129083 FINAL REPORT

I. Proceeding of the last stage of the project

In the first chapter, I aim to summarize our proceedings of the last 11 months. Due to the extremely strict regulations of Japan's border control, we could visit our partner (Professor Takasuka) only in August 2022. In contrast, annual bidirectional visits were planned in our original work plan. A scientific meeting was held at Hokkaido University with three scientific workgroups: our group from Semmelweis University, Professor Takasuka's group, and Professor Ryotah Uehara's group whose main topic is the mechanism of cell division. We aimed to seek other overlapping research interests and new project ideas for the foreseeable future. Tamás Mészáros introduced our department's (Molecular Biology Department, Semmelweis University, Budapest) scientific scope and our wide variety of current research projects. During this visit, we proceeded with two new projects:

- Project 1

Mass spectrometry measurements were done at Hokkaido University related to a new project in collaboration with Professor Beatrix Horvath (Department of Molecular Genetics, Utrecht University, Utrecht, The Netherlands). We set out to study the function of AT5G60250.1, a C3HC4-type RING finger holding putative ubiquitin ligase protein in DNA damage response. To demonstrate the enzyme activity and identify the protein interacting partners of AT5G60250.1, we created various wheat germ extract-based in vitro translation compatible vector constructs and optimized the conditions of the in vitro ubiquitination assay. Having the needed constructs, we demonstrated:

i) The AT5G60250.1, which is deposited in the RIKEN stock collection does not have ubiquitin ligase activity due to C392R mutation.

ii) Restoring the mutated cysteine by in vitro mutagenesis results in the wild-type AT5G60250.1, which presents ubiquitination activity in the in vitro translation system.

iii) The wild-type AT5G60250.1 interacts with H2A.X and H2B in pull-down assays.

Next, we studied if AT5G60250.1 is capable for ubiquitination of any of the above two histones. It has been described that some of the ubiquitin ligases cannot ubiquitinate the isolated histones, they only accept them for substrates as components of the nucleosome. To reveal if AT5G60250.1 could ubiquitinate histones of the nucleosome, we composed two types of nucleosomes (H2A.X-H2B-H3.1-H4 and H2A.10-H2B-H3.1-H4) by in vitro translation and applied them as putative substrates of ubiquitination. The first experimental setups indicated no ubiquitination, but these measurements call for further investigations. Most of the above-described results are integral parts of a manuscript in preparation.

- Project 2

Petra Bankó, who is Ph.D. student at Hokkaido University, is working with Arabidopsis thaliana to assemble its chromatin with histones and other histone variants using the in vitro wheat germ extract-based platform, that can later serve as a template for the functional analysis of chromatin modifying enzymes. Histone variants make up a big portion of chromatin, carrying out specialized roles for the fine-tuning of chromatin, so they are indispensable components

for screening histone modifiers. She successfully assembled eight types of chromatins using canonical histones: H2A.10, H2B.9, H3.1, H4; and 4 histone variants: H2A.X, H2A.W, H3.3, and CENH3. Two histone variants, H2B.7 and H2A.Z, did not assemble into chromatin, which might require an extra chromatin chaperone. She analyzed the produced chromatin with supercoiling assay and micrococcal nuclear assay. She presented the results at the annual conference of The Molecular Biology Society of Japan in December 2022 in Yokohama. Petra visited Hungary in September 2022, where she conducted experiments related to Project 1 and 2. Petra presented her results at the scientific forum of the Institute of Biochemistry and Molecular Biology at Semmelweis University, as well. The 129083 grant was the main supporter of her experiments. In the following months, she is finalizing her new results in a publication and plans to defend her thesis in 2023.

II. Participants

In 2019, Brigitta Kállai constructed FLAG-Ubiquitin coding vector and expressed the protein in bacterial cells, and purified it. Petra Bankó, Alexandra Tar and David Schein joined to the project in February 2019 as research students. David helped to set up the dataset of human histone modifier enzymes, Alexandra and Petra was participating in the cloning and chromatin assembly experiments. Petra Bankó was awarded a 3-month summer internship by Campus Mundi Scholarship and spent this time in Professor Takasuka's laboratory cloning several human histone deacetylase enzymes. Ágnes Bitskey joined the workgroup for 3 months ,and she was also participating in the cloning of human histone modifier enzymes.

In 2020, two students, whose work was fundamental at the beginning of the project, successfully graduated and left the workgroup. Firstly, Petra Bankó graduated from the Bioengineer master's course at Budapest University of Technology and Economics, in January 2020. She was awarded a MEXT Scholarship and started her Ph.D. in September 2020 in the Takasuka Laboratory, at Hokkaido University, Japan. Her presence there is making the bond stronger between our two workgroups. In June 2020, Alexandra Tar accomplished her Bioengineer master's degree at Budapest University of Technology and Economics, as well. In her thesis 'Cloning and production of human histone modifying enzymes by in vitro translation', she summarized her experiments of constructing kinase and phosphatase clones. Unfortunately, her laboratory work was suspended in March 2020 due to the COVID-19 regulations. Despite these difficulties, she successfully finished her thesis and later joined the workgroup of Tamás Mészáros as a Ph.D. student.

In 2021, two students joined our workgroup. Kamel Bendali, who started to perform his 6-week-long practice in February 2021. He was participating in the cloning of human histone modifier enzymes, namely ubiquitin ligases. Anett Fürtön started to work in September 2021, she successfully designed and constructed a new pEU plasmid, containing the Strep-II affinity tag.

Tamás Mészáros continuously provided his expertise and his vast variety of laboratory equipment for the project. The essential practical help of Anna Gyurkovics and the logistical

support from Mária Gránicz, Józsefné Bombicz, and Valéria Mile continuously aided the project during these four years.

III. Summary

In the field of epigenetics, there is a great demand for reconstituted chromatin that can serve as a substrate for enzyme modification assays and drug development. In our original work plan, we aimed to reconstitute human chromatin in vitro and study human histone modifiers using assembled human chromatin as the substrate. Unfortunately, the work plan has changed dramatically due to the unexpected effects of the COVID-19 pandemic. Nevertheless, we redesigned the research focus to be compatible with the local and global restrictions of the pandemic. Thus, the work with human histone modifiers is still ongoing, proceedings will be assigned to the 129083 grant in the close future. Despite the difficulties, we presented our research at various conferences in Europe and Japan, successfully published 3 papers, and established new cooperative projects.

Initially, a dataset of the human histone modifying enzymes (writers and erasers) was built based on online databases (HIstome, HEDD, NCBI, UniProt): types of enzyme, name, coding gene, sizes of gene and protein, modified histone, modified residue, and type of modification on histone tail, relevant publications were added to our database. The cloning of histone modifier kinases and phosphatases was started, additionally, the cloning of acyltransferases and methyltransferases was continued in Professor Takasuka's workgroup in Japan. Later, ubiquitin ligases were added to the database, since their relevance is emerging in human diseases and our workgroup has previous experiences in ubiquitination assays. Gene specific primers of 25 kinases, 10 phosphatases, and 16 ubiquitin ligases were designed. Human cDNA libraries were made from RNA extracts of epithelial, colon, and fibroblast cells by cDNA Synthesis Kit. The target genes were amplified from the abovementioned human cDNA libraries by high-fidelity DNA polymerase and inserted into 6xHis affinity-tag containing pEU0 vector suitable for the wheat germ-based, cell-free translation.

Due to the fact, that some reagents arrived 6 months after we ordered, we had limited time before my first visit to Japan in August 2019. We only had the chance to set up a few trial samples for the first mass spectrometry analysis: one control chromatin and five modified chromatin samples, that were modified by the selected five histone modifiers (CDK3, CDK5, GSK3, BUB1, RNF168). We proved that the modifier enzymes can be expressed by in vitro translation and purified by His-tagged-based affinity purification for further experiments. The mRNAs of five histone modifier enzymes were co-translationally expressed by cell-free translation with the human core histones (H2A, H2B, H3.1, H4) and pBSK plasmid. During my visit to Japan, I performed mass spectrometry analysis on the modified chromatin sample set. This gave us preliminary results: i.) histones can be found unmodified in the control sample, ii.) histone H3.1 is not found in any samples, iii.) histone modifier enzymes can be detected. These data led us to the conclusion that purification of the in vitro assembled chromatin and/or the histone modifier enzymes might increase the efficiency of the assay. We concluded that optimization of the protease digestion process during sample preparation would be

necessary. A scientific meeting was held with Professor Takasuka's team, where we presented our current results and discussed our plans.

Restrictions due to the COVID-19 pandemic in 2020 suspended the life and the project for several months, and altered the work and budget plans. Semmelweis University had a ban on students entering the University, followed by restrictions on the physical presence of employees (between March and May 2020, and later in the autumn). The students who were working on the project were unable to enter the laboratory, foreign travel was forbidden for the Semmelweis University employees, and package/mail sending was prohibited for months/years, international conferences were canceled. Despite the difficulties, we successfully finalized a publication presenting our current proceedings, entitled 'Reconstitution of Drosophila and human chromatins by wheat germ cell-free co-expression system', which was published in BMC Biotechnology in 2020. Besides, that this in vitro reconstructed human chromatin will be the substrate of the following human histone modifying assays, it will aid other researchers to characterize the unrevealed structure, function, and regulation of the human chromatin dynamics, to investigate epigenetic drugs. In the publication, we show a novel, single-step, in vitro co-expression-based chromatin assembly method by using wheat germ protein synthesis, which results in a native-like, unmodified chromatin. Both Drosophila and human chromatins were reconstituted using in vitro translation by the addition of the mRNAs of core histones and chromatin assembly factors (dAcf1/dISWI chromatin remodeling complex, and nucleosome assembly protein, dNAP1), circular plasmid, and topoisomerase. Unexpected differences were described with respect to the required ratio of histone-coding mRNAs and reaction time. Regarding further usage of the assembled chromatin, Drosophila melanogaster linker histone H1 incorporation was proven during the in vitro chromatin assembly by MNase assay, which opened a possibility for investigating the effect of the human histone H1 on the chromatin assembly.

To achieve a more diverse experimental setup for the in vitro chromatin studies, the pEU3 vector family was expanded. His12 and FLAG affinity tag coding vectors can increase the specificity of protein interaction studies, Halo affinity tag vector provides covalently linkable ligands for pull-down assays, and double-tagging (GST-His6, and GST-biotin) vectors can further broaden the possibilities of protein-protein interaction studies. We published a paper describing these vector constructs, namely 'A novel family of expression vectors with multiple affinity tags for wheat germ cell-free protein expression' (BMC Biotechnology). These vectors were added to the Addgene's plasmid deposit to make it accessible to the scientific community (https://www.addgene.org/Tamas_Meszaros/).

In May 2021, we published our further results in FEBS OpenBio entitled 'De novo reconstitution of chromatin using wheat germ cell-free protein synthesis'. The main advantages of the in vitro chromatin reconstitution by wheat germ-based translation were highlighted: the in vitro expressed histones are homogenous and do not possess posttranslational modifications, thus, they can serve as a good substrate, and well-described histone modifications can be reproduced in our system. Importantly, Mg^{2+}-isolated (purified) chromatin was shown to be stable over several repeats of freeze-thawing cycles and can be

stored stably over several months at 80°C. Furthermore, its ability to serve as a substrate for histone modifier enzyme assays was proved by histone modification reaction by adding in vitro-synthesized dGCN5 (histone acetyltransferase) or dSet8 (histone methyltransferase) in the presence of radiolabeled acetyl-CoA or S-adenosyl methionine, respectively.

Summarily, between 2018-2022, in cooperation with the Takasuka group, wheat germ-based chromatin assembly methods for humans and Drosophila have been established. Our common work resulted in 3 publications (Okimune et al., BMC Biotechnol., 2020; Endo et al., FEBS Open Bio 2021; Okimune et al., FEBS Open Bio, 2021). We established two methods, a posttranslational and a cotranslational, single-step nucleosome assembly protocol. As it was mentioned above, purification of the assembled chromatin seemed to be essential. Currently, the chromatin substrate for upcoming epigenetic enzyme screening is successfully prepared by combining Mg^{2+}-precipitation and sucrose density gradient methods in the Takasuka laboratory (publication in preparation). Furthermore, Arabidopsis chromatin reconstitution was successfully established with canonical histones and histone variants (publication in preparation). Shortly, human and Arabidopsis chromatin modifying enzymes will be tested on the purified chromatin substrate. The developed methods are planned to be patented and the proceedings to set up a high throughput assay are continued.