DEPARTMENT OF MOLECULAR AND BIOMEDICAL SCIENCES

The research program of the Department of Molecular and Biomedical Sciences is focused mainly on basic research in protein biochemistry, molecular and cellular biology, and genetics. The primary goal of our investigations is the acquisition of new understanding of mammalian pathophysiology with the aim of improving human and animal health.

Toxinology

One of our traditional research topics in the field of toxinology is the study of molecular mechanisms of toxic action of secreted phospholipases A_2 ($sPLA_2$s) from animal venoms. In particular, we are focused on those endowed with presynaptic neurotoxicity (β-neurotoxins). The knowledge that we are gaining by studying toxic $sPLA_2$s is helping us to discover the pathophysiological roles of orthologous mammalian $sPLA_2$s, for example, their role in the development of neurodegenerative diseases such as Alzheimer's disease (AD).

In this year we continued with the characterization of the interaction of ammodytoxin (Atx), a neurotoxic snake venom $sPLA_2$, with its mitochondrial receptor, cytochrome c oxidase (CCOX), to deepen our understanding of the motoneuron poisoning by Atx and to shed light on the pathophysiological role of a mammalian group IIA $sPLA_2$ (GIIA), an orthologue of Atx, in this organelle. With this aim, we monitored the intracellular trafficking of derivatives of recombinant Atx, rat GIIA and their enzymatically inactive (D49S) mutants in PC12 cells by transmission electron and fluorescence confocal microscopy. From the obtained results, we concluded that $sPLA_2$ molecules do not require enzymatic activity to enter and traffic within the cell, including entering the mitochondria. A draft article has been prepared to report on this observation. We also continued to investigate the effect of both $sPLA_2$s, Atx and rat GIIA, on mitochondria in PC12 cells. By flow cytometry, we found that GIIA(D49S) slightly reduced mitochondrial potential, whereas GIIA had no significant effect on it. Using ^{125}I-GIIA, we determined the binding affinity of GIIA to a mitochondrial protein with an apparent molecular mass of 20 kDa (R20). Given that GIIA, like Atx, inhibits CCOX activity, we hypothesize that R20 is a subunit of CCOX IV (CCOX-IV). As we were not able to confirm this with the help of anti-CCOX-IV antibodies, we approached to identify R20 following its isolation from porcine mitochondria. We have synthesized a GIIA-affinity gel, but the conditions for reversible receptor binding to it have not yet been found. A precise description of the action of GIIA on CCOX is crucial for the use of our findings in medicine, both for the early diagnosis of Alzheimer's disease (AD) as well as for the subsequent treatment of this severe neurodegenerative disease. Namely, it has been shown that GIIA in AD is overexpressed and becomes toxic to mitochondria, which is similar to the effects observed in Atx poisoned nerve endings.

It has been demonstrated that certain $sPLA_2$s specifically bind to nicotinic acetylcholine receptors (nAChRs). Therefore, we have become involved in the research of a specific lung cancer where cancer cells overexpress α7 nAChR, and where natural selective α7 nAChR antagonists are being intensively studied for the development of new target drugs. By recombinant DNA technology, we have prepared an array of $sPLA_2$s, such as human GV and GX, Atx and rat GIIA, and their enzymatically inactive mutants, GV(H48Q), GX(H48Q), Atx(D49S) and GIIA(D49S). In collaboration with pharmacologists from the University of Leuven, Belgium, we determined the effect of these proteins on α7 and muscle-type nAChRs. Measurements are still ongoing, but the most interesting result was that GV(H48Q) selectively binds only to the α7 form of nAChR but not to the muscle form. In parallel, we also developed a research model of lung cancer, an A549 lung adenocarcinoma cell line with the overexpression of α7 nAChR, to test the effects of GV(H48Q) and other α7 nAChR antagonists on cell viability, cytotoxicity, proliferation, and apoptosis.

We have prepared two review articles on the topic of $sPLA_2$s. The first, which presents the antiviral activity of both endogenous mammalian and exogenous $sPLA_2$s, i.e. those found in animal toxins, has already been published (J. Pungerčar et al., *Biochimie*, 189 (2021), 40–50). The second one, on the action of $sPLA_2$s as a consequence of their protein receptor binding, has been accepted for publication (A. Ivanušec et al., *Int. J. Biol. Sci.*, in press). A monograph was also published by the renowned publishing house CRC Press where I. Križaj participated in the preparation of a chapter on $sPLA_2$ toxins from snake venoms (B. Lomonte and I. Križaj, 2021).

In 2021 we continued with an extensive study of snake venom proteins that affect the process of blood coagulation – haemostasis. As part of research project J1-2475, we are investigating a unique anticoagulant homologue of serine protease from the venom of the nose-horned viper (*Vipera ammodytes ammodytes*, *Vaa*), VaaSPH-1, in the direction of developing completely new and safe drugs with anticoagulant action. We searched for the best possible conditions for the expression of VaaSPH-1 in HEK293 suspension cells. In parallel, in collaboration with a US partner from the University of North Carolina (NCATSU), USA, we optimized a three-dimensional *in silico* model of the complex between activated coagulation factor VIII (FVIIIa) and VaaSPH-1, to design low-molecular-mass FIX antagonists for their testing *in vitro*. We have also prepared an invited review article on the topic of serine pseudoproteases (N. Zupanič et al., *FEBS J.*, in press).

After the publication of a detailed description of the VaaSP-VX serine protease, which promotes blood clotting by activating both FV and FX, we began to isolate and characterize a very similar molecule in *Vaa* venom, VaaSP-6. In contrast to VaaSP-VX, we know the entire cDNA sequence of VaaSP-6, which makes it possible to obtain protein in recombinant form. If the recombinant VaaSP-6 exhibits the same unique procoagulant activity as VaaSP-VX, it could replace dilute Russell's viper venom (dRVV) that is currently used for the clinical determination of lupus anticoagulants (LA test) but is quite unreliable.

We concluded with the extensive genetic, biochemical and physiological characterization of the VaaMPIII-3 protein from the long-nosed viper venom (Figure 1). By analysing its gene structure, we have unequivocally proved that the protein belongs to a special subclass of metalloproteinases from snake venoms, subclass P-IIIe, whose introduction we proposed. We have written the article that is ready to be sent for publication (K. Požek et al., in preparation).

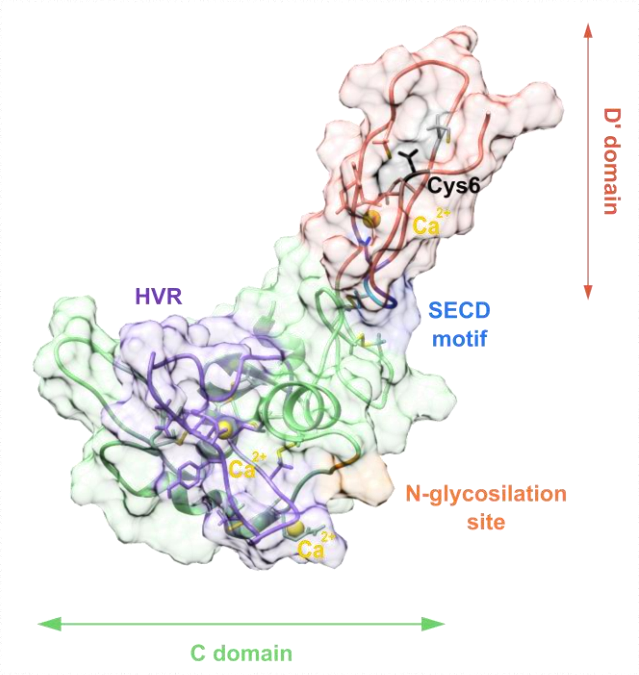

Figure 1: ***Three-dimensional homologous model of VaaMPIII-3 protein****. The structural model of the VaaMPIII-3 protein was prepared by homologous modelling according to the crystal structure of the most similar AaHIV protein from the venom of the crotalid snake Agkistrodon acutus (PDB code: 3HDB). The truncated disintegrin-like part of the molecule (D') is dark orange and the Cys-rich part (C) is green. Cys6, integrin-binding motif (SECD), N-glycosylation site and hypervariable region (HVR) are shown in black, blue, orange and purple.* Ca^{2+} *ions are represented by yellow beads and disulphide bonds by yellow rods.*

Together with colleagues from the Centre for Clinical Toxicology and Pharmacology, University Medical Centre Ljubljana (UMCL), we investigated an interesting clinical effect in patients envenomed by the nose-horned viper, namely deep, transient and reversible thrombocytopenia of functional thrombocytopenia, as part of research project J3-2534. In thromboembolic diseases, such as myocardial infarction and ischemic stroke, platelets play a central role. Existing antiplatelet drugs have one common side effect – a reduced number of platelets whose activity is inhibited. This condition poses a high risk of life-threatening bleeding (haemorrhage), especially in interventional cardiology and angiology that use an antithrombotic approach. Our findings may pave the way for the development of a new group of antiplatelet agents that would reduce the risk of dangerous bleeding in such an approach in interventional cardiology and angiology, and increase the effectiveness of vasodilation and clot removal. As shown, reversible thrombocytopenia in patients envenomed by the *Vaa* can be caused by snake proteins similar to type C lectins (PLTCs, named also snaclecs). Several PLTCs were isolated from *Vaa* venom, and two of them, PLTC3/2 and PLTC1/7, in homogeneous form and in sufficient quantity for detailed characterization. Among other things, we measured their effect on blood clotting and characterized their binding to platelet receptors. High-affinity PLTC3/2 was found to bind to the GPIb receptor (CD42b). The better-labelled PLTC3/2 is ready for an *in vivo* study in mice that will be carried out in cooperation with partners from the Faculty of Veterinary Medicine, University of Ljubljana (VF/UL).

Within the research network, with the experts from UMCL, University Hospital and University of Split, University of Zagreb (UZ) and from our group, we analysed samples of patients who were envenomed by the *Vaa* and treated with different antidotes. In the publication, we propose new guidelines for effective immunotherapy in the treatment of *Vaa* envenomation (T. Kurtović et al., *Toxins*, 13 (2021), 279).

Lipid metabolism and signalling

Our research in the field of lipid metabolism and signalling is focused on the role of lipid droplets in cellular stress. These organelles are essential for fat storage and energy production in various tissues, but emerging evidence has shown that lipid droplets are also crucial for the cellular response to imbalances in energy and redox status. We are particularly interested in the connections between fat storage, inflammatory signalling, autophagy and ferroptosis. This work is of general interest for the wide field of molecular and cell biology, but is particularly applicable to the expanding range of pathophysiological conditions associated with dysregulated lipid metabolism, including cancer, metabolic diseases and neurodegeneration.

In 2021, our experimental work in the scope of the postdoctoral research project Z3-2650 was focused on the study of the roles of lipid droplets in the production of inflammatory lipid mediators (E. Jarc Jovičić et al., *bioRxiv*, doi.org/10.1101/2021.11.25.470010). The results show that lipid droplets not only store polyunsaturated fatty acids (PUFAs) in the form of neutral lipids, but also control their entry into lipid mediator production pathways, thereby implicating lipid droplets in inflammation, immunity and tumourigenesis. We demonstrate that adipose triglyceride lipase (ATGL) promotes the incorporation of lipid droplet-derived PUFAs into phospholipids, which are then targeted by cytosolic PLA_2. This work is important because it identifies a central role of fat storage for the supply of PUFAs into oxygenation pathways. Namely, the control of the availability of PUFAs has been traditionally attributed to PLA_2-dependent membrane phospholipid hydrolysis. Our data suggests that targeting lipid droplet turnover – instead of PLA_2s, which have been proven as unsuitable targets for pharmacological intervention in several clinical trial – could be a valid strategy for reducing inflammation and inflammation-related tumorigenesis.

We continued our work on the research project J7-1818, focused on targeting lipid droplets to reduce cancer cell resistance to stress. We tackled the question of the cooperation between autophagy and lipid droplets in cancer. Promising preliminary results on ferroptosis (Figure 2) supported the acquisition of another postdoctoral research grant Z3-3211 devoted to the study of the interplay between lipolysis and lipophagy in the modulation of ferroptosis in cancer.

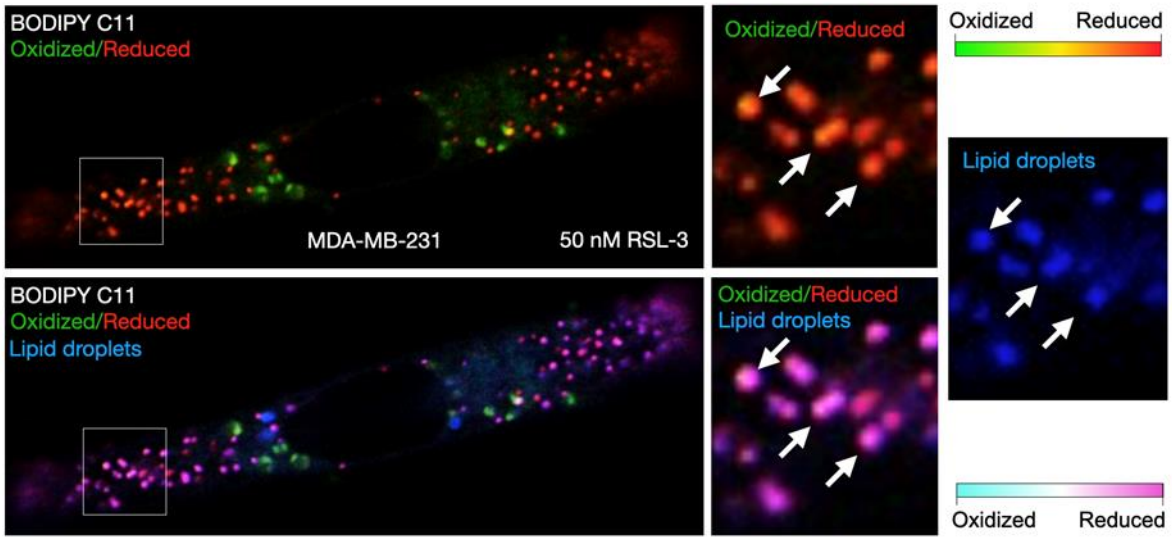

Figure 2: ***Lipid droplets are antioxidant organelles that protect lipids from oxidation****. Lipid droplets in MDA-MB-231 breast cancer cells with compromised redox defences (via glutathione peroxidase 4 (GPX4) inhibition by RSL-3) were stained with the LipiBlue dye (blue) (Dojindo Molecular Technologies) and imaged by live-cell confocal microscopy. The level of lipid peroxidation was estimated by staining cells with the oxidation-sensitive lipophilic probe BODIPY C11 581/591 (Thermo Fischer Scientific). Lipid droplets predominantly contain the reduced form of the dye (red, magenta), suggesting that they provide an antioxidant environment that protects the dye from oxidation (A. Kump et al., in preparation).*

We have contributed to the study of accumulation of lipid droplets in astrocytes under stress, led by the colleagues from the Medical Faculty, University of Ljubljana (MF/UL). We investigated the dynamics of astrocytic lipid droplet accumulation during nutrient and oxidative stress or noradrenaline exposure, and suggested a neuroprotective role of lipid droplets that accumulated during stress conditions (T. Smolič et al., *Glia*, 69 (2021), 1540–1562).

Scientific community accepted our work on lipid droplets very well as evident from the rapid increase of the number of citations and lecture invitations, including the presentation at the joint workshop of the EpiLipidNET COST Action and the German Research Council Priority Program on Ferroptosis. We received several awards for our works. The most prestigious, the *FEBS Letters Best Poster Award*, was obtained by our PhD student Špela Koren at the 45th FEBS Congress.

High-throughput genetics and functional genomics in yeast *Saccharomyces cerevisiae*

Most traits, including those important for microbial biotechnologically, are polygenic. Therefore, for the past several years our research has been focused on the polygenic traits in the yeast *S. cerevisiae*. In this model organism, we have been studying fundamental aspects of genetics that are also important from a biomedical point of view, as well as developing new, biotechnologically interesting yeast strains.

In the field of the biotechnological application of yeast, we completed and published a study in which we identified and characterized three new causal genes for lipid storage content in common yeast (K. Pačnik et al., BMC Genomics, 22 (2021), 110). We also modified this trait of the yeast as a biotechnologically established organism, which in the future will be able to largely replace the use of other sources of lipids or similar molecules (e.g. oil for energy needs or palm oil for the food industry), in another study published this year (S. Arhar et. al., Microb. Cell Fact., 20 (2021), 147). Here, we made six changes to the yeast genome for the purpose of metabolic engineering. We showed that with only such a relatively small change in the genome a strain capable of accumulating lipids up to 65% of the dry weight could be obtained (Figure 3).

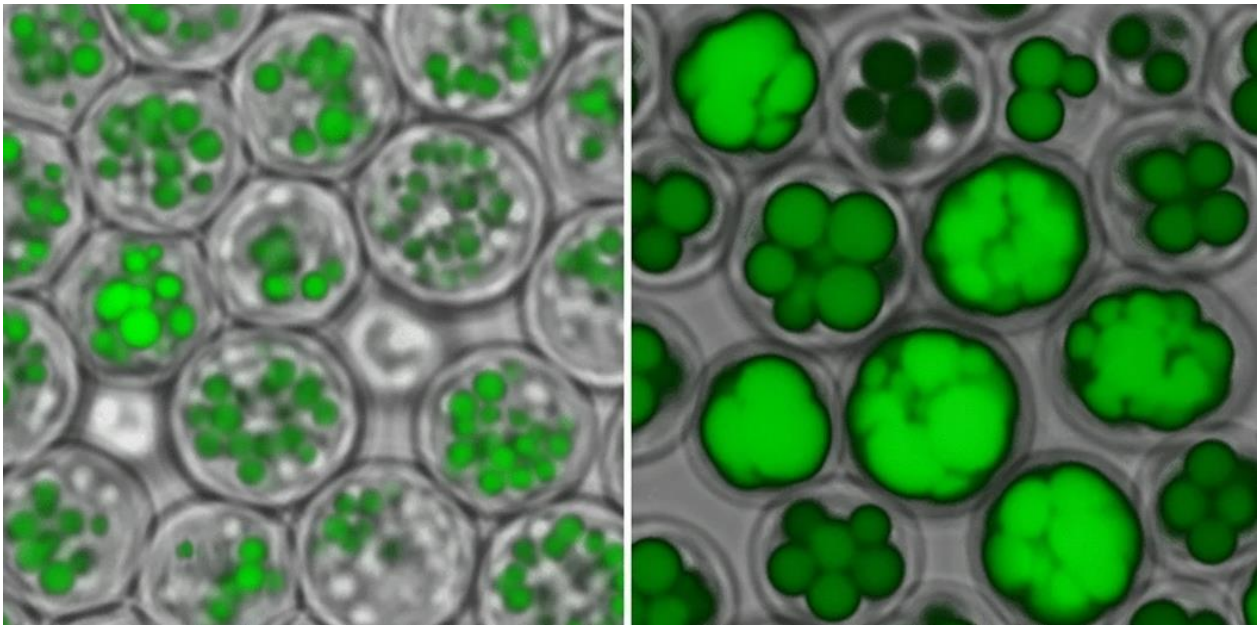

Figure 3: ***Yeast microscopy under storage lipid accumulation conditions****. Left: wild-type strain; right: genetically modified strain. Storage lipids in lipid droplets were stained with the BODIPY 493/503 dye.*

We continued to investigate the molecular mechanisms of the pathogenicity of the SARS-CoV-2 virus. In collaboration with colleagues from the Institute of Microbiology and Immunology (IMI) of the Medical Faculty in Ljubljana, we followed the emergence of virus variants. Specifically, we focused on the variants of the ORF8 protein encoded by viral genomes in Slovenia. Binary protein-protein interactions between variants of the ORF8 viral protein and human proteins were analysed by the two-hybrid yeast system method. We showed that ORF8 specifically interacts with the extracellular domains of three proteins important for the human immune response: TGFB1, ITGB1, and ADAM9. We found that a truncated version of the ORF8 protein encoded by the genomes of a strain of the virus that was prevalent in Slovenia and the world in the middle of 2021 (i.e. the alpha variant) loses the ability to form these interactions. In collaboration with colleagues from IMI and the Clinic for Infectious Diseases of UMCL, we proved that this change does not lead to the loss of pathogenicity of the virus. This has called into question previously published hypotheses that mutations in the ORF8 protein gene lead to attenuation of SARS-CoV-2 and some other β-coronaviruses.

Evolutionary genomics

The cave salamander or proteus (*Proteus anguinus*) is an animal with exceptional morphological and physiological adaptations to the subterranean environment, with regenerative ability, high resistance to prolonged starvation, and a lifespan that may exceed 100 years. To explain so very interesting features of this organism we initiated the analysis of its genome. The genome sequence, estimated to almost 50 Gb, is still unknown. The international Proteus Genome Research Consortium (http://proteusgenome.com) has been established to tackle the challenge of sequencing the proteus genome and its transcriptomes, and funds to initiate the work raised. In J1-2469 project, led by our colleagues at the Biotechnical faculty UL (BF/UL), we participate with the analysis of genomic and transcriptomic data. Until now, we have obtained initial genomic and transcriptomic data from multiple tissues. DNA sequencing is entering the next phase, the phase of a real-time single molecule sequencing (SMRT), and also sequencing of short DNA transcripts (Figure 4).

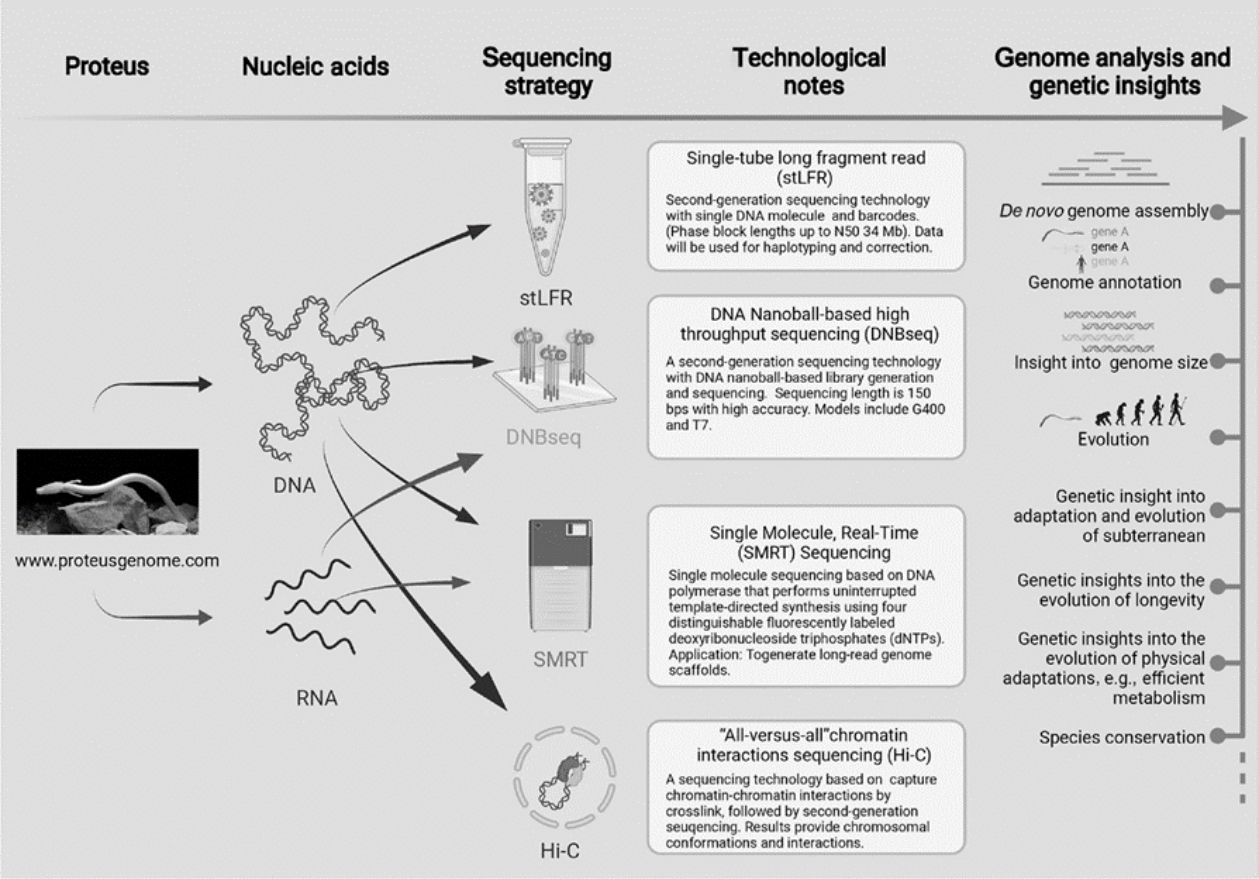

Figure 4: ***Schematic illustration of the proteus genome sequencing plan****. Both genomic DNA and RNA are isolated from Proteus anguinus tissue, and sequenced. The proteus genome structure will provide genetic insights into its evolution and adaptation to the subterranean environment, longevity, metabolism and regeneration, and will aid preservation of this unique animal. The figure is adapted from R. Kostanjšek* et al*. (Ann. NY Acad. Sci., (2021), in press).*

The genomes of salamanders are exceptionally large (more than 15-fold larger than the human genome) and possess high degrees of repetitive sequences, which makes both sequencing and assembly challenging. Repetitive DNA, made mostly of diverse transposable elements (TEs), is estimated to 90% of the proteus genome size (~40,5 Gb). Assembly of the genome sequence is

thus extremely difficult task. In 2021, we analysed TEs in the proteus transcriptome and assembled a large collection of TEs (species-specific repetitive library). Ultimate ambition of the proteus project is that we foster, by insight into the proteus genome, medical advancements in the area of aging, tissue regeneration, and therapy of metabolic disorders. The first paper on this subject has been accepted for publication (R. Kostanjšek et al., *Ann. NY Acad. Sci.*, in press).

Further, we analysed the origin, diversity and domain architecture of aerolysin superfamily of protein toxins in basal metazoans (sponges, ctenophores and cnidarians). These toxins that form pores in cell membranes represent one of the most fundamental defence systems of organisms. Proteins of the aerolysin superfamily contain a pore-forming aerolysin domain and a receptor-binding domain (RBD). In contrast to the highly conserved pore-forming domains, RBDs are highly variable, and their structural variations lead to differences in target recognition and, consequently, of the way of action. In numerous genomes and transcriptomes of basal metazoans we discovered unexpectedly large diversity and many novel domain architectures of aerolysin superfamily (D. Kordiš, manuscript in preparation).

Other subjects

In 2021, we also participated in different projects out of the thematic framework of our department.

We are partners in several research projects funded by the Slovenian Research Agency (ARRS). In the project J1-2482 (the leading institution is BF/UL), we determined the impact of environmentally relevant nano- and microplastics on terrestrial vertebrates by mass spectroscopy, and started preparing an article based on the obtained results. As part of the application project L4-1839 (leading institution: BF/UL), we developed a procedure for the isolation and identification of antimicrobial defensins in chestnut honey for medical use.

We also informally collaborated with several groups at home and abroad. Colleagues from the Ruđer Bošković Institute and UZ were assisted in researching the mechanism of formation and morphogenesis of biomineral nanostructures of the *Archa noae* shell. We performed structural identification of protein components of the shell that are potentially involved in the biomineralization process. The publication is in the process of being reviewed by the journal *Colloid Surf. A: Physicochem. Eng. Asp.* (I. Sondi et al., submitted). In a study led by colleagues from the Faculty of Electrical Engineering UL (FE/ UL), we analysed the protein crown composition of nanoparticles in a proteomic approach to explain their impact on the human immune system. The results were described in an article under review in the journal *Int. J. Biol. Sci.* (K. Strojan et al., submitted), and another article is in preparation. At the invitation of our colleagues from the same faculty, we also prepared and published a review article on electroporation of cell membranes (K. Balantič et al., *Acta Chim. Slov.*, 68 (2021), 753–764). We participated in a study of colleagues from VF/UL who were interested in the usefulness of insect proteins for food purposes. Using mass spectroscopy, we searched for potential human allergens in protein preparations. We have already managed to publish this analysis (B. Premrov Bajuk et al., *Animals*, 11 (2021), 1942). To a group from the MF/UL, we came to the aid of research into glioblastoma multiforme (GBM), the most common and deadly form of brain tumour. We collaborated with the confocal microscopic analysis of NB3F18 nanobody as a candidate for selective targeting of glioblastoma cells (Figure 5). A publication on this topic is in the final stages of preparation. We also participated in the preparation of two publications in the field of analysis of trans fatty acids

in nutrition (N. Zupanič et al*., Nutrients*, 13 (2021), 207 and A. Kušar et al*., Public Health Nutr.*, 24 (2021), 12–21).

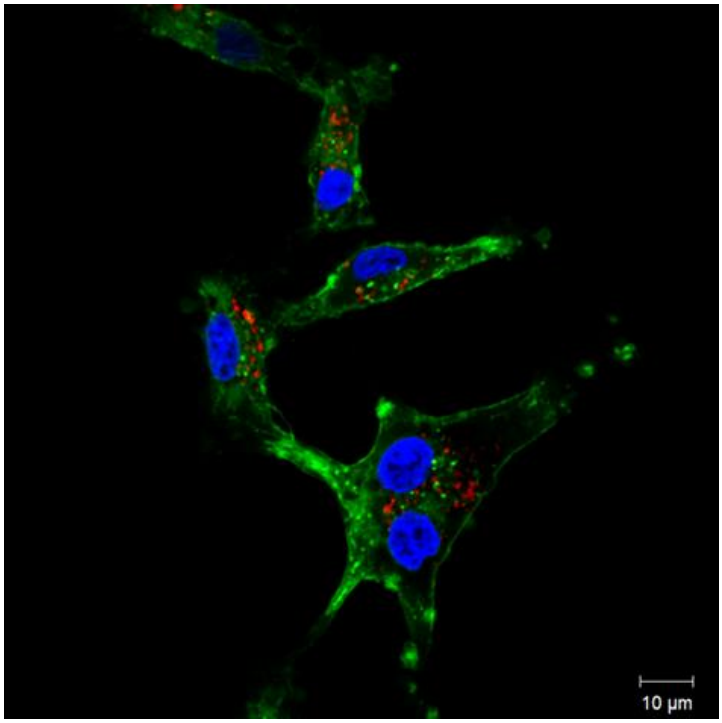

Figure 5: ***Confocal microscopy of glioblastoma cells****. The NB3F18 nanobody (red) recognizes the target antigen on U87MG glioblastoma cells. The membranes are coloured green (BioTracker Green) and nuclei blue (DAPI).*

Following the outbreak of the covid-19 pandemic in 2020, we began a two-year expanded programme activity aimed at researching various aspects of the functioning and evolution of SARS-CoV-2. We are completing the planned research and preparing publications.

Most important publications in the past year

1) Lomonte, B. and Križaj, I. (2021): Snake venom phospholipase A_2 toxins. In: Handbook of Venoms and Toxins of Reptiles, 2nd Edn. (Stephen P. Mackessy; Ed.). ISBN 9780367149741. CRC Press; Taylor & Francis Group, Boca Raton, Florida, USA; pp. 389–411
2) Kurtović, T., Karabuva, S., Grenc, D., Dobaja Borak, M, Križaj, I., Lukšić, B., Halassy, B. and Brvar, M.: Intravenous *Vipera berus* Fab fragments and intramuscular *Vipera ammodytes* $F(ab')_2$ fragments in *Vipera ammodytes* envenomed patients. Toxins, 13 (2021), 279
3) Pungerčar, J., Bihl, F., Lambeau, G. and Križaj, I.: What do secreted phospholipases A_2 have to offer in combat against different viruses up to SARS-CoV-2? Biochimie, 189 (2021), 40–50
4) Pačnik, K., Ogrizović, O., Diepold, M., Eisenberg, T., Žganjar, M., Žun, G., Kužnik, K., Gostinčar, C., Curk, T., Petrovič, U. and Natter, K.: Identification of novel genes involved in neutral lipid storage by quantitative trait loci analysis of *Saccharomyces cerevisiae*. BMC Genomics, (22) 2021, 110
5) Arhar, S., Gogg-Fassolter, G., Ogrizović, M., Pačnik, K., Schwaiger, K., Žganjar, M., Petrovič, U. and Natter, K.: Engineering of *Saccharomyces cerevisiae* for the accumulation of high amounts of triacylglycerol. Microb. Cell Fact., (20) 2021, 147