared are clearly identified). This information was very useful since it allowed focusing on those experiments that were performed by mgr Piotr Cywoniuk and on his contribution to each of the three evaluated articles. I strongly stress this point since it is crucial for reviewing PhD theses presented in the form of co-authored, published articles.

In the article entitled "Hybrid splicing minigene and antisense oligonucleotides as efficient tools to determine functional protein/RNA interactions" new assays for experimental validation of potential MBNL-binding sites are described. The assays are based on the application of antisense oligonucleotides combined with specifically designed "hybrid" splicing minigenes. Mgr Piotr Cywoniuk was responsible for the *in cellulo* part of the work. He has found that approx. 20-nucleotides-long 2'-*O*-Methyl RNAs with phosphorothioate backbone are the most effective antisense oligonucleotides that block protein-RNA interactions. He designed several oligomers targeting MBNL1-binding sites in the intronic and exonic regions of *Atp2a1*, *Pphln1*, *Nfix*, *Ldb3* and *Mbnl1* pre-mRNAs and found that only MBNL1-binding sites localized in introns could be verified using this approach. Subsequently, he prepared a series of *Atp2a1* gene-based minigenes in which an intronic MBNL-binding cassette was replaced with elements derived from other studied transcripts with potential MBNL1-binding sites. Mgr Piotr Cywoniuk has shown that transposition of a particular pre-mRNA fragment into the context of downstream intron into the hybrid minigene restores response of an alternative exon of this minigene to the MBNL1 activity. He has also shown that subsequent treatment with antisense oligonucleotides specific to the analyzed hybrid fragment strongly inhibits the interaction of MBNL1 with targeted RNAs. Together with Dr Taylor, with whom mgr Piotr Cywoniuk shares the article's first-co-

authorship, they have identified serine/arginine-rich splicing factor 1 as a potential modulator of MBNL activity in the regulation of splicing of two alternative exons.

In my opinion, the contribution of mgr Piotr Cywoniuk to the article published in *Scientific Reports* is the most valuable part of his dissertation. Although the article has been subjected to strict peer-reviewing I would like to have some detailed aspects of the study clarified during the public defense of the thesis. First, the authors used different types of antisense oligonucleotides of various lengths determining the Kd values for their interactions with RNA targets by the filter binding assay. No computer calculated ΔG energy values of duplex formation were taken into consideration. I wonder whether ΔG values, and possibly their comparison with Kd values, would have supplied some additional information; at least in terms of the role of the target RNA structure in the binding of antisense oligonucleotides? Second, all antisense oligonucleotides without the PS modification revealed no inhibitory effect on the splicing of ex22 in the cell; could the Author speculate on the possible explanation of this observation? Third, the Author hypothesized on a plausible mechanism of alternative splicing changes caused by antisense oligonucleotide binding; what kind of experiments could be suggested to verify these propositions?

In the second article entitled "Mechanistic determinants of MBNL activity" the authors compare several features of three MBNL paralogs and their splicing isoforms, such as splicing regulatory potential, subcellular localization and contribution to myotonic dystrophy pathomechanism. In this article mgr Piotr Cywoniuk compares the distribution of three alternative exons in MBNL1, 2 and 3 mRNAs among human tissues at the fetal or adult developmental stage and in samples derived from DM1 and DM2 patients. To this end, the RT-PCR technique has been used. Importantly, in this work the assay combining antisense oligonucleotide strategy with hybrid splicing minigenes, described in the first article, has also been applied. This new approach allowed mgr Piotr Cywoniuk to confirm that all three MBNL paralogs bind the same RNA sequence but with different strength.

The third article entitled "MBNL splicing activity depends on RNA binding site structural context" aims to elucidate the impact of the organization of YGCY consensus motifs in pre-mRNAs on the affinity of MBNL proteins and splicing activity. The authors show that binding preferences of these proteins strongly depend on the number and composition of YGCY motifs within the secondary structure context of pre-mRNA. The results show that

MBNL1 preferentially binds to single-stranded RNA regions containing four YGCY sequence motifs and that the affinity decreases when such motifs are located on opposite sides of RNA hairpin structure. Moreover, the authors have found a competitive relationship between the activity of MBNL1 and MBNL3 which strongly depends on the organization of YGCY motifs. In the work described in this article mgr Piotr Cywoniuk was responsible for the quantity-control experiments of particular MBNL protein levels using western blotting technique. For that purpose he has also employed a method based on visualization of eGFP-fused proteins directly in acrylamide gels.

To conclude this review, the results presented in the thesis shed a new light on the mechanism of alternative splicing regulation by MBNL proteins and on the pathomechanism of myotonic dystrophy disorder. The major achievement of mgr Piotr Cywoniuk is the elaboration of a new experimental strategy that uses antisense oligonucleotides and hybrid splicing minigenes for the determination of functional protein/RNA interactions. He has tested this strategy on MBNL-specific RNA binding regions but it could be potentially applied for other RNA-binding proteins. Moreover, hybrid minigenes could be used to evaluate the efficiency of potential therapeutic agents for inhibiting the interaction between MBNL proteins and expanded short nucleotide repeats. The new strategy has successfully been applied in the studies described in the three articles, which were published in highly prestigious scientific journals. These remarkable achievements and very high quality of mgr Piotr Cywoniuk's results documented in the articles included in his dissertation prompted me to ask for considering awarding him an appropriate scientific award.

Taking all the above into account, I recommend that the Scientific Board of the Faculty of Biology, The Adam Mickiewicz University in Poznań, would proceed with further procedural steps to confer the PhD degree on mgr Piotr Cywoniuk.