HABILITATION THESIS

Structural basis for human mitochondrial DNA replication and repair

Dr. Michał Roman Szymański

Intercollegiate Faculty of Biotechnology
University of Gdańsk and Medical University of Gdańsk

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1. Name and surname: **Michał Roman Szymański**

2. Diplomas and scientific degrees - with the name, place and year of obtaining them and the title of the doctoral dissertation:

PhD in Biochemistry and Molecular Biology (with distinction) 08/2007 - 12/2011
Molecular Biophysics Educational Track
Biochemistry and Molecular Biology Department, University of Texas Medical Branch w Galveston, Texas, USA
PhD Mentor: Wlodek M. Bujalowski Ph.D.
PhD Thesis Title: Helicase-initiated Assembly of Macromolecular Machines Involved in DNA Replication and Repair.

Bachelor of Science in Biochemical and Biophysical Sciences (with distinction) 01/2002 - 05/2007
Minor in Chemistry
Biology and Biochemistry Department, University of Houston, Houston, Texas, USA
Mentor: Dr. H.J. Yeo Laboratory
Thesis Title: Structural and functional characterization of membrane protein from *Haemophilus influenza.*

3. Information on the employment in scientific units:

Structural Biology Group Leader 10/2017 – obecnie
Intercollegiate Faculty of Biotechnology
University of Gdansk – Medical University of Gdansk, Poland

Research Scientist 02/2016 – 09/2017
Dr. Whitney Y. Yin Laboratory
Pharmacology and Toxicology Department
University of Texas Medical Branch w Galveston, Texas, USA

Jeane B. Kempner Postdoctoral Fellow [#] 05/2013 – 01/2017
Dr. Whitney Y. Yin Laboratory
Pharmacology and Toxicology Department
University of Texas Medical Branch w Galveston, Texas, USA
[#] Full postdoctoral stipend from Jeane B. Kempner Foundation

Jeane B. Kempner Postdoctoral Fellow [#] 01/2012 – 04/2013
Dr. Wlodek M. Bujalowski Laboratory
Biochemistry and Molecular Biology Department
University of Texas Medical Branch w Galveston, Texas, USA
[#] Full postdoctoral stipend from Jeane B. Kempner Foundation

Graduate Student 08/2007 - 12/2011
Dr. Wlodek M. Bujalowski Laboratory
Biochemistry and Molecular Biology Department
University of Texas Medical Branch w Galveston, Texas, USA

Welch Research Assistant * **01/2005 - 05/2007**
Dr. H.J. Yeo Laboratory
Biology and Biochemistry Department
University of Houston, Houston, Texas, USA
** Full pre-doctoral research stipend from Welch Foundation*

Research Assistant $ **01/2004 - 12/2004**
Polyorganix Inc.,
Biochemical Laboratory and Service Company
Houston, Texas, USA
$ Position within the COOP program at the University of Houston promoting student internships in biotechnology companies.

4. Indication of the achievement resulting from art. 16 sec. 2 of the Act of 14 March 2003 on academic degrees and academic title and on degrees and title in the field of art (Journal of Laws No. 65, item 595, as amended):

a) title of the achievement:

Structural basis for human mitochondrial DNA replication and repair

b) presented achievement includes a series of 4 related articles:

i) Szymanski, M.R., Yu, A., Gmyrek, A.M., White, M.A., Molineux, I.J., Lee, J.C., Yin W.Y. A novel domain in human EXOG converts apoptotic endonuclease to DNA- repair exonuclease. ***Nature Communications.*** 2017 May 3;8:14959.
Recommended by F1000.
IF_{2017}: 12.4; $MNiSW_{2016}$: 45; Citations (WoS):2; Citations (Google Scholar): 3

ii) Li, M., Mislak, A.C., Foli, Y., Agbosu, E., Bose, V., Bhandari, S., **Szymanski, M.R**., Shumate, C.K., Yin, W., Anderson, K.S., Paintsil, E. DNA Polymerase-γ R953C Mutant Linked to ART-Associated Mitochondrial Toxicity. ***Antimicrobial Agents and Chemotherapy.*** 2016 Aug 22;60(9):5608-11.
IF_{2016}: 4.3; $MNiSW_{2016}$: 40; Citations (WoS): 2; Citations (Google Scholar): 3

iii) Sohl, C.D*****., **Szymanski, M.R***., Mislak, A.C., Shumate, K.C., Amiralaei, S., Schinazi, F.R., Anderson K.S., Yin W.Y. (2015). Probing the Structural and Molecular Basis of Nucleotide Selectivity by Human Mitochondrial DNA Polymerase γ**. *Proceedings of National Academy of Sciences of the United States of America*.** 112(28):8596-601.
***equal contribution**
IF_{2015}: 9.4; $MNiSW_{2015}$: 45; Citations (WoS): 9; Citations (Google Scholar): 10

iv) Szymanski, M.R., Kuznetsov, V.B., Shumate, C., Meng, Q., Lee, Y-S., Patel, G., Patel, S.S., Yin W.Y. (2015). Structural basis for processivity and antiviral drug toxicity in human mitochondrial DNA replicase. ***EMBO J.*** 34(14):1959-70.
IF_{2015}: 9.6; $MNiSW_{2015}$: 45; Citations (WoS): 14; Citations (Google Scholar): 20

c) description of the scientific/artistic purpose of the above work and results achieved, discussing their possible use:

Mitochondria are key to the physiology of eukaryotic organisms. A primary function of the mitochondria is energy production in the form of ATP. However, mitochondria also participate in many basic cellular processes such as homeostasis of calcium and iron, biosynthesis of heme, pyrimidines and steroids, fatty acid β-oxidation and apoptosis (Orrenius, Gogvadze and Zhivotovsky 2007, Ott et al. 2007). All these essential activities are correlated with the integrity of mitochondrial DNA (mtDNA), therefore, maintaining the integrity of the mitochondrial genome is essential for the proper functioning of cells. There are many proteins involved in mtDNA replication and repair, but only two: DNA polymerase gamma (Pol γ) and 5′-exo / endonuclease (EXOG), were shown to be present exclusively in the mitochondria (Akhmedov and Marin-Garcia 2015, Alexeyev et al. 2013, Kazak, Reyes and Holt 2012). While Pol γ is involved in both mtDNA replication and repair, EXOG is critical to the repair of the human mitochondrial genome. Inactivation of POLG gene is embryonic lethal emphasizing importance of mtDNA replication and repair in maintenance of mitochondrial and cellular homeostasis (Hance, Ekstrand and Trifunovic 2005). To date, more than 300 point mutations associated with mitochondrial diseases have been identified in POLG gene. In addition, mutations in Pol γ are the most common cause of hereditary mitochondrial disorders (Copeland 2012, Copeland and Longley 2014, Stumpf, Saneto and Copeland 2013). The function of EXOG, appears to be similarly important as inactivation of EXOG gene is embryonic lethal in mice (Szczesny, B. and Szymanski, M.R., data not published). EXOG-depletion causes accumulation of unrepaired intermediates in the mtDNA (single strand breaks, SSBs) that induce mitochondrial dysfunction. Moreover, unlike nucleases FEN1 and DNA2, the presence of EXOG in mitochondria is critical for cellular viability (Tann et al. 2011). While these exciting findings shed a new light on mtDNA metabolism, the molecular mechanism by which Pol γ and EXOG perform their critical functions in mtDNA replication and repair is not well understood.

The main goal of studies presented as my habilitation dissertation is the functional and structural characteristisation of Pol γ and EXOG proteins responsible for the replication and repair of human mitochondrial DNA. The research was aimed at understanding the fundamental principles that govern the actions of these enzymes in human mtDNA replication and repair as well as the mechanistic basis for toxicity of antiviral drugs and mutations in mtDNA, related to wide spectrum of mitochondrial diseases and the normal aging process. To provide mechanistic insights a combination of biochemical, biophysical and structural biology techniques was used.

Structural basis for processivity and antiviral drug toxicity in human mitochondrial DNA replicase. Szymanski, M.R., Kuznetsov, V.B., Shumate, C., Meng, Q., Lee, Y-S., Patel, G., Patel, S.S., Yin W.Y. (2015). ***EMBO J.*** 34(14):1959-70.

In comparison to nuclear polymerases with specialized functions (DNA replication or repair), in the mitochondria, Pol γ is responsible for both DNA replication and repair. Pol γ belongs to the A-family of DNA polymerases, and the human enzyme consists of the Pol γA catalytic subunit and the dimeric accesory subunit Pol γB. Pol γA harbors all holoenzye activities: 5′-3′ polymerase, 3′-5′ exonuclease, and 5′-deoxyribose phosphate (dRP) lyase (Johnson et al. 2000, Lim, Longley and Copeland 1999). Pol γB has no enzymatic activity but regulates all Pol γA activities within the holoenzyme (Gellon et al. 2008, Johnson et al. 2000, Longley et al. 1998a) increasing its processivity and affinity for DNA, as well as accelerating the synthesis reaction rate (Johnson et al. 2000, Lee et al. 2010). Pol γB also increases 5′dRP lyase activity and reduces exonuclease activity (Pinz and Bogenhagen 2006). The polymerase and

exonuclease active sites are separated from each other by ~35 Å, while the active site of 5'dRP lyase was never described structurally, and its activity demonstrated in biochemical experiments, is rather low as compared to other polymerases with dRP lyase activity. This raises an interesting question: how are all activities of Pol γ regulated by Pol γB?

The error frequency during DNA replication catalysed by Pol γ is 10^{-5}, which is comparable with other DNA polymerases of high accuracy (Lee and Johnson 2006, Longley et al. 2001). However, Pol γ has a lower ability than its nuclear counterparts to distinguish errors using its exonuclease domain. Decreased ability of Pol γ to remove errors is particularly important in removing dideoxynucleotides incorporaded by Pol γ and is a side effect of antiretroviral treatment with the use of Nucleoside Reverse Transcriptase Inhibitors (NRTIs) (Johnson and Johnson 2001, Johnson et al. 2001, Lee, Hanes and Johnson 2003). Inhibition of Pol γ by NRTIs leads to moderate to life-threatening drug toxicity that clinically manifests as neuropathy, lactic acidosis, and hepatic failure (Koczor and Lewis 2010, McKenzie et al. 1995). What are the structural reasons for the lack of NRTI differentiation by Pol γ?

Mutations in the catalytic subunit of Pol γA lead to a broad spectrum of disease manifestations such as progressive external ophthalmoplegia, myopathy, epilepsy, neonatal hypotonia, encephalopathy and Alpers syndrome (Chan and Copeland 2009, Graziewicz et al. 2004, Zeviani and Taroni 1994). Mice with mutations in exonuclease domain of Pol γA show an increased accumulation of point and deletion mutations in mtDNA and are characterized by reduced lifespan and premature aging (Trifunovic et al., 2004). Understanding the structure of the Pol γ apoenzyme proved to be insufficient to explain why mutations in Pol γA cause a disease phenotype and how they affect the function of the holoenzyme in the replication or repair of mtDNA (Lee, Kennedy and Yin 2009). We assumed that DNA and nucleotides induce conformational changes significantly change the structure of Pol γ. Therefore, in order to better understand the molecular mechanism of mtDNA replication and repair process and to rationalize the phenotypes of Pol γ mutants associated with mitochondrial diseases, we set out to solve the holoenzyme structure "captured" in the replication process.

In order to crystallize the mtDNA replicating complex, we optimized the overproduction of the catalytic Pol γA subunit in insect cells with the use of the baculovirus expression system. The accesory subunit, Pol γB, was overproduced in *Escherichia coli*. Both proteins were purified in tandem by successive applications to affinity columns and then to a gel filtration column. Pol γA and Pol γB purified in this fashion were combined and Pol γ holoenzyme was isolated by re-applying to a gel filtration column. The complex was concentrated and directly used for crystallization. Considering the published reports, as well as predicted size of the holoenzyme, we tested different lengths of DNA substrates (primer/template) between 34 and 20 nt. Eventually, the 24/28 nt substrate (primer/template) proved to be the best to obtain high quality crystals. Additionally, to avoid stimulation of DNA replication, it was important to use $CaCl_2$ instead of $MgCl_2$. In order to preserve DNA duplex, we used a Pol γA mutant without exonuclease activity. We have published two structures: a holoenzyme complex with DNA, $CaCl_2$ and dCTP nucleotide, and a holoenzyme complex with DNA, $CaCl_2$ and first-generation antiviral drug, Zalcytabine. Structures could not be resolved using Molecular Replacement methods using the apoenzyme structure as a model. Heavy metal soaks also proved to be unproductive to solve the phase problem. Only after both Pol γA and Pol γB were derivatized with selenomethionine, the high-quality electron density maps appeared and allowed to rebuild regions that underwent structural changes induced by nucleotide, Zalcitabine and DNA binding. Our structures are the first to show the multi-subunit DNA replicase captured during DNA synthesis and illustrate how the two subunits of human mitochondrial Pol γ coordinate functions within the holoenzyme to achieve high processivity and regulation of the polymerase *vis-à-vis* exonuclease activity. The structures also suggest thr mechanism of communication between the polymerase and the exonuclease domain in the holoenzyme. Structural

analysis suggests that highly conserved region, consisting of amino acids 835-858 that forms the hairpin loop, can physically and functionally serve as a control point for the proper function of the enzyme. Mutations in R852 and R853, found in this region drastically reduce the activity of polymerase and were identified in patients with mitochondrial diseases (Davidzon et al. 2006, González-Vioque et al. 2006, Kasiviswanathan et al. 2009, Vasta et al. 2012). In addition, our structures provide mechanistic insight into the causes of disease-related mutations in Pol γ and allow us to map mutations that occur in patients and correlate them with changes in the enzyme activity. This has diagnostic significance because it can answer the question whether mutations with a specific phenotype, for example those manifestded at a young age, group in the specific regions of the holoenzyme structure. Therefore, base on our structures, we can predict the pathogenic potential and disease phenotype of new mutations in Pol γ. Currently, in collaboration with clinicians, we analyze such mutations. In addition, the structures we describe show that Pol γ binds Zalcitabine inhibitor and the native substrate in almost identical fashion, explaining, at the molecular level, the susceptibility of Pol γ to inhibition by antiviral drugs (NRTIs).

Probing the Structural and Molecular Basis of Nucleotide Selectivity by Human Mitochondrial DNA Polymerase γ. Sohl, C.D*., **Szymanski, M.R***., Mislak, A.C., Shumate, K.C., Amiralaei, S., Schinazi, F.R., Anderson K.S., Yin W.Y. (2015). ***Proceedings of National Academy of Sciences of the United States of America.*** 112(28):8596-601.
***equal contribution**

Introduced in 1996 highly active antiretroviral therapy (HAART) effectively inhibits HIV-1 (Human Immunodeficiency Virus, type 1) replication and improves the survival and quality of life of patients infected with HIV. HAART relies on the simultaneous use of three antiretroviral agents, most often two nucleoside reverse transcriptase inhibitors (NRTIs) and one protease inhibitor. This combination shows very high efficacy and significantly improved the treatment of people infected with HIV. In order to be effective, antiretroviral treatment must be carried out throughout life, resulting in the accumulation of side effects and complications related to the toxicity of antiretroviral drugs (Vidal et al. 2011). Examples of complications are potentially fatal lactic acidosis, neuropathy, lipodystrophy syndrome, cardiomyopathy, pancytopenia (Brinkman et al. 1998, Moyle 2000, Moyle et al. 2002). Nucleoside Reverse Transcriptase Inhibitors (NRTIs) are the cornerstone of HAART and were the first to be used in the treatment of people infected with HIV. NRTIs are analogues of natural nucleosides, but lack the 3'OH group, which is necessary to generate phosphodiester bonds between nucleotides in the growing DNA chain. In such fashion NRTIs inhibit the activity of HIV reverse transcriptase (HIV-RT) responsible for converting genetic material of virus (RNA) into DNA, to enable further production of virus particles after integration with the human genome. However, NRTIs not only inhibit viral reverse transcriptase, HIV-RT, but can also inhibit certain DNA polymerases in human cells (Copeland, Chen and Wang 1992). For example, NRTIs inhibits the action of DNA polymerase α and β, but the most sensitive to inhibition by NRTIs is the mitochondrial Pol γ (Kohler and Lewis 2007, Sohl et al. 2015). Inhibition of Pol γ responsible for the replication of mtDNA leads to a reduction in mtDNA level, causes mtDNA damage and oxidative stress, contributing to the previously discussed side effects associated with the use of NRTIs (Chan and Copeland 2009, Chan et al. 2007, Kline et al. 2009). Interestingly, the adverse effects of NRTIs, resulting from blocking the activity of Pol γ, are not perfectly correlated with the effectiveness of these drugs against HIV-RT. For example, NRTI emtricitabine [(-) - 2,3'-dideoxy-5-fluoro-3'-thioritidine, (-) - FTC] and its (+) - FTC enantiomer are eually efficient in inhibiting HIV-RT. At the same time, (+) - FTC is much more toxic than (-) – FTC to humans. We asked whether using a combination of

structural biology methods, biochemical and kinetic approaches can we understand the mechanistic similarities and differences between Pol γ and HIV-RT. The identification of structural differences between the intended target, HIV-RT, and adverse reaction target, Pol γ, could provide invaluable insights to aid in designing more effective drugs with lower toxicity.

In this study we presented the key structural and mechanistic differences in interations betwween NRTIs, Pol γ and HIV-RT. We show that Polγ and HIV-RT use different mechanisms of substrate and inhibitor binding and incorporation. The residue I948 in Pol γA, which does not play a significant role in binding and incorporation of the natural substrate, is important for the (-) - FTC binding selectivity. At the same time, homologous residue, R72 in HIV-RT, is important for both inhibitor and substrate binding. These conclusions are fundamental in drug design: we have shown that modification of 5-F in (-) - FTC interacts with the amino acid residue necessary for normal HIV-RT activity, at the same time, because of steric clash is "repelled" by homologous amino acid in Pol γ. Our work, for the first time, describes the mechanism of differential selectivity (-) - FTC and provides the structural basis for the development of new, urgently needed NRTIs with increased selectivity and lower toxicity. It is also worth mentioning that this research direction is still carried out by my team in cooperation with the laboratory Prof. Whitney Yin, from UTMB, United States.

DNA Polymerase-γ R953C Mutant Linked to ART-Associated Mitochondrial Toxicity. Li, M., Mislak, A.C., Foli, Y., Agbosu, E., Bose, V., Bhandari, S., **Szymanski, M.R**., Shumate, C.K., Yin, W., Anderson, K.S., Paintsil, E. ***Antimicrobial Agents and Chemotherapy.*** 2016 Aug 22;60(9):5608-11.

The works discussed above describe both the vulnerability of the Pol γ to NRTIs and the side effects associated with the use of HAART. Interestingly, not all patients undergoing the same treatment experience side effects to the same extent, suggesting that toxicity may be caused by genetic predisposition or other environmental conditions (Naviaux et al., 1999). Different variants of the POLG gene may affect the activity of Pol γ and thus the pharmacokinetics of certain drugs, therefore, it was suggested that changes in toxicity associated with HAART may partly result from the polymorphisms observed in the POLG gene (Carr and Cooper 2000, Feng et al. 2001, Johnson et al. 2001). In other words, the genetic makeup of the patient affects the beneficial or adverse effects of specific treatment (Dalal, Shankarkumar and Ghosh 2015, Vidal et al. 2011). To understand the role of Pol γ variants in toxicity associated with HAART, we performed a retrospective analysis of data and samples collected during case-controled study of HAART-induced mitochondrial toxicity. As a result, we identified a patient who developed symptoms of mitochondrial toxicity after 10 years of using a lamivudine inhibitor (3TC). We found that the patient had heterozygous mutation C2857T→R953C in exon 18, as a result of which the amino acid R953 in Pol γ was mutated to cysteine. Considering that R953 is in the active site of Pol γ and is highly conserved even among distant species, mutations in this area can lead to the loss of mtDNA and are associated with mitochondrial diseases. We have shown that, compared to the control group, the patient with the R953C mutation had a significantly lower mtDNA copy number. Interestingly, the affinity of the R953C mutant for DNA is not significantly different from the affinity of the wild type and is consistent with the low nanomolar affinity previously described for Pol γ (Lim et al. 2003, Sohl et al. 2013). To understand how the R953C mutation contributes to HAART-induced toxicity, we investigated the incorporation of natural nucleotide (dCTP) and the active triphosphate form of the 3TC inhibitor. In a series of kinetic experiments, we have shown that Pol γ R953C mutant has a decreased ability to bind dCTP (8-fold) and reduced ability to distinguish the drug from the substrate (4 times). To explain reduced ability of Pol γ R953C mutant to discriminate between (-) - 3TC and dCTP, we first modelled the structure and later solved the crystal structure of Pol γ R953C

in complex with DNA and dCTP. Both the model and the crystal structure show that amino acid R953 is located in the O-helix which is a part of the enzymes active site and takes part in dNTPs binding during DNA synthesis. Since the interaction of R953 with residues E1056 and Y986, can stabilize O-helix and affect the polymerase activity, the R953C mutation in Pol γ can interfere with the interactions network, causing a small O-helix shift, thereby reducing nucleotide affinity and causing loss of the ability to distinguish the drug from substrate. Our findings provide structural explation for the relationship between Pol γ R953C mutation and HAART-induced toxicity. Based on our results and previously published data, we hypothesized that Pol γ mutations and / or polymorphisms may predispose patients to mitochondrial induced mitochondrial toxicity. We suggested that, given the rapidly decreasing costs genotype testing and the rapid development of pharmacogenetics, an individual approach to the patient is expected to maximize the therapeutic effect and minimize the side effects resulting from the administration of NRTIs.

A novel domain in human EXOG converts apoptotic endonuclease to DNA- repair exonuclease. **Szymanski, M.R**., Yu, A., Gmyrek, A.M., White, M.A., Molineux, I.J., Lee, J.C., Yin W.Y. ***Nature Communications.*** 2017 May 3;8:14959.
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It has only recently become clear that DNA Base Excision Repair pathway (BER) is the main DNA repair pathway in the mitochondria (Driggers, LeDoux and Wilson 1993, Larsen, Rasmussen and Rasmussen 2005, Mandavilli, Santos and Van Houten 2002). However, most of our knowledge about mitochondrial BER repair (mtBER) is based on observations from nuclear BER. Although mitochondrial BER is much less understood, it must be carried out in a different way than BER in the nucleus (Krokan and Bjørås 2013, Larsen et al., 2005). The reason for this difference is that in the mitochondria there is no specialized polymerase responsible for the repair of mitochondrial DNA, and the replicase, Pol γ, has two functions: it is necessary for mtDNA replication and repair (Longley et al. 1998a, Longley et al. 1998b). In addition, Pol γ has weak dRP lyase activity and is unlikely to cope with an increased number of Abasic sites (AP) without the help of other enzymes (Pinz and Bogenhagen 2000, Stierum, Dianov and Bohr 1999). In addition, Pol γ is ineffective in single nucleotide gap filling and has no strand displacement activity, i.e. all the functions necessary to carry out the canonical BER (He et al. 2013). Despite these limitations, human mitochondria cope well with DNA repair due to the presence of the 5′ exo / exonuclease, EXOG (Tann et al. 2011). EXOG is found exclusively in the mitochondria. Its absence causes accumulation of damage in mitochondrial DNA but not in nuclear DNA, increases oxidative stress and leads to mitochondrial dysfunction (Tann et al., 2011). The ectopic expression of the EXOG coding gene increases the resistance of proliferating myoblasts to oxidative stress (Szczesny et al. 2013). Importantly, EXOG is found in complex with other mitochondrial BER repair enzymes: APE1, Pol γ and Ligase III, called "mitochondrial repairosome". It was also shown that the interactions between proteins within the "repair complex" are enhanced by oxidative stress (Szczesny et al., 2014).

Based on the amino acid sequence, EXOG belongs to the ββα-Me nuclease family that contains a group of non-specific endonucleases, e.g. EndoG (Cymerman et al. 2008). The non-specific activity of EndoG is perfectly suited for the function of this enzyme in apoptosis that requires the digestion of single-stranded DNA (ssDNA), double-stranded DNA (dsDNA) and RNA, but is incompatible with the precise exonuclease activity required to modify the 5′ end of DNA in the BER pathway. EXOG is a paralog of EndoG that harbors both endonuclease and 5′-exonuclease activity. However, it is not well understood how these activities are regulated.

In addition, even though EXOG can function as a key 5′-exonuclease in the mtBER, the mechanism of its action in DNA repair is not well understood. Research in this field was hindered by the problems in obtaining suficient quantities of well-folded and active enzyme. EXOG is a membrane protein that localizes in the inner mitochondrial membrane via its N-terminal transmembrane domain (Cymerman et al., 2008). In order to isolate EXOG, we removed both the amino acids responsible for its location in the inner mitochondrial membrane as well as predicted disordered region at the N-terminus (ΔN58-EXOG). Based on previous reports, we developed an efficient method of purification of EXOG from inclusion bodies (Kieper et al. 2010), which allow us to obtain correctly folded and active enzyme.

To gain molecular insights to EXOG function in human mitochondrial BER, we solved the crystal structure of EXOG in the presence of Mg^{2+} ions to 1.81 Å. The apoenzyme is a symmetric homodimer built from a core catalytic domain that resembles the structure of apoptotic endonuclease, EndoG, and the C-terminal Wing domain built of three helix bundle. The active site located in a positively charged cleft between core and Wing domains contains the catalytic amino acid H140 and the Mg^{2+} ion coordinated in perfect octahedral geometry via five water molecules and δ-N of N171. To confirm the presence of metal in the active site, we solved the second structure of EXOG in the presence of the Mn^{2+} ion (2.6 Å). Then, by examining enzymatic activity of EXOG, we demonstrated that EXOG cuts dinucleotides from the 5′ end (Cymerman et al. 2008, Tann et al. 2011). This activity distinguishes EXOG from typical exonucleases that hydrolyze the phosphodiester bond between the first and second nucleotide (producing mononucleotides). Using kinetic methods, we have shown that only one monomer of EXOG dimer is active at a given time (half-site activity) and that dissociation of the reaction product is a very slow process. To determine if both monomers of EXOG dimer are capable of DNA binding we generated an inactive nuclease mutant (EXOG-H140A) and using isothermal titration calorimetry (ITC) we have shown that EXOG dimer can bind two DNA molecules with different affinities. This suggests that at certain DNA and EXOG concentrations only one monomer of EXOG can bind DNA. This explains the half-site activity of EXOG observed in kinetic experiments.

To obtain structural insight into enzymatic properties of EXOG, an inactive nuclease enzyme (EXOG-H140A) was used for co-crystallization with double stranded DNA (10 bp) substrate. The structure of the EXOG-dsDNA complex in the presence of Mg^{2+} ion was solved at 1.85 Å by the molecular replacement using EXOG apoenzyme as a model. To verify the presence and coordination of the metal ion in the active site, we solved another structure of EXOG-dsDNA-Mn^{2+} complex (2.6 Å). The metal ion, coordinated by five molecules of water and δ-N N171 in the EXOG-Mg^{2+} structure, in the EXOG-dsDNA-Mg^{2+} complex is still in octahedral coordination, but with three water molecules, δ-N N171, bridging oxygen between the 2nd and 3rd nucleotide and the non-bridging oxygen Op of the +2nt. The structure of the EXOG-dsDNA-Mg^{2+} complex shows that binding to DNA does not change the structure of the core of the enzyme, however, the two wing domains assume a different conformation. Consequently, after DNA binding, the two active sites have a different conformation: one is "closed" and the other "open". In addition, the open wing domain in DNA complex is less ordered as compared to the apo enzyme structure. This may be critical for the interactions of EXOG with other proteins involved in mtBER. The analysis of the EXOG-dsDNA structure show that DNA binding using the wing domain ensures precise positioning of the DNA substrate for the incision between 2nd and 3rd nucleotide. This mechanism allows EXOG to cut DNA every two nucleotides. In addition, the analysis of the EXOG structure in the complex with DNA shows that amino acids of the wing domain interact with the 5′ phosphate, stabilizing the interactions with the DNA, leading to slow product release. We verified these observations using point mutations and the wing domain deletion mutant (EXOG-ΔC68). We have shown that the EXOG-ΔC68 eliminates the residues involved in 5′ phosphate binding of both the substrate and the product. Furthermore, the active site of deletion mutant is completely open, and the

enzyme loses the ability to control two-nucleotide DNA hydrolysis as well as specificity for BER DNA substrates. Interestingly, the endonuclease activity in EXOG-ΔC68 increases by eight-fold compared to the native enzyme and is similar to that of EndoG. Our results suggest that the emergence of the wing domain in EXOG has transformed nonspecific nuclease into exonuclease specific for the mtBER.

In the canonical BER process, the action of repair enzymes can be stopped by the formation of covalent bonds with the oxidized ribose at the 5′ end of the damaged DNA. Since the active site of EXOG is distant from the 5′ end and the DNA substrate is cut between 2 and 3 nucleotides, we suggested that EXOG could process any damaged at 5′ end, regardless of the chemical nature, bypassing the potential blockade of the DNA repair pathway. Based on our results, we proposed a new model of BER that allows the repair of mitochondrial DNA via controlled "cut-and-fill" mechanism generating the optimal substrate for Pol γ without the need for strand displacement activity.

5. Other scientific and research achievements:

a) Scientific work at the Biology and Biochemistry Department, University of Houston, Texas, USA before obtaining PhD:

Area of research: Structural and functional characterization of membrane proteins.

Primary project focused on the purification and crystallization of HMW1B (High-Molecular Weight adhesin), a membrane protein from the bacterium *Haemophilus influenzae*, a common respiratory pathogen and important cause of morbidity in humans. We aimed to solve the structure of HMW1B by X-ray crystallography to yield insights into how this bacterium interacts with its host which could lead to new drug targets. I obtained crystals that diffracted to 2.8Å at the Argonne National Laboratory which directed the project to labeled proteins purification and further exploration of the HMW1B structure. My work led to the publication in JBC that describes how HMW1 adhesin binds to the HMW1B channel, provides insights into how the bacterium interacts with its host.

Duret, G., **Szymanski, M.,** Choi, K.J., Yeo, H.J., and Delcour, A.H. (2008). The TpsB translocator HMW1B of haemophilus influenzae forms a large conductance channel. ***Journal of Biological Chemistry***. *283*, 15771-15778.

b) Scientific work during my PhD studies at the Biochemistry and Molecular Biology Department, University of Texas Medical Branch w Galveston, Texas, USA:

Area of research: Helicase-initiated Assembly of Macromolecular Machines Involved in DNA Replication and Repair.

All cellular organisms rely on multi-component molecular machines to perform various functions essential for life. Each process requires elaborate temporal and spatial orchestration of assembly and disassembly of numerous proteins, multiple cofactor molecules, and nucleic acids. Helicases have been found as integral component of the multi-protein molecular machines and play a key role in the assembly processes. Comprehensive understanding of these complex processes is pivotal for understanding the fundamental biological reactions and, in turn, the mechanisms of disease pathogenesis in humans. Consequently, this knowledge is critical to preventing, diagnosing, and treating diseases that affect millions of people. To this end, we established the quantitative framework to study the helicase initiated assembly of macromolecular machines involved in DNA replication and

repair. Specifically, we examined the molecular basis for interactions between proteins involved in the assembly of the pre-primosome, the bacterial replication restart machinery from *E. coli*. We used quantitative thermodynamic approaches to examine interactions of the PriA helicase with ssDNA, dsDNA and DNA gap structures. In addition, we defined the location of the ATP- and the DNA-binding sites on the N-terminal domain of PriA and uncovered the allosteric interactions between these sites. Subsequently, we used biochemical and biophysical methods to determine the binding constant and stoichiometry of PriB–DNA complex. In addition, we tackled the energetics of the interactions between the PriA helicase and PriB protein. These studies included an application of the fluorescence anisotropy titrations, analytical ultracentrifugation methods, fluorescence energy transfer (FRET), isothermal titration calorimetry (ITC), UV-crosslinking and helicase activity assays. Consequently, we published over 10 peer-reviewed publications and 19 abstracts that shed the light on the molecular mechanism of assembly of these crucial macromolecular complexes.

Szymanski, M.R., Jezewska, M.J., Bujalowski, W. (2013). The *Escherichia Coli* Primosomal DnaT Protein Exists in Solution as a Monomer – Trimer Equilibrium System. ***Biochemistry.*** 52, 1845-57.

Szymanski, M.R., Jezewska, M.J., Bujalowski, W. (2013). Energetics of the *Escherichia Coli* DnaT Protein Trimerization Reaction. ***Biochemistry.*** 52, 1858-73.

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Roychowdhury, A., **Szymanski, M.R.,** Jezewska, M.J., and Bujalowski, W. (2009). Mechanism of NTP hydrolysis by the Escherichia coli primary replicative helicase DnaB protein. 2. Nucleotide and nucleic acid specificities. ***Biochemistry****. 48*, 6730-6746.

Roychowdhury, A., **Szymanski, M.R.,** Jezewska, M.J., and Bujalowski, W. (2009). Escherichia coli DnaB helicase-DnaC protein complex: allosteric effects of the nucleotides on the nucleic acid binding and the kinetic mechanism of NTP hydrolysis. 3. ***Biochemistry***. *48*, 6747-6763.

In addition to the main PhD project (described above), I initiated and implemented several projects in cooperation with other research groups from around the world. These projects are described below:

Area of research: Molecular Basis of Virus-Host Protein Interactions. During the viral infection, the antiviral protein network of the host and protein network of the infecting virus clash and interplay to generate intricate characteristics leading to viral replication, pathogenesis and eventually oncogenesis. For most viruses, however, little is known about the interactions between viral and cellular proteins. Understanding the molecular mechanisms of virus-host protein interactions will allow for examination of strategies the viruses employ to manipulate host cells and reveal novel targets for therapeutic intervention.

i) The Dengue Virus is a member of the *Flaviviridae* family and the etiological agent of dengue fever, a disease affecting 100 million people each year. The small, 10.7-kbp positive strand RNA genome of the dengue virus encodes only 10 proteins: capsid, premembrane, envelope, NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5 translated as a single polyprotein, subsequently cleaved by viral and cellular proteases into functional entities. One of these proteins, NS5 is a viral replicative RNA-dependent RNA polymerase that can catalyze *de novo* synthesis of RNA (*i.e.* synthesis of the complementary RNA strand). The interactions with the ssRNA are sufficient to initiate and continue the nucleic acid synthesis and play a vital role in the function of the enzyme as one of the major elements that determine the degree of fidelity of the RNA synthesis. Despite its paramount importance for understanding the RNA recognition process by the full-length dengue RNA polymerase, the direct and quantitative analyses of the enzyme interactions with the nucleic acid have not been addressed. Our studies, published in JBC,, provide the first quantitative analyses of the full-length Dengue virus polymerase interactions with the nucleic acid and provide the molecular basis for development of novel targets for therapeutic intervention against the Dengue Virus.

Szymanski, M.R., Jezewska, M.J., Bujalowski, P.J., Bussetta, C., Ye, M., Choi K.H., Bujalowski, W. (2011). Full-Length Dengue Virus RNA Dependent RNA Polymerase – RNA/ DNA Complexes. Stoichiometries and Energetics of Intrinsic Affinities, Cooperativities, Base and Conformational Specificities. ***Journal of Biological Chemistry****.* 286, 33095-33108.

ii) **Classical Swine Fever Virus (CSFV)** or hog cholera causes serious losses in the pig industry because it is highly pathogenic and may cause widespread deaths. We have shown that the Pestiviruses, such as Classical Swine Fever Virus (CSFV), use the viral protein, called N^{pro}, to subvert host cell antiviral responses. We determined that N^{pro} contains a novel metal-binding TRASH motif consisting of Cys-X_{21}-Cys-X_3-Cys (where X is any amino acid) at its C-terminus. We also found that N^{pro} coordinates a single zinc atom as determined by graphite furnace–atomic absorption spectrophotometry and inductively coupled plasma–mass spectrometry. Mutational and biochemical analyses show that the cysteine

residues in the TRASH motif are required for zinc binding and protein stability. Individual substitutions of the cysteines in the TRASH motif of CSFV N^{pro} abolished the interaction of N^{pro} with IRF3 and resulted in the loss of virus-mediated IRF3 degradation in CSFV-infected cells. Thus we show a direct functional relationship between the ability of N^{pro} protein to coordinate a zinc atom and its ability to interfere with the pathway leading to antiviral responses such as destruction of viral RNA, inhibition of cellular transcription, translation, and ultimately cell death. The publication describing the results of our research is the result of international cooperation and was published in the *Journal of Molecular Biology*:

Szymanski, M.R., Fiebach, A.R., Tratschin, J.D., Gut, M., Ramanujam, V.M., Gottipati, K., Patel, P., Ye, M., Ruggli, N., and Choi, K.H. (2009). Zinc binding in pestivirus N(pro) is required for interferon regulatory factor 3 interaction and degradation. ***Journal of Molecular Biology***. *391*, 438-449.

i) Swine Fever Virus (ASFV) is the etiological agent responsible for acute highly lethal, hemorrhagic fever in domestic swine; however, the exact way in which the virus infects the host cell is unclear. African Swine Fever Virus encodes specialized replication protein, called polymerase X, that allows the virus to repair damaged DNA, caused by the host immune system during the viral infection. Elucidation of the mechanism in which the ASFV pol X interacts with the nucleic acid is a prerequisite for understanding the recognition processes of the nucleic acid. This is of particular importance in the case of the DNA repair polymerases, which must recognize specific structure of the damaged DNA. These studies have been published in two back-to-back publications in *Biophysical Chemistry* in 2011 and explain how the efficient repair of the viral DNA allows the virus to subvert host cell antiviral response.

Jezewska, M.J., **Szymanski, M.R.,** and Bujalowski, W. (2011). Interactions of the DNA polymerase X from African Swine Fever Virus with the ssDNA. Properties of the total DNA-binding site and the strong DNA-binding subsite. ***Biophysical Chemistry.*** *158*, 26-37.

Jezewska, M.J., **Szymanski, M.R.,** and Bujalowski, W. (2011). Kinetic mechanism of the ssDNA recognition by the polymerase X from African Swine Fever Virus. Dynamics and energetics of intermediate formations. ***Biophysical Chemistry***. *158*, 9-20.

Research area: Structural and functional characteristics of proteins involved in DNA replication and repair.

i) Human replication proteins action and their involvement in cancer. The faithful propagation of genetic material is essential to life. Each time a cell divides a complete copy of the genetic information stored in its chromosomes must be accurately passed to each daughter cell. Hence, DNA replication must be tightly regulated to ensure the highest precision and accuracy. However, the replication process is constantly compromised due to environmental factors and cellular metabolism which induce DNA damage. In humans, DNA polymerase beta maintains genome integrity, performs base excision repair (BER) and is required for accurate DNA replication, recombination, and drug resistance. This essential enzyme has been correlated with a number of cancer types including colorectal cancer, gastric cancer and prostate cancer; therefore, it is essential to understand its mechanism of interaction with DNA. In the paper published in *Biophysical Chemistry,* in 2011, we have examined the interactions of DNA polymerase beta with DNA.

Jezewska, M.J., **Szymanski, M.R.,** and Bujalowski, W. (2011). The primary DNA binding subsite of the rat pol beta. Energetics of interactions of the 8-kDa domain of the enzyme with the ssDNA. ***Biophysical Chemistry***. *156*, 115-127.

ii) Kinetic mechanism of the ssDNA entry Into the cross-channel of the RepA helicase. RepA is a hexameric DNA helicase essential for replication of RSF1010, a broad-host nonconjugative plasmid that confers bacterial resistance to sulfonamides and streptomycin. Using the fluorescence intensity and anisotropy stopped-flow, as well as analytical ultracentrifugation methods, we examined the kinetic mechanism of recognition and the cross-channel entry of ssDNA into the hexameric RepA. We have shown that association of RepA with the ssDNA is a minimum four-step mechanism and that in the recognition step, the ssDNA initially binds to the binding site located outside of the helicase cross-channel. The entry of the nucleic acid to the crosschannel is accomplished due to large conformational transitions of the enzyme. Subsequently the hexameric structure closes over the bound DNA, although only partially. These findings show that the entry of the DNA into the cross-channel is a multistep process with different reactions occurring in very different time ranges. The results of these studies were presented in the these publications:

Andreeva, I.E., **Szymanski, M.R.,** Jezewska, M.J., Galletto, R., and Bujalowski, W. (2009). Dynamics of the ssDNA recognition by the RepA hexameric helicase of plasmid RSF1010: analyses using fluorescence stopped-flow intensity and anisotropy methods. ***Journal of Molecular Biology***. *388*, 751-775.

Andreeva, I.E., Roychowdhury, A., **Szymanski, M.R.,** Jezewska, M.J., and Bujalowski, W. (2009). Mechanisms of interactions of the nucleotide cofactor with the RepA protein of plasmid RSF1010. Binding dynamics studied using the fluorescence stopped-flow method. ***Biochemistry***. *48*, 10620-10636.

c) Current work and future plans:

In 2017 I receivieved two prestigeus research grants that enabled me to open an independent research group in Poland (POLONEZ grant from National Science Centre, Poland and FIRST TEAM grant from Fundation for Polish Science). In October of 2017 I started working as a Structural Biology Group Leader at the Intercollegiate Faculty of Biotechnology of the University of Gdansk and Medical University of Gdansk. In addition, in December 2018, as one of three Polish scientists, I was awarded the prestigious EMBO Installation Grant from the European Organization of Molecular Biology to support my research activities. Currently, I lead a group of four researchers: two Postdocs and two PhD students. Research in my group focuses on understanding the mechanisms of the formation and structure-function relatoinsihps of macromolecular complexes involved in key cellular processes. The main research methods used by my group are X-ray crystallography and electron microscopy as well as biochemical and biophysical aproaches to study the interactions of proteins, nucleic acids and their complexes. In the First Team project titled "Targeting mitochondrial DNA repair for anti-cancer therapies" (2018-2021), we focus on understanding the structure and function of mitochondrial membrane nuclease, EXOG. Research in this project may contribute to the development of specific inhibitors against EXOG. Inhibition of EXOG in cancer cells may increase the number of mutations in mitochondrial DNA, thereby increasing their sensitivity to traditional chemotherapy. The project is carried out in colaboration with Prof. Bartosz Szczęsny from the University of Texas Medical Branch in Galveston, Texas, United States.

In the NCN project entitled "Unraveling the molecular basis of DNA damage recognition and processing in human mitochondria" we aim to study the molecular mechanism of recognition and cleavage of damaged DNA by EXOG. The results obtained during the implementation of this project were used in the ERC Starting Grant 2019 application, submitted in October 2018.

In addition, I continue to colaborate with a group of prof. Andrew Fire from Stanford University, California, United States, to understand the sequence and structural features of RNA templates replicated by transcription polymerses. I also continue colaboration with a group of prof. Marc Morais from the University of Texas Medical Branch in Galveston, Texas, United States, on the mechanism of action of molecular motors responsible for placing a negatively charged nucleic acid in the closed space of the viral capsid. The mechanism of action of these ATPases is to convert the chemical energy derived from ATP hydrolysis into the targeted mechanical motion required for the translocation of the genome into the interior of the virion. In order to obtain a pseudoatomic model of the motor complex and to understand the mechanism of action of these molecular motors, we used a combination of analitical ultracentrifugation, small-angle X-ray scattering (SAXS), cryo-electron microscopy (cryo -EM) and X-ray crystallography as well as enzymatic biochemistry. We are also continuing cooperation with the Prof. Wlodek Bujalowski, University of Texas Medical Branch in Galveston, Texas, USA, to determine the structure of the E. coli primosome by X-ray crystallography and cryo-electron microscopy.

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