Structure and function of nucleoprotein complexes in DNA replication

Summary of Professional Accomplishments

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Attachment 3
to the application for commencement of the procedure
for the conferment of the post-doctoral degree of doctor habilitated
Katarzyna Ewa Węgrzyn, PhD

Summary of Professional Accomplishments

1. *Name.*

   Katarzyna Ewa Węgrzyn

2. *Diplomas, degrees conferred in specific areas of science or arts, including the name of the institution which conferred the degree, year of degree conferment, title of the PhD dissertation*

   2009 Doctor of biological sciences in the field of biochemistry

   The degree awarded by the Intercollegiate Faculty of Biotechnology of the University of Gdańsk and the Medical University of Gdańsk based on the thesis entitled "The Influence of Bacterial Partitioning Proteins on the Localization and Stability of Plasmid RK2," conducted at the Department of Molecular and Cellular Biology of the Intercollegiate Faculty of Biotechnology.
   Supervisor: p. dr hab. Igor Konieczny.
   The thesis received distinction from the UG-AMG IFB Council.

   2005 Master degree in biotechnology

   The title awarded by the Intercollegiate Faculty of Biotechnology of the University of Gdańsk and the Medical University of Gdańsk. The master's thesis entitled "Localization of Plasmid RK2 Minireplicons in *Escherichia coli* Bacterial Cells" was conducted at the Department of Molecular and Cellular Biology of the Intercollegiate Faculty of Biotechnology under the supervision of dr Monika Witosińska and prof. dr. hab. Igor Konieczny.

3. *Information on employment in research institutes or faculties/departments or school of arts*

2012 – present

assistant professor, Intercollegiate Faculty of Biotechnology, the University of Gdańsk

2009-2012

assistant, Intercollegiate Faculty of Biotechnology, the University of Gdańsk

4. *Description of the achievements, set out in art. 219 para 1 point 2 of the Act*

4.1. Cycle of scientific articles related thematically

The scientific achievements that form the basis of this application are as follows:

I. Description of the structure of broad-host-range plasmids' Rep proteins and determination of the mechanism of action of these proteins in *Caulobacter crescentus* cells.

II. Discovery of the interaction between Rep proteins and *Ap*Orc1 protein with the single-stranded DNA of the DUE region of origin and description of the structure of nucleoprotein complexes of RepE protein.

III. Determination of the influence of DNA interactions on the proteolysis of Rep proteins.

Achievements I-III are documented in publications constituting the cycle of works entitled **"Structure and function of nucleoprotein complexes in DNA replication."**

In the papers documenting achievements I-III, an asterisk (*) denotes co-authors who made an equal contribution to the creation of the respective work alongside me. A cross (#) indicates co-authors who, together with me, fulfill the role of corresponding author.

Statements from co-authors of the publications specifying the individual contributions of each author to the creation of specific publications are included in Attachment No. 3A.

I. Description of the structure of broad-host-range plasmids' Rep proteins and determination of the mechanism of action of these proteins in *Caulobacter crescentus* cells.

At the time I embarked on the research topic related to the analysis of Rep protein structures, it was known that plasmid replication initiators, Rep proteins, were composed of two winged-helix (WH) domains. However, the structure of replication initiators of broad-host-range plasmids was unknown. The research I conducted allowed me to describe the three-domain structure of the protein TrfA encoded by RK2plasmid and determine the significance of all three domains in the process of initiating replication in the broad-host-range plasmid RK2. Since TrfA protein is encoded in two forms (a shorter 33kDa TrfA-33 and a longer 44kDa TrfA-44), which are capable of initiating plasmid replication to varying degrees in different bacterial species, I investigated the mechanism of action of these two initiator forms in *Caulobacter crescentus* cells. I characterized the replication process of the broad-host-range plasmid in *C. crescentus* during the cell cycle.

This achievement has been described in two original papers:

1. **Wegrzyn, K.***, Zabrocka, E.*, Bury, K., Tomiczek, B., Wieczor, M., Czub, J., Uciechowska, U., Moreno-Del Alamo, M., Walkow, U., Grochowina, I., Dutkiewicz, R., Bujnicki, J.M., Giraldo, R., Konieczny, I.; (2021) Defining a novel domain that provides an essential contribution to site-specific interaction of Rep protein with DNA. *Nucleic Acids Research*, 49 (6), pp. 3394-3408
(IF_{2021} 19,160; $MEiN_{2021}$ = 200)

2. **Węgrzyn, K.**, Witosińska, M., Schweiger, P., Bury, K., Jenal, U., Konieczny, I.; (2013) RK2 plasmid dynamics in Caulobacter crescentus cells - Two modes of DNA replication initiation *Microbiology*-SGM 159:1010-1022.
(IF_{2013} 2,835; $MNiSW_{2013}$ = 30)

II. Discovery of the interaction between Rep proteins and ApOrc1 protein with the single-stranded DNA of the DUE region of origin and description of the structure of nucleoprotein complexes of RepE protein.

I investigated and discovered that, just as it has been demonstrated for chromosomal replication initiators, DnaA proteins, plasmid replication initiators (Rep) form complexes with single-stranded DNA (ssDNA), and their formation depends on DNA

sequence and is essential for the initiation of replication. I demonstrated that this interaction is crucial for the formation of a stable open complex during the initiation of replication. I also discovered a similar interaction for the initiator from the archaeon *Aeropyrum pernix*, Orc1 protein. I obtained crystal structures of the RepE protein from plasmid F with ssDNA and the triple complex dsDNA-RepE-ssDNA. The structure of the triple complex and the biochemical analyses, that I conducted, provide the first direct evidence confirming the widely discussed loop-back model for DNA replication initiation.

This achievement has been described in three original papers.

1. **Węgrzyn, K.**, Fuentes-Perez, M.E., Bury, K., Rajewska, M., Moreno-Herrero, F., Konieczny, I., (2014) Sequence-specific interactions of Rep proteins with ssDNA in the AT-rich region of the plasmid replication origin *Nucleic Acids Res.* 42 (12), pp. 7807-7818.
(IF_{2014} 9,112; $MNiSW_{2014}$ = 40)

2. **Wegrzyn, K.#**, Oliwa, M., Nowacka, N., Zabrocka, E., Bury, K., Purzycki, P., Czaplewska, P., Pipka, J., Giraldo, R., Konieczny, I.# (2023) Rep protein accommodates together dsDNA and ssDNA which enables a loop-back mechanism to plasmid DNA replication initiation. *Nucleic Acids Research*, DOI: 10.1093/nar/gkad740
(IF_{2023} 14,90; $MEiN_{2023}$ = 200)

3. **Wegrzyn, K.#,** Konieczny, I.; Archaeal Orc1 protein interacts with T-rich single-stranded DNA. (2021) *BMC Research Notes*, 14 (1), art. no. 275
(IF_{2021} 0,521 ; $MEiN_{2021}$ = 70)

and three review papers:

4. Rajewska, M., **Węgrzyn, K.,** Konieczny, I.; AT-rich region and repeated sequences - the essential elements of replication origins of bacterial replicons (2012) *FEMS Microbiology Reviews* 36 (2), pp. 408-434.
(IF_{2012} 13,231; $MNiSW_{2012}$ = 45)

5. Zabrocka, E., **Wegrzyn, K.**, Konieczny, I.; Two replication initiators - one mechanism for replication origin opening? (2014) *Plasmid* 76, pp. 72-78.
(IF_{2014} 1,578; $MNiSW_{2014}$ = 15)

6. **Wegrzyn, K.*#, Gross**, M.*, Uciechowska, U., Konieczny, I.#; Replisome assembly at bacterial chromosomes and iteron plasmids (2016) Front Mol Biosci. 3:39.
(IF_{2016} 1,774; $MNiSW_{2016}$ = -)

III. Determination of the influence of DNA interactions on the proteolysis of Rep proteins

The research conducted by Prof. Igor Konieczny's team, in which I participated, revealed that the proteolytic activity of the Lon protease towards Rep proteins is dependent on substrate interactions with DNA. Continuing these studies, I discovered that the Lon protease also needs to bind to DNA for the proteolysis of Rep proteins. I identified the amino acid residues of the Lon protease essential for forming the nucleoprotein complex and demonstrated that substitutions of these residues result in a lack of proteolytic activity towards plasmid replication initiators.

The results of these studies have been described in an original paper:

1. Karlowicz, A.*, **Węgrzyn, K.***, Gross, M., Kaczynska, D., Ropelewska, M., Siemiatkowska, M., Bujnicki, J.M., Konieczny, I.; (2017) Defining the crucial domain and amino acid residues in bacterial Lon protease for DNA binding and processing of DNA-interacting substrates *J Biol Chem.* 292 (18), pp. 7507-7518.
(IF_{2017} 4,010; $MNiSW_{2017}$ = 35)

and review publication:

2. Karlowicz A.*, **Węgrzyn K.***, Dubiel A., Ropelewska M., Konieczny I., (2016) „Proteolysis in plasmid DNA stable maintenance in bacterial cells." Plasmid 86:7-13.
(IF_{2016} 1,545; $MNiSW_{2016}$ = 15)

The publications describing the presented achievements I-III, published between 2012 and 2023, constitute a cycle of works titled "Structure and Function of Nucleoprotein Complexes in DNA Replication." The total citation impact factor (IF) for the papers comprising the presented cycle is **68.666**. The total number of points assigned by the Ministry of Science and Higher Education (MNiSW) for the papers comprising the presented cycle is **650 points** (in accordance with the attachment to the communication from the Minister of Science and Higher Education dated December 18, 2019).

The above cycle of publications presents the results obtained during my work from 2009 to 2023 at the Laboratory of Molecular Biology (formerly the Department of Molecular and

Cellular Biology) of the Itercollegiate Faculty of Biotechnology of the University of Gdańsk and the Medical University of Gdańsk. It also includes results of my work during scientific internship at the laboratory of Prof. Fernando Moreno-Herrero at the National Center for Biotechnology in Madrid, Spain, and study visits to the laboratory of Prof. Marcin Nowotny at the International Institute of Molecular and Cell Biology in Warsaw.

During the course of my doctoral project, I focused on the localization of plasmid DNA molecules in bacterial cells and their partitioning into daughter cells (Attachment 4, point II 1 in the summary of my complete scientific achievements). The research was conducted using the plasmid RK2 as a model system, which is present in many Gram-negative bacteria, including animal and human pathogens. The experience and skills acquired during my doctoral project served as a foundation, and I decided to further develop and utilize them in my ongoing scientific work on the structure and significance of nucleoprotein complexes in DNA metabolism, particularly in the replication process.

The process of DNA replication is essential for the survival of every cell and has been the subject of research by numerous scientific teams for many years. Advanced research techniques have made it possible to increasingly understand and describe the course of this process in greater detail. In the case of bacterial chromosomes, DNA replication initiation primarily depends on the availability and activity of the replication initiator protein, DnaA, as well as other replication proteins such as helicase (e.g., DnaB), gyrase, and primase. The replication of plasmid DNA, especially iteron-containing plasmids with repeated sequences (iterons) in the replication *origin*, relies on the activity of the plasmid-encoded initiator, Rep protein, and often the chromosomal initiator DnaA. The activity of other host replication proteins is also essential for initiating and properly executing the plasmid DNA replication process. In the initiation of replication, the correct sequence and orientation of motifs in the *origin* region, which is the replication starting point, are of utmost importance. The significance of the *origin* region's structure has been described in the work **Rajewska, Węgrzyn, Konieczny, 2012, FEMS Microbiology Reviews**, of which I am a co-author (position 4 documenting achievement II, forming the basis of this application).

It is extremely important for the DNA replication process to occur at the appropriate stage of the cell cycle. The precise initiation of this process is associated with the availability and activity of replication initiation proteins, which form specific nucleoprotein complexes at the replication starting point. In the case of plasmids as extrachromosomal genetic elements, it was not known to what extent their replication is correlated with the replication of the host cell's

chromosomal DNA. Research conducted using the broad-host-range plasmid RK2 and bacteria of *Caulobacter crescentus*, which undergo asymmetric cell division (the maternal cell compartment is well distinguishable from the daughter cell compartment), allowed me to investigate this dependency. The results of my studies were described in the publication **Węgrzyn et al., 2013, Microbiology-SGM** (position 2 documenting achievement I). By synchronizing *C. crescentus* cells through density gradient centrifugation and isolating cells in the G1 phase of the cell cycle, it was possible to track the localization and copy number of the plasmid, the level of DNA synthesis, and the formation of nucleoprotein complexes at the replication *origin* in different cell cycle phases. Microscopic observations using a fluorescent DNA labeling system in cells, the Fluorescent Repressor-Operator System (FROS), revealed that RK2 plasmid molecules form clusters that are asymmetrically located, primarily in the maternal cell compartment, rather than the daughter cell compartment. My analysis of the copy number of plasmid DNA showed that cluster contain two copies of plasmid DNA, regardless of the cell cycle phase or compartment. Detection of DNA synthesis in synchronized cultures of *C. crescentus* cells showed that plasmid DNA, like the chromosome, primarily replicates in the S phase of the cell cycle. **The results obtained by me and my colleagues, for the first time, demonstrated plasmid DNA replication in relation to the bacterial cell cycle.** Interestingly, despite many years of research, the relationship between plasmid DNA metabolism and host cell metabolism is not entirely clear. However, current research conducted by me and our team indicates that this relationship exists and has a particularly significant impact on plasmid DNA metabolism during stress growth conditions.

The analyses I conducted using the Chromatin Immunoprecipitation (ChIP) method, which I optimized for detecting complexes at the replication *origin* regions of plasmids and bacterial chromosomes, revealed that, unlike the chromosome, the level of plasmid DNA synthesis does not correlate with the number of complexes formed by the host replication initiation protein DnaA at the plasmid replication *origin*. I detected DnaA protein bound at the plasmid *origin* throughout all phases of the cell cycle, whereas at the chromosomal DNA *origin*, it was primarily detected in the S phase, coinciding with the highest DNA synthesis level. I observed differences in the number of nucleoprotein complexes at the plasmid *origin* during the cell cycle of *C. crescentus*, particularly in relation to the plasmid-encoded replication initiation protein TrfA. Therefore, the occurrence of DNA replication in plasmid RK2 depended on the appropriate amount of TrfA protein binding to the iterons. TrfA protein is encoded on the plasmid in two forms: a longer one with a mass of 44 kDa (TrfA-44) and a shorter one with a mass of 33 kDa (TrfA-33). In *Escherichia coli* cells, both forms are replicationally active but

require cooperation with the bacterial DnaA protein. In contrast, in *Pseudomonas aeruginosa* cells, only TrfA-44 is capable of initiating the replication of plasmid RK2 without the involvement of DnaA protein. The method of initiating plasmid RK2 replication in *C. crescentus* cells was unknown. Therefore, I cloned and purified DnaA and DnaB proteins from this bacterium and examined the activity of the TrfA-33 and TrfA-44 initiators at the RK2 plasmid *origin*. I also conducted *in vivo* studies on the activity of the two forms of TrfA protein. **Obtained by me results,for the first time indicated the possible existence of two different mechanisms for initiating replication of plasmid RK2 in these bacterial cells, one dependent on DnaA protein activity and the other independent of it.** These results were published in the work **Węgrzyn et al., 2013, Microbiology-SGM**.

Plasmid replication initiators, Rep proteins, are primarily composed of two winged helix (WH) domains. In the case of TrfA-33 protein, the replication initiator of plasmid RK2, there is an additional N-terminal part of the protein for which the structure was previously unknown. Due to the instability of purified TrfA protein, obtaining a sample with a concentration suitable for structural analysis was impossible. Therefore, we employed a bioinformatics approach in our structural investigations. Collaboration with prof. Janusz Bujnicki's team from the International Institute of Molecular and Cell Biology in Warsaw resulted in a model of the TrfA-33 protein, which included two WH domains and a short preceding fragment. Unfortunately, the use of methods based on homology modeling did not allow us to obtain a complete model of the protein. To obtain a full-length model of the TrfA-33 protein, we employed a new approach. In collaboration with dr Bartłomiej Tomiczek from the Laboratory of Molecular Evolution at University of Gdansk and prof. Jacek Czub's team from the Gdańsk University of Technology, we conducted an evolutionary analysis of the TrfA protein's origin and employed a multi-step approach to obtain the structural model of the protein and its complex with dsDNA. Phylogenetic analysis allowed us to identify a new family of TrfA-like proteins characterized by the presence of an additional sequence preceding the WH domains. To prepare the protein model, we used homology modeling (for WH domains), *ab initio* modeling (for the N-terminal domain), and biochemical data (from mass spectrometry analyses of TrfA-dsDNA complexes and my analyses of dsDNA complex formation by TrfA protein variants). In the initial stage, we prepared a preliminary model of the protein, based on which I, along with the team, designed thirteen variants containing single amino acid substitutions. For nine of these variants, I constructed expression vectors and purified all variants and next tested their ability to interact with dsDNA using the real-time interaction analysis technique - surface plasmon resonance (SPR). The SPR analysis allowed me to determine the kinetic

constants of DNA interaction for all purified proteins. I also tested the ability of all these proteins to initiate plasmid DNA replication *in vitro*. These data were then used to verify and optimize the preliminary model and then identify the most probable model of the full-length TrfA-33 protein in complex with dsDNA. The model we obtained confirmed our earlier biochemical studies, indicating that the N-terminal sequence of TrfA-33 independently forms a distinct domain. **This was the first structural model of a Rep protein with a three-domain structure.** I decided to examine whether the binding of all domains is necessary for stable binding of the protein to DNA. The SPR analyses I conducted, confirmed that the N-terminal domain independently forms a complex with dsDNA, but only the protein containing both the N-terminal domain and the two WH domains can stably bind the iteron sequence. Interestingly, while individual domains bind dsDNA independently of the sequence, the entire TrfA protein specifically binds only to iteron sequences. **The results I obtained were the first to demonstrate that DNA sequence specificity in the replication origin of broad-host-range plasmids depends on the interaction of all domains of the initiator protein.** These results were published in the work **Węgrzyn, Zabrocka et al., 2021, Nucleic Acids Research** (position 1 documenting achievement I, forming the basis of this application).

Despite years of research on DNA replication initiator proteins in both prokaryotic and eukaryotic cells, the mechanism of replication of genetic material, especially its initiation, is not fully understood. Recent studies have shown, among other things, that the bacterial protein DnaA, in addition to interacting with specific DnaA-box sequences located in the chromosome's replication *origin*, also interacts with single-stranded DNA (ssDNA), which appears in the DNA unwinding element (DUE) region when the double helix of DNA is unwound. Such activity of other replication initiators was not known. I decided to investigate this issue, conducting research on both plasmid proteins (Rep proteins: TrfA, RepE) and the protein from the archaeon *Aeropyrum pernix* (*Ap*Orc1). In the studies on the interaction of Rep proteins with ssDNA, I utilized both biochemical techniques and the more advanced method of imaging nucleoprotein complexes – atomic force microscopy (AFM). AFM allows for the visualization of individual biomolecules and direct observation of the formation of protein-DNA complexes. Due to the collaboration established with **Prof. Fernando Herrero-Moreno from Madrid, Spain**, a specialist in AFM analysis of protein-DNA complexes, **I was able to visit his laboratory three times** (October 3-27, 2011; July 8-21, 2012; and August 5-18, 2012) to conduct analyses of Rep protein interactions with ssDNA in the DUE region of plasmids. The results of my research in Madrid were consistent with those obtained in Gdańsk. Rep proteins interacted with ssDNA in the DUE region, but only with one specific strand. Since the DUE region contains repeated

sequences (13-mers for plasmid RK2; 8-mers for plasmid F), I examined whether the interaction of Rep proteins with this region requires the presence of all repeats. I demonstrated that changing the sequence of even one of the four repeats disrupts the formation of the nucleoprotein complex, resulting in the lack of plasmid DNA replication. Furthermore, analyses I conducted using size-exclusion chromatography and AFM showed the formation of triple complexes between dsDNA containing iterons, Rep protein, and a ssDNA fragment containing the sequence of one of the strands of the DUE region. The results I obtained were published in the work **Wegrzyn et al., 2014, Nucleic Acids Research** (position 1 documenting achievement II). **This was the first report on the interaction of Rep proteins with ssDNA.** I summarized the results of these and earlier studies in review articles **Zabrocka, Węgrzyn, Konieczny, 2014, Plasmid** (position 5 documenting achievement II), in which I am a co-author, and in the work **Wegrzyn*#, Gross*, Uciechowska, Konieczny# 2016, Front Mol Biosci.** (position 6 documenting achievement II), in which I am equal first author and one of the two corresponding authors.

Until this point, the conducted studies had not determined whether a single molecule of the Rep protein binds both iterons and ssDNA or if some molecules interact only with dsDNA while others with ssDNA. The structure and function of these complexes were also unknown. The question of whether other replication initiators, such as those in Archaea cells, form nucleoprotein complexes with ssDNA in the DUE region remained open. I addressed these issues after returning from an extended medical leave (01.2014-08.2014) and maternity leave (09.2014-02.2015). First, I investigated whether replication initiators of the chromosome in Archaea possess the ability to interact with ssDNA in the DUE *origin* region.. I cloned and purified the Orc1 protein from the archaeon *Aeropyrum pernix* (*Ap*Orc1). Using techniques such as electrophoretic mobility shift assay (EMSA), surface plasmon resonance (SPR), and biolayer interferometry (BLI), I analyzed the interaction of this protein with ssDNA containing the sequence of the region considered as the site of open complex formation during initiation of replication. My analyses demonstrated that the *Ap*Orc1 protein binds ssDNA and exhibits specificity for sequences rich in thymidine residues. Therefore, it is possible that the formation of such a complex plays a role in the process of replication initiation in organisms other than bacteria. **These studies were funded with grant MINIATURA, I obtained from the National Science Center** , and the results were published in the work **Wegrzyn# and Konieczny, 2021 BMC Research Notes** (position 2 documenting achievement II), in which I served as the corresponding author.

The next question I aimed to answer was what structure the Rep protein complexes form with ssDNA and what role these complexes play in the DNA replication initiation process. Since obtaining the structure of nucleoprotein complexes requires protein purification at high concentrations, which was not possible for the TrfA protein, I continued my research on the structure of Rep nucleoprotein complexes using the RepE protein from the F plasmid, which I could purify at the required concentration. **These studies were funded by the National Science Center under the SONATA project, where I served as the project leader and principal investigator.** The results obtained in the framework of this project were described in the paper **Wegrzyn# et al., 2023 Nucleic Acids Research** (position 3 documenting achievement II), in which I am the first author and one of the corresponding authors. Due to collaboration with **Prof. Marcin Nowotny's team from the International Institute of Molecular and Cell Biology in Warsaw, I was able to conduct crystallization of the purified RepE protein with ssDNA in his laboratory.** My visits to the laboratory in Warsaw (11-12.07.2017 and 11-12.12.2018) allowed me to familiarize myself with the entire process of obtaining the protein's structure, from preparing the crystallization mixture to harvesting crystals and assessing their quality. The data obtained for the crystals of the RepE-ssDNA complex enabled us to determine the structure at a resolution of 1.6 Å. The obtained structure and mass spectrometry data of RepE-ssDNA complexes allowed me to design variants of the RepE protein with single amino acid substitutions that could be relevant for ssDNA interaction. Based on literature data, I also designed a RepE protein variant (R205E/R206E/R207E) that should be defective in interaction with dsDNA. For these variants, I constructed expression vectors, purified the proteins, and conducted phenotype analyses. Analyses using electrophoretic mobility shift assay (EMSA) and SPR showed that out of the twelve purified RepE protein variants, three (containing substitutions N22E, F146E, and Y172E) were incapable of interacting with ssDNA DUE. The remaining proteins, except for the I180E variant, which retained its ssDNA-binding ability, exhibited reduced ssDNA interaction. Interestingly, all these proteins were capable of interacting with dsDNA containing the iteron sequence at a level similar to the wild-type protein. Simultaneously, the RepE R205E/R206E/R207E variant, as expected, showed disrupted interaction with dsDNA but was able to bind to ssDNA. **My results unequivocally demonstrated that the binding of dsDNA and ssDNA by Rep proteins is independent.** In my earlier work from 2014, I showed that interaction with ssDNA is essential in the plasmid DNA replication process. Conducting *in vitro* replication analysis and KMnO4 footprinting analysis for the prepared RepE protein variants, **allowed me to demonstrate that the Rep-ssDNA complex plays a role in maintaining a stable open complex in the DUE region of**

the plasmid *origin*. Similar studies were also carried out for the second model system, plasmid RK2, and its encoded protein TrfA. Analysis of TrfA variants defective in ssDNA binding but interacting with dsDNA showed that these proteins were unable to form a stable open complex in the DUE region of plasmid RK2 origin. **These studies were conducted by Ph.D. student Monika Oliwa, under my supervision as an auxiliary promoter.**

In the work Wegrzyn et al., 2014, I demonstrated that Rep proteins can form a triple complex. An open question remained whether a single molecule of the protein can simultaneously interact with both dsDNA and ssDNA in the DUE. This issue is currently being discussed in the literature, also in the context of the bacterial replication initiator DnaA. Two models of the nucleoprotein complex formed by the replication initiator and DNA in the origin region are proposed. In one model, it is suggested that a certain pool of protein molecules interacts with specific sequences in dsDNA, and then, after the replication bubble is formed, other protein molecules interact with one of the strands in this region. The second model assumes that some of the initiator molecules simultaneously bind to sequences in dsDNA and one of the strands in the DUE region (the so-called "loop-back model"). However, neither of these models has been supported by structural data so far. Using my research model, I decided to investigate this issue. Again, in collaboration with Prof. Nowotny's team, I prepared crystallization mixtures in his laboratory containing RepE protein, a double-stranded fragment containing the iteron sequence, and a ssDNA fragment from the DUE region. The obtained crystals allowed for solving the structure of the triple complex with a resolution of 3.2Å. I also conducted biochemical analyses using the obtained variants of the RepE protein defective in interacting with ssDNA but capable of binding dsDNA, and a variant that could only bind dsDNA but not ssDNA. I showed that the ability of Rep protein to interact with ssDNA and dsDNA cannot occur *in trans* in two different protein molecules but must be active within a single molecule. **The structural and biochemical data obtained by me are the first direct evidence that the loop-back model is highly probable.** These results have been described in the paper **Wegrzyn et al., 2023, Nucleic Acids Research** (position 3 documenting achievement II).

The formation of nucleoprotein complexes by initiator proteins is essential for initiating the process of DNA replication. Equally important is how long such a complex persists in the cell. Mechanisms are needed to regulate the stability of these complexes at the *origin* site. One of these mechanisms involves the proteolysis of replication initiation proteins by cellular proteases. Studies conducted using purified *E. coli* cellular proteases and the plasmid replication initiator TrfA demonstrated that the presence of DNA and TrfA's binding to DNA stimulate

protein degradation by the Lon and ClpAP proteases. At the same time, my EMSA analyses showed the ability of the protease itself to interact with DNA. These results were published in the work by Kubik, **Wegrzyn**, Pierechod, Konieczny (2012) in Nucleic Acid Research (Attachment 4, position II 3 in the complete scientific achievements list). Continuing these studies, I designed point mutations in the Lon protease that could result in the loss of DNA interaction. The designed changes were introduced into the protein gene sequence, and the Lon variants were purified as part of a master's project for which I was the supervisor. Then I examined the impact of presence of DNA on the degradation of replication initiators, both from plasmids (TrfA, RepE) and phage (λO), by the obtained Lon protease variants. I showed that the substitutions introduced in the ATPase domain of the protease led to a loss of ability to interact with DNA and inhibited proteolytic activity towards the tested substrates. Interestingly, I did not observe such an effect for IbpB protein, which does not interact with DNA. Additionally, the universal substrate α-casein was degraded by both wild-type and Lon protease variants. My analysis of substrate degradation rates by the examined Lon variants revealed that the protein containing four substitutions in the ATPase domain (R306E/K308E/K310E/K311K) could degrade FITC-casein faster than the wild-type protein, which could be due to differences in the oligomeric state of these proteins. The results of these studies were published in the paper **Karłowicz*, Wegrzyn*, Gross, M., Kaczynska, D., Ropelewska, M., Siemiatkowska, M., Bujnicki, J.M., Konieczny (2017) J Biol Chem.** (position 1 documenting achievement III), where I am one of the co-first authors. The research on the importance of proteolysis in plasmid DNA metabolism was presented in the review paper **Karlowicz A., Węgrzyn K., Dubiel A., Ropelewska M., Konieczny I., (2016) Plasmid**, where I am one of the first two authors. This is the second of the works describing achievement III.

Based on the results of my research and numerous studies by other authors, it is evident that DNA replication and its regulation are complex processes. So far, the exact role of the bacterial protein DnaA in plasmid DNA initiation remains unknown. Additionally, there is a lack of structural data that would reveal the composition of the full replication origin complex, which includes at least several molecules of the initiator protein, histone-like proteins (HU, IHF), SSB protein, helicase, gyrase, primase, and components of the replication complex.In my further research, I would like to focus on these issues.

4.2. Other Scientific achievements

Research on the analysis of nucleoprotein complexes has allowed me to expand my research capabilities related to biomolecular interactions. I have gained significant expertise in studying

the kinetics of biomolecular interactions in real-time using the Surface Plasmon Resonance (SPR) technique. I successfully apply these and other interaction analysis techniques in various research projects, including the search for new compounds with potential therapeutic applications. I conduct such studies as **an researcher in a project funded by the Polpharma Foundation, led by Prof. Dr. Sylwia Rodziewicz-Motowidło from the Faculty of Chemistry at the University of Gdańsk.** Within the scope of these project, including the SPR analysis of peptide and protein interactions conducted by me, we have identified peptides that block the interaction between PD-L1 and PD-1 proteins. This interaction is responsible for inhibiting the proliferation of T lymphocytes and plays a crucial role in cancer development. **The results of this work were published in a collaborative paper** (Bojko, M., **Węgrzyn, K.,** Sikorska, E., Kocikowski, M., Parys, M., Battin, C., Steinberger, P., Kogut, M.M., Winnicki, M., Sieradzan, A.K., Spodzieja, M., Rodziewicz-Motowidło, S.; Design, synthesis and biological evaluation of PD-1 derived peptides as inhibitors of PD 1/PD-L1 complex formation for cancer therapy. (2022) Bioorganic Chemistry, 128, art. no. 106047), **where I am the second author** (Attachment 4, item II 11 of the complete scientific achievements).
I also conduct research with similar subjects in other projects led by Prof. Sylwia Rodziewicz-Motowidło and in collaboration with teams outside the University of Gdańsk. I have described my scientific activities in these studies in section 7 of this Summary of Professional Accomplishments.

5. *Presentation of significant scientific or artistic activity carried out at more than one university, scientific or cultural institution, especially at foreign institutions.*

The analysis of nucleoprotein complexes formation can be conducted using various biochemical, microscopic, and structural techniques. Since my research primarily involved biochemical methods in such analyses, I decided to expand my research toolbox with the innovative, at that time, technique of Atomic Force Microscopy (AFM). AFM allowed for the analysis of individual biomolecules and direct observation of the formation of protein-DNA complexes. One of the research groups specializing in the analysis of protein-DNA complexes using AFM is **the team of Prof. Fernando Morreno Herrero at the National Center for Biotechnology in Madrid, Spain.** Due to prof. Moreno Herrero invitation, **I was able to undertake a one-month visit in 2011, followed by two two-week research visits in 2012 to his laboratory (Attachment 3C).** These visits were made possible through funding I obtained in a competition for young scientists from the funds of the Intercollegiate Faculty of Biotechnology of the University of Gdańsk and the Medical University of Gdańsk. During these

research visits, I conducted analyses of nucleoprotein complexes formation by plasmid replication initiators, TrfA and RepE proteins, with fragments of ssDNA containing the sequence of DUE region. In these analyses I used DNA fragments with wild-type sequences and those with introduced modifications. **During these research prepared the appropriate reaction mixtures, prepared the AFM imaging samples, scanned the prepared surfaces, and then analyzed the obtained data using appropriate software.** These analyses, for the first time, demonstrated that plasmid replication initiators specifically bind to one of the strands in the DUE region and confirmed earlier observations, which I made using biochemical techniques. **As a result of the data obtained in Gdańsk and during my stay in Madrid, a publication was prepared and published in a high-impact factor journal, Wegrzyn et al., 2014 Nucleic Acids Research, where I am the first author.** This publication is the first paper documenting Achievement II in the submitted application. My stay in Prof. Fernando Moreno Herrero's laboratory and my experience with AFM analysis **allowed me to introduce AFM techniques to the Intercollegiate Faculty of Biotechnology of the University of Gdańsk and the Medical University of Gdańsk.** Initially, I organized workshops on Atomic Force Microscopy, and later, the AFM microscopy station was established in the Laboratory of Specialized Laboratories of MWB, which is now equipped with three different AFM microscopes for biological applications.

Continuing the research on the complexes of plasmid DNA replication initiator proteins with ssDNA in the DUE region of the replication *origin*, I focused on the structural analysis of these complexes. To conduct the structural analysis of the Rep protein complexes with ssDNA, I initiated collaboration with **Prof. Marcin Nowotny's team at the International Institute of Molecular and Cell Biology in Warsaw**, which specializes in studying the structure of protein-DNA complexes. **In 2017 and 2018, I made visits to Prof. Nowotny's laboratory, where I prepared the RepE protein purified by me for crystallization in the presence of ssDNA and ssDNA-dsDNA, respectively.** As a result of the work conducted during these visits, crystals of nucleoprotein complexes were obtained, and their structures were solved. The obtained structures, in combination with experiments conducted in the Laboratory of Molecular Biology, **allowed me to demonstrate that DNA replication initiation in bacterial cells most likely occurs according to the loop-back model.** The results obtained have been described in the publication **Wegrzyn et al., 2023 Nucleic Acids Research**, which is the third item documenting Achievement II in the submitted application. **I aim to leverage the experience gained through collaboration with Prof. Nowotny's team and further develop it in my future research on the structure of replicative nucleoprotein complexes.**

6. *Presentation of teaching and organizational achievements as well as achievements in popularization of science or art*

From the beginning of my work at the Intercollegiate Faculty of Biotechnology, didactic activites hasbeen an integral part of my work. I am involved in teaching activities for students majoring in Biotechnology, primarily at the undergraduate level. As part of my teaching duties, I conduct lectures, seminars, as well as laboratory and classroom exercises.From 2005 to 2019, I led laboratory exercises in Genetic Engineering, for which **I wrote a script titled 'Genetic Engineering Laboratory - Exercise Materials.'** I prepared the script both **in Polish and English** as part of a project co-financed by the European Social Fund, under the project title: 'Increasing the activity of IFB UG and MUGed students in actions improving graduates' attractiveness on the job market.' In 2019, after the introduction of a new curriculum at IFB, in which I was involved in its preparation, the program content of the laboratory exercises in the Genetic Engineering Laboratory became part of the new laboratory exercises: Biomolecules - Structure, Synthesis, and Properties, Unicellular Organisms - Genetics, and Unicelullar Organisms - Metabolism. For the new laboratory classes, I modified the previously conducted exercises and prepared new ones, covering topics such as ELISA techniques and protein detection in silver-stained polyacrylamide gels. I also created new supplementary materials for students.

I also conduct seminars in Methodology I (formerly Genetic Engineering Methodology), Methodology II (formerly Molecular Biology Methodology), and Emerging Trends in Biotechnology. These classes cover both basic and advanced techniques in genetic engineering, molecular biology, and biotechnology. During these sessions, **I employ teaching methods based on new technologies and innovative pedagogical solutions that engage students in the learning process** (group and individual tutoring, peer-feedback, blended learning, rubrics).In order to familiarize students with some of the advanced molecular biology techniques discussed in these courses and enable them to gain direct experience with these methods, **I organized microscopy workshops in collaboration with Olympus in 2012 and atomic force microscopy workshops in cooperation with Labsoft in 2015.**

From 2016 to 2018, **I organized and conducted original workshops titled 'Molecular Biology Methodology – Exercises,' which were co-financed by the University of Gdańsk's Educational Innovation Fund, obtained through a competition.** Since 2019, these workshops have been permanently included in the curriculum for the Biotechnology program at IFB as elective courses. I also used my knowledge of nucleoprotein complex research methodology, along with Prof. Dr. Igor Konieczny, to prepare a script titled 'Molecular Biology

of Nucleic Acids – Experimental Methodology,' which is used by students in the second-cycle Biotechnology program.

Since 2019, I have been co-teaching original computer-based lectures and workshops on Unicellular Organisms – Genetics with Prof. Michał Obuchowski. During these classes, students become acquainted with designing and introducing genetic modifications (genome editing, gene cloning).

My teaching responsibilities also include lectures primarily on gene cloning and genetic engineering (Milestones in Biotechnology: Cloning; Unicellular Organisms – Genetics: Gene and Genome Modifications; Unicellular Organisms – Life Environment: *Caulobacter crescentus*).

From 2010 to 2023, I served as an supervisor to **28** undergraduate students working on their bachelor's theses and **11** students preparing their master's theses. I also acted as the chairperson and a member of the Diploma Examination Committee for the undergraduate Biotechnology program, as well as a reviewer for master's theses. On several occasions, I served as a supervisor for student internships conducted in the Laboratory of Molecular Biology (formerly the Department of Molecular and Cellular Biology). In **three doctoral projects**, I served as an auxilary supervisor, and two doctoral dissertations have already been defended (Marta Gros on 8th March 2019; Andrzej Dubiel on 23rd October 2020), while one is currently in preparation (Monika Oliwa).

In recognition of my scientific and educational activities, **I received the Medal of the National Education Commission in 2017**.

In addition to classes and workshops aimed at the academic community, **I also try to popularize science**. As part of the Baltic Festival of Science, I organized workshops and open demonstrations titled 'Is DNA Blue?' In the years 2016-2018, as a member of the Faculty team for organizing promotional and educational events, **I co-organized popular science events at the Faculty**. This year, **I also served as one of the two advisors to students participating in the international synthetic biology competition iGEM** organized by The iGEM Foundation. This competition involves young science enthusiasts striving to develop solutions to current issues related to the environment, civilization, and humanity, using synthetic biology. It aims to popularize the use of genetic engineering to improve the quality of life. At the invitation of the IFB student team participating in the competition, I supported them with my expertise and knowledge in the field of bacterial genetic engineering.

Since the beginning of my career as an academic teacher, **I have placed a strong emphasis on improving my teaching competencies by participating in training programs and initiatives**

dedicated to educators, as well as attending scientific conferences focused on academic teaching. Among other activities, I took part in projects organized by the Ministry of Science and Higher Education, such as the 'Masters of Teaching' and 'Masters of Teaching – Implementation' programs.Within the first of these projects, **in April 2019, I conducted a one-week study visit to Ghent University in Belgium**. During this visit, I had the opportunity to learn about and practice the method of group tutoring applied there. In the academic year 2021/2022, I completed a course and **obtained a certificate as an academic tutor**. In the role of a tutor, I had the chance to assist students in various ways, including helping them prepare their theses and popular science articles (Matulewska, Węgrzyn, Ziętkiewicz (2022) Tutoring Gedanensis 7(2)/2022 (13 – 22)).In the academic years 2022-23, **I also participated in the advanced program 'Advanced Qualification in Teaching,'** dedicated to academic teachers, which was co-organized by the University of Groningen in the Netherlands.

In my role as an academic teacher, I engage in activities related not only to teaching but also **to the organization of education at the University of Gdańsk**, particularly within the Intercollegiate Faculty of Biotechnology. In the academic year 2018/2019, **I was appointed to the Faculty Team for the Development of a New Educational Program and actively participated in the development of an innovative curiculum** based on thematic modules rather than traditional subjects (concept-based learning). The developed curiculum was appreciated by the Rector of the University of Gdańsk and received a **First-degree Team Award**. The innovative educational program co-created by me was also recognized by the State Accreditation Commission, which awarded the Biotechnology program the Certificate of Excellence.

Following the implementation of the new educational program, **I was appointed as the coordinator of one of the Bachelor's Degree Modules in the Biotechnology** program in May 2019 and, in 2020, as **a member of the Program Board for the Biotechnology** program at the Intercollegiate Faculty of Biotechnology of the University of Gdańsk and Gdańsk Medical University. In 2022, at the request of the Educational Research Institute (IBE) and the University of Gdańsk, I prepared a synthetic description of qualification characteristics for the Bachelor's Degree program in Biotechnology at the University of Gdańsk.

My **organizational activities** also include participation in the Intercollegiate Faculty of Biotechnology Council and the Discipline Council for Biotechnology at the University of Gdańsk, to which I was elected. Additionally, I was appointed by the Dean as a member of the University Disciplinary Commission for Student Affairs at the University of Gdańsk and the

Faculty Commission for the Safety of Confined Use of Genetically Modified Microorganisms (GMM) and Genetically Modified Organisms (GMO)

Teaching training programs in which I have participated:

2010 - Pilot Course in Academic Didactics, University of Gdańsk

2015-2018 - Laboratory of Didactic Initiatives training, University of Gdańsk

2019-2020 - Participation in the Ministry of Science and Higher Education project 'Masters of Didactics'; one-week study visit to Ghent University, Belgium

2019-2021 - Participation in the Ministry of Science and Higher Education project 'Masters of Didactics – Implementation'

2021-2022 - Academic Tutoring Course, Center for Didactic and Tutoring Excellence, University of Gdańsk; Academic Tutor Certificate

2022 - Training programs by the Institute of Regional Development Foundation as part of the 'Accessible University of Gdańsk' project: Support for Students with Cognitive Disorders; Working with Students with Psychological Difficulties

2022-2023 - Participation in the Ministry of Education and Science and University of Groningen (Netherlands) project 'Advanced Qualification in Teaching

Teaching conferences:

06.2019 - 7th Nationwide Academic Didactics Conference IDEATORIUM

K. Węgrzyn, 'Utilization of high-specialization research equipment in the teaching of Molecular Biology Methodology – practical laboratory exercises' (poster)

09.2020 – Ministry of Science and Higher Education conference as part of the 'Masters of Didactics' project

K. Węgrzyn, 'The educational innovation project: Introduction of peer assessment activity into the course Seminar I - Experimental publications in molecular biology and biotechnology' (oral presentation)

05.2021 - Biochemical Society Conference: Evolving molecular bioscience education

06.2021 – Ministry of Science and Higher Education conference: Masters of Didactics. Improving the teaching competences of academic teachers;

K. Węgrzyn, 'Teaching together - effective involvement of Students in the teaching process' (oral presentation)

07.2021 - FEBS Conference

K. Węgrzyn, R. Czajkowski, S. Jafra, P. Koszałka, A. Lipińska, S. Ołdziej, W. Żmudzińska, 'Boosting the learning of Biotechnology by Concept-based teaching' (oral presentation)"

Roles held at the University of Gdańsk:

1. Member of The diploma examination committee for undergraduate studies in Biotechnology.
2. Member of The university disciplinary committee for University of Gdańsk students (term of office: 2012-2016; 2016-2020; 2020-2024).
3. Member of The team responsible for organizing promotional and educational events at IFB UG-MUG (2016-2018).
4. Member of the Council of the Intercollegiate Faculty of Biotechnology (term of office: 2016-2020; 2020-2024).
5. Member of the Faculty Committee for the Safety of Genetically Modified Microorganisms (GMM) and Genetically Modified Organisms (GMO).
6. Member of the Discipline Council for Biological Sciences (2020-2022).
7. Member of the Discipline Council for Biotechnology (since February 2023).
8. Member of the Program Council for the Biotechnology program.
9. Member of the Committee for the Assessment of Academic Staff Activity at MWB UG-GUMed.
10. Member of the University of Gdańsk Tutoring Center (tutor)

7. Apart from information set out in 1-6 above, the applicant may include other information about his/her professional career, which he/she deems important

Scientific and research activities not presented in point 4:

As a result of my research on the structure and function of nucleoprotein complexes, I have gained significant experience in analyzing biomolecular interactions. I have used this experience in research conducted in collaboration with other research teams both in Poland and abroad. I primarily conduct biomolecular interaction analyses using techniques such as electrophoretic mobility shift assays (EMSA), microscale thermophoresis (MST), and most importantly, surface plasmon resonance (SPR), which enables real-time kinetic studies of interactions. The collaborations I have established have resulted in the publication or

submission for publication of several papers (Attachment 4, Section II), with additional works in progress.

I am particularly interested in research related to the discovery of new compounds, molecules, or peptides that can block interactions between cellular proteins, which lead to pathological conditions. The techniques I use for interaction analysis allow for the rapid and precise identification of molecules with potential therapeutic applications, which can then be further tested *in vivo*. Research conducted in collaboration with **prof. dr hab. Sylwia Rodziewicz-Motowidło's team at the Faculty of Chemistry, University of Gdańsk**, on the interactions between BTLA and HVEM proteins, involved in immune system regulation, has led to the identification of peptides containing fragments of BTLA and glycoprotein D sequences that block this interaction. The results of these studies have been published in the following papers: **International Journal of Molecular Sciences (2020) 21 (2), art. no. 636** (Attachment 4, Section II 8), **Bioorganic Chemistry (2022) 122, art. no. 105748** (Attachment 4, Section II 10), **and Biomed Pharmacother. (2023) 165:115161** (Attachment 4, Section II 15), in which **I am a co-author**. Similarly, we have identified peptides that block the interaction between PD-L1 and PD-1 proteins (**Bioorganic Chemistry (2022) 128, art. no. 106047**) (Section 4.2 of the Summary of Professional Accomplishments).

The search for molecules with potential therapeutic effects has also formed the basis for collaboration with **prof. dr hab. Maciej Bagiński's team at the Gdańsk University of Technology**. In the course of research on blocking the interactions of TRF1 and TIN2 proteins, which are essential for the proper functioning of telomeres and constitute a potential target in anticancer therapy, peptidomimetics with potential applications have been identified. The results of this research have been partially published in the **Chemistry journal (2023) e202300970, doi: 10.1002/chem.202300970** (Attachment 4, Section II 14), in which **I am a co-author**. **A patent application has also been submitted**: "Inhibitors of interactions between TRF1-TIN2 or TRF2-TIN2 telomeric proteins for use in anticancer therapy," Patent Office of the Republic of Poland (RO/PL), Ref. No. PZ/8885/RW/PCT International application number: PCT/PL2022/050017 (Attachment 4, item III).

Recently, I have also been invited to collaborate with **prof. John Matsoukas' team at the University of Patras (Greece)** to search for potential new drugs to combat SARS-CoV-2 virus infection. The results obtained so far have been published in the paper **Kelaidonis K et al., (2023) Int J Mol Sci. 24(9):8454**, and another manuscript has been submitted to the Advanced Science journal and is currently under review.

The significance of the search for new drugs, especially in anticancer or antiviral therapies, is demonstrated not only by the interest in this issue among numerous research groups but also by biotechnological and pharmaceutical companies. In order to search for new therapeutics, **I conducted SPR analyses for Recepton Sp. z o.o., a company based in Gdańsk**, as part of the project POIR.01.01.01-00-0129/18 'Technology platform designed for the development and study of therapeutics used in cancer immunotherapy. Development of small molecules for the immune checkpoint blockade in combination cancer immunotherapies'.

Awards and scholarships received after obtaining a doctoral degree:

2010 - Scholarship from the University of Gdańsk Development Foundation

2012 - Scholarship as part of the project 'Educating the Best: A Comprehensive Development Program for Doctoral Students, Young Doctors, and Academic Teaching Staff at the University of Gdańsk, Component: Scholarship and Training Support for Doctoral Students and Young Doctors'

2016 - Elsevier-Plasmid Prize for the poster titled 'Disclosing the multiple roles of plasmid replication initiator during DNA replication initiation,' presented at the International Plasmid Biology Conference, 18-23 September 2017, Cambridge, UK

2017 - Medal of the National Education Commission

2020 - Awarded a grant by COST (European Cooperation in Science and Technology); reference number COST-TS-ECOST-TRAINING_SCHOOLCA15126-270120-111550 to participate in the FEBS HyThaBio conference and training in Grenoble, France

2019 - Rector's Team Award I degree for developing a new and innovative Education Program at the Intercollegiate Faculty of Biotechnology of the University of Gdańsk and the Medical University of Gdańsk

2022 - Rector's Team Award I degree from the University of Gdańsk for scientific achievements supported by scientific publications

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(Applicant's signature)