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, and to develop novel and more effective therapeutic treatments of envenomation.

The molecular mechanism of action of ammodytoxin A (AtxA), a potent β-neurotoxic $sPLA_2$ from the venom of the nose-horned viper (*Vipera a. ammodytes*; *Vaa*), has already been largely described. One of the questions, which still needs some clarification, is the role of the phospholipase activity in β-neurotoxicity. To this end, we prepared enzymatically inactive mutant of AtxA, AtxA(D49S). In collaboration with the research group led by Professor R. Frangež from the Veterinary Faculty, University of Ljubljana (VF/UL), we concluded in 2023 the comparative electrophysiological characterization of AtxA and its enzymatically inactive mutant. We demonstrated that the effects of AtxA independent of enzymatic activity cannot be studied with classical electrophysiological measurements on the isolated neuromuscular preparation. Our results also suggested that the inhibition of cytochrome c oxidase activity by AtxA is not involved in the rapid neuromuscular blockade by this β-neurotoxin, but that its pathological consequences are rather long-term (M.C. Žužek et al., *Toxicon*, submitted).

AtxA has been used as a tool to study the mechanism of regeneration of the nerve terminal after the trauma. In consortium, led by Professor C. Monteccucco from the Department of Biomedical Sciences, University of Padua, and the Institute of Neuroscience, National Research Council, Padua, Italy, we published a research paper on the action of an agonist of a G-protein-coupled chemokine receptor CXCR4. We demonstrated that a small molecule agonist of CXCR4, dubbed NUCC-390, induces a rapid regeneration of the motor axon terminal with functional recovery of the neuromuscular junction. Our results qualify NUCC-390 as a promising novel therapeutic agent capable of improving recovery from the paralysis caused by the snakebite of neurotoxic vipers (M. Stazi et al., *Journal of Neurochemistry* (2023), doi: 10.1111/jnc.15803).

It has been demonstrated that certain $sPLA_2$s specifically bind to nicotinic acetylcholine receptors (nAChRs). The binding of ACh or other agonists, such as nicotine and its derivatives, to nAChRs has been linked to uncontrolled cell division, prevention of apoptosis and induction of angiogenesis, ultimately supporting tumour growth and metastasis. However, antagonists of nAChRs showed opposite effects on the cells, indicating their potential value in cancer therapy. Among the naturally occurring nAChR antagonists, found in various venoms, snake venom $sPLA_2$s were also shown to suppress ACh-elicited ion currents. For this reason, we investigated the anti-cancer effect of an array of human $sPLA_2$s and their single-point enzymatically inactive mutants to assess their lung cancer therapeutic potential. In collaboration with pharmacologists from the University of Leuven, Belgium, we have been determining the effect of these proteins on α7- and muscle-type nAChRs. The most interesting result was obtained with GV(H48Q), which was absolutely selective for α7-nAChR. We used then GV(H48Q) to assess its effects on viability, cytotoxicity, proliferation and apoptosis of various lung cancer cell lines as well as one non-cancerous lung cell line. We demonstrated that GV and GV(H48Q) are able to prevent the ACh-induced cell proliferation and viability. In parallel, we were also involved in a similar study with

another group of α7-nAChR antagonists, 3-alkylpyridinium salts (APS). The paper describing the effects of APS7 and APS8, either free or packed in gelatine nanoparticles, on human lung cancer cells is under revision (A. Joukhan et al., *Marine Drugs*, submitted). The second paper on this subject has been submitted for publication (V. Kononenko, *Journal of Controlled Release*, submitted). We prepared also a review paper on the role of nAChR in cancer – it has been accepted for publication (T. Bele et al., *BBA – Molecular Basis of Disease*, in press).

In 2023, we continued the study of snake venom proteins that affect the process of blood coagulation – haemostasis. In the scope of the research project J1-2475, funded by the Slovenian Research and Innovation Agency (ARIS), we have been investigating a unique anticoagulant homologue of a serine protease from the venom of the nose-horned viper (*Vipera a. ammodytes*, *Vaa*), VaaSPH-1, in direction of developing completely new and safe drugs with anticoagulant activity. We searched for the best possible conditions for the expression of VaaSPH-1 as well as its binding protein, blood coagulation factor VIIIa (FVIIIa), in mammalian HEK293-F cells. In parallel, we have been designing low-molecular-mass FIX antagonists. We have a promising peptide candidate to test its action *in vitro*. On this topic, we published an invited review article on serine pseudoproteases (N. Zupanič et al., *FEBS Journal* 290 (2023), 2263–2278), in which we highlighted and discussed a previously neglected possibility of the non-enzymatic functions of these SP molecules. Our paper was featured on the cover page (Figure 1).

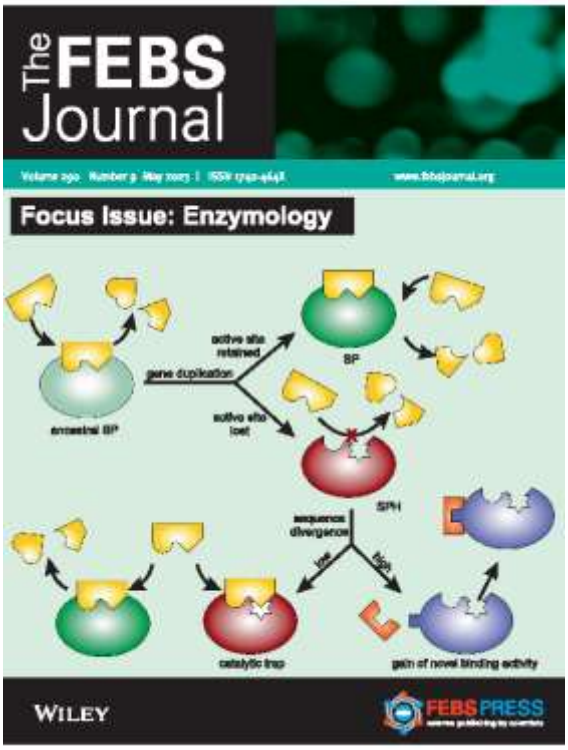

Figure 1: ***The cover page of the FEBS Journal issue advertising our paper on evolution of novel biological functions in serine pseudoproteases****. The cover page is from FEBS J. 290(9) (2023).*

Following the publication of a detailed description of the serine protease VaaSP-VX, which promotes blood clotting by activating FV and FX, we isolated a structurally very similar molecule VaaSP-6 from the *Vaa* venom. Since the entire cDNA sequence of VaaSP-6 is known, we will produce this protein recombinantly in order to characterize it. Hopefully, the recombinant VaaSP-6 will exhibit the same unique procoagulant activity as VaaSP-VX, so that it could replace the unreliable dilute Russell's viper venom (dRVV) assay currently used in clinics for the determination of lupus anticoagulants (LA test).

In the last year, we also experimentally concluded our ARIS research project J3-2534. In this project, together with colleagues from the Centre for Clinical Toxicology and Pharmacology of the University Medical Centre Ljubljana (UMCL), we investigated an interesting clinical effect observed in patients envenomed by the nose-horned viper, namely a profound, transient and reversible thrombocytopenia of functional platelets. Platelets play a central role in thromboembolic diseases such as myocardial infarction and ischemic stroke. Existing antiplatelet drugs have a common side effect – a reduced number of platelets whose activity is inhibited. This condition carries a high risk of bleeding (haemorrhage), especially in interventional cardiology and angiology that use an antithrombotic approach. Our results could pave the way for the development of a new group of antiplatelet agents that would reduce the risk of dangerous bleeding in interventional

cardiology and angiology, and increase the efficacy of vasodilatation and clot removal. We have demonstrated that reversible thrombocytopenia in patients envenomed by the *Vaa* is induced by proteins similar to type C lectins (snaclecs). We have isolated several snaclecs from the *Vaa* venom and showed that snaclec 3/2 in particular induces severe thrombocytopenia through its interaction with the GPIb platelet receptor. In collaboration with our partners from the VF/UL, we have performed an *in vivo* study in a mouse model of arterial thrombosis to validate the potential of snaclec 3/2 to prevent clot formation and arterial occlusion after experimentally induced vascular injury, and to determine its potential for medical applications. The first set of results has already been published (M. Dobaja Borak et al., *Thrombosis Research* 229 (2023), 152–154). However, the preparation of two further research papers on this topic (M. Dobaja Borak et al., *Thrombosis Research*, submitted; M.C. Žužek et al., in preparation) and a review paper (K. Požek et al., in preparation) has already been initiated.

Within the research network, comprising 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 *Vaa* and treated with different antidotes. A publication is underway (T. Kurtović et al., in preparation).

Our scientific achievements in the field of toxinology were very well recognised also in 2023. I. Križaj has been invited as a lecturer at the Société Française pour l'Etude des Toxines (SFET) annual meeting in Paris from the 30th November to the 1st December 2023. He was also invited to act as a Guest Editor in two well-established journals, *Toxins* (I. Križaj, *Toxins* 15 (2023), 212) and the *International Journal of Molecular Sciences* (P. Veranič and I. Križaj, *International Journal of Molecular Sciences* 24 (2023), 13667). However, the most prestigious recognition for the outstanding research work of I. Križaj and his team was the Zois Award, the highest science award in Slovenia (Figure 2).

Figure 2: ***Dr. I. Križaj receiving the Zois award****. At the ceremony in Cankarjev dom, on the 28th November 2023, I. Križaj received the Zois Award, the highest national science award. Left from Dr. Križaj is Dr. Nataša Vaupotič, the president of the Zois Awards Committee. On the right side is the Minister of Higher Education, Science and Innovation, Dr. Igor Papič. The justification of Dr. Križaj's decoration is available at https://www.gov.si/novice/2023-11-28-podeljene-najvisje-drzavne-nagrade-v-znanstvenoraziskovalni-in-razvojni-dejavnosti/.*

Lipid metabolism and signalling

Lipids are essential for life. They constitute the membranes of all cells and organelles, and serve as the most efficient form of energy storage. However, owing to their structural and functional diversity and the complexity of their assemblies, the roles of lipids and lipid metabolism in health and disease remain poorly defined. Lipid droplets are recently recognized organelles that could help us understand the mechanisms underlying lipid function at the cellular level and in various pathophysiological conditions associated with dysregulated lipid metabolism. These dynamic fat storage organelles are involved in essential cellular processes, ranging from energy production and membrane homeostasis to infection and inflammation. Our work in this field has been focused on answering the following questions: (1) Are lipid droplets involved in the generation of lipid signalling molecules involved in intercellular communication? (2) What is the role of the lipid droplets in cellular lipid trafficking and the regulation of membrane integrity and function? and (3) How does autophagy cooperate with lipid droplet breakdown mechanisms during nutrient stress?

In our recent report (E. Jarc Jovičić et al., *Molecular Metabolism* 76 (2023), 101791), we described a novel mechanism of fatty acid trafficking between membrane phospholipids and triglycerides stored in lipid droplets, which is critical for the generation of potent lipid mediators that promote tumour growth and inflammation. We demonstrated that certain types of fatty acids are enriched in lipid droplets of cancer cells and discovered the molecular mechanism of their release from lipid droplets (Figure 3). This research uncovers a previously unrecognized central role for lipid droplets in fatty acid metabolism and signalling, and significantly expands the current membrane-centric paradigm of lipid mediator production.

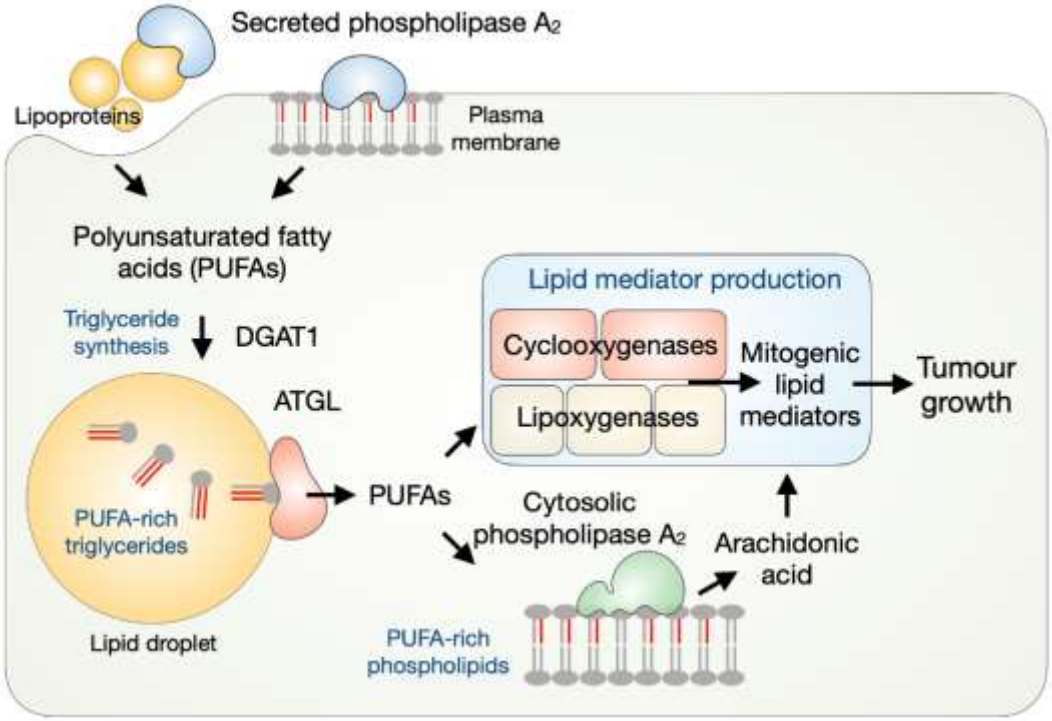

Figure 3: ***Mechanism of lipid droplet-controlled generation of lipid signals that promote tumour growth****. Our study shows that lipid droplets control the supply of ω-3 and ω-6 polyunsaturated fatty acids (PUFAs) for the production of lipid mediators, which in turn drive cancer cell proliferation. The esterification of PUFAs into triacylglycerols (TAGs) and their release from lipid droplets are necessary for PUFA entry into lipid mediator production pathways (E. Jarc Jovičić et al., Mol. Metab. 76 (2023), 101791).*

In an invited review (M. Danielli et al., *Frontiers in Cell and Developmental Biology* 11 (2023), 1104725) published in a special issue entitled “The evolving role of lipid droplets: Advancements and future directions”, we highlighted the concept of lipid droplets having multifaceted, context-dependent and often contradictory roles, ranging from the production of lipid signalling molecules to the control of membrane lipid peroxidation and cell death, including ferroptosis. We explored how these evolving concepts in lipid droplet biology can improve our understanding of cell adaptability and resilience to stress and how they can be harnessed in the fight against cancer.

Our report on autophagy and lipid droplets highlights the complex interplay between autophagy/lipophagy and lipid droplets in the regulation of cellular homeostasis and adaptation to stress (M. Jusović et al., *Cancers* 15 (2023), 4857). Our findings suggest a cooperation between autophagy and lipid droplet metabolism in protecting cancer cells against starvation (Figure 4). Using a strategy of combined targeting of autophagy and lipid droplet biogenesis, we impaired two essential processes for cancer cell resistance, thereby revealing a potential novel approach for cancer treatment. Our currently unpublished work on the mechanisms that control lipid droplet breakdown and direct lipids to other organelles is in preparation for publication (Š. Koren et al., in preparation). Our ongoing work on the capacity of lipid droplets to control cellular lipid fluxes and manage ferroptotic cell death is in preparation for publication (A. Kump et al., in preparation; L. Perne et al., in preparation). These results were recently presented at two renowned conferences (EMBO Lipid Droplets Workshop; EMBO Ferroptosis Workshop) and were accepted exceptionally well by our peers. For our studies on ferroptosis and lipid droplets, we were awarded an EMBO Advanced Collaboration Grant, which financed the expansion of our work into lipidomics and epilipidomics. Our studies on lipid droplets were also presented through invited talks at several other conferences and institutions, including the 63rd ICBL Meeting in Spain and the Institute of Physiology at the Czech Academy of Sciences.

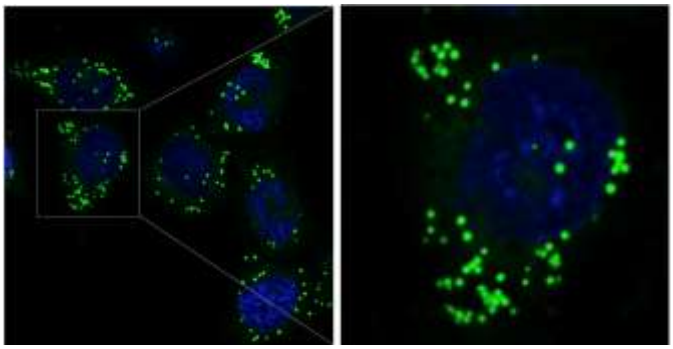

Figure 4: ***Lipid droplets and autophagy work together to protect cells from severe nutrient deficiency****. In our work (M. Jusović et al., Cancers 15 (2023), 4857), we demonstrate that lipid droplets accumulate in starving cancer cells in an autophagy-dependent manner and that combined targeting of autophagy initiation and lipid droplet biogenesis leads to cancer cell death (BODIPY-stained lipid droplets are green, cell nuclei are blue).*

Through a collaboration with several European groups working on lipids, we published a review discussing the current state and challenges of organelle lipidomics (M.J. Sarmento et al., *Cellular and Molecular Life Sciences* 80 (2023), 237).

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

The budding yeast *Saccharomyces cerevisiae* is a well-established model organism for basic research and a cell factory in biotechnology. In biotechnological applications it is also important for synthetic biology given its highly efficient homology recombination-based assembly of DNA fragments, called *in yeasto* assembly.

Within the ARIS project L4-3181, *Hierarchical DNA assembly for advanced applications in biopharmaceuticals production and cell therapy*, we have been developing a toolbox for hierarchical DNA assembly by combining *in vitro* and *in yeasto* assembly approaches. We successfully developed an experimental pipeline for assembly of genomes of recombinant bacteriophages with modified host-range. This pipeline will be of great importance for the industrial

partner and co-financer of the said project. The results were presented in an invited lecture at a scientific conference and a research paper (G. Žun et al., *Yeast* 40 (2023), 32–41).

With our partners in the ERACoBioTech project OLEOFERM (https://oleoferm.eu/), we found a new biotechnologically useful strain of the oleaginous yeast species *Yarrowia lipolytica* with a promising potential for lipid production from short-chain fatty acids (SCFAs) using yeast cell factories. We have performed genomics and transcriptomics analyses with the aim to identify the metabolic routes involved in this biotransformation (Figure 5). The results have been compiled and are planned to be published in 2024.

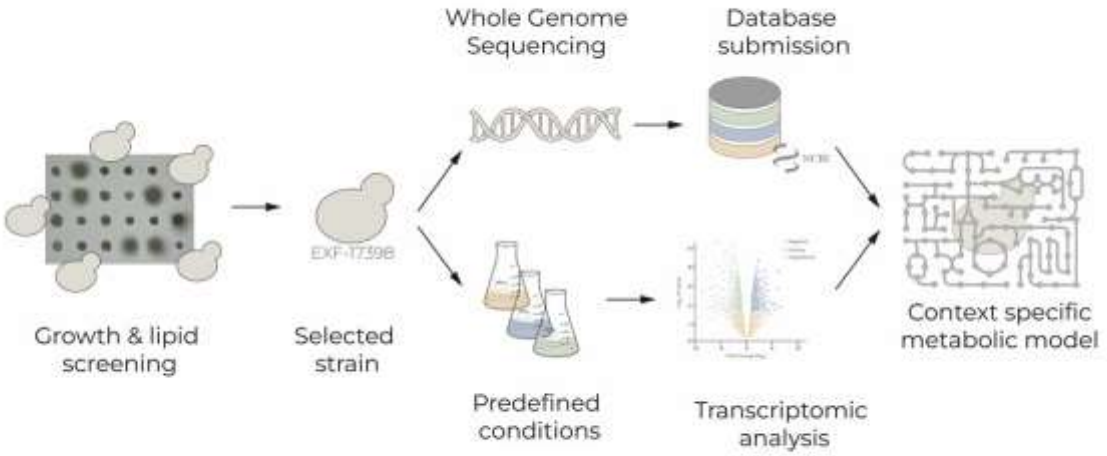

Figure 5: ***Scheme of strain selection, genomic and transcriptomic analysis to identify metabolic routes involved in microbial oil production from short-chain fatty acids****.*

Within the ARIS project J4-4560, *Engineering of polygenic traits in S. cerevisiae*, we have started developing custom-made methods for haploid selection and a novel assay for protein secretion screening. Preliminary results were presented at local student conferences.

Evolutionary genomics

Peptidases of the papain family play a key role in protein degradation, regulated proteolysis, and the host–pathogen arms race. Although the papain family has been the subject of many studies, knowledge about its diversity, origin, and evolution in Eukaryota, Bacteria and Archaea is limited. In the past year, we addressed this gap by tracing the birth and expansion of the papain family with a phylogenomic analysis using publicly available information from numerous prokaryotic and eukaryotic proteomes, transcriptomes and genomes (D. Kordiš and V. Turk, *International Journal of Molecular Sciences* 24 (2023), 11761). We found that the papain family was present in the last universal common ancestor and expanded greatly during eukaryogenesis through massive gene innovation and diversification, which resulted in eight ancestral C1A lineages in the ancestor of eukaryotes. These eight ancestral eukaryotic C1A peptidase lineages are cathepsins B, C, X, L, H and F, 26/29 kDa peptidase and type 1 long C1 peptidase, which are present in all eukaryotic supergroups (Figure 6). The papain family expanded further during

eukaryotic evolution, especially through extensive gene duplications in the ancestral cathepsin L and B lineages. Together, we demonstrated that diversification of the papain family predates the origin of eukaryotes and that a burst of innovation during eukaryogenesis led to a eukaryotic ancestor with a complex set of ancestral C1A lineages. The findings of this comprehensive study provide guidelines for future structural and functional studies of the papain family.

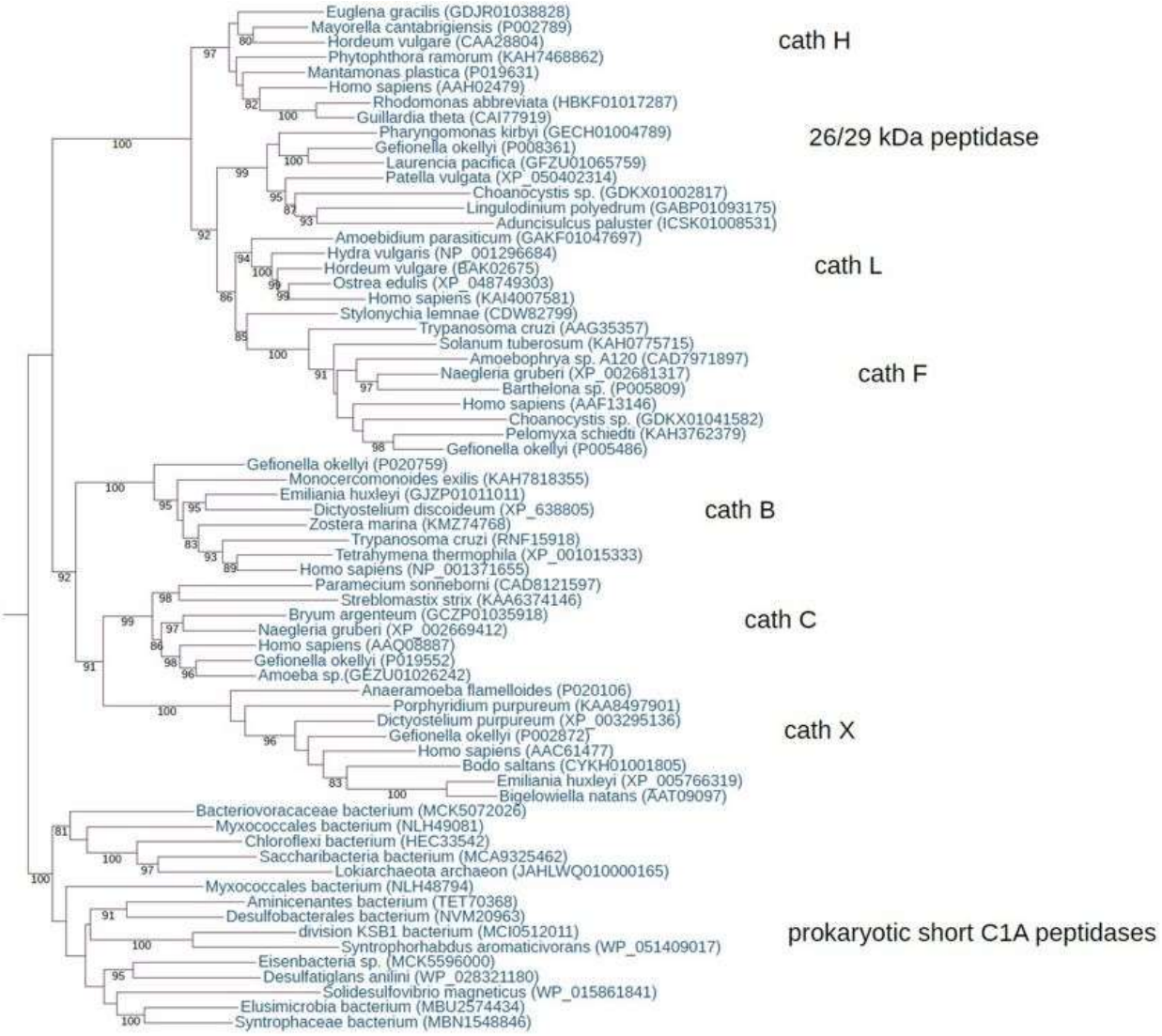

Figure 6: ***Early diversification of the papain family in the eukaryotic ancestor****. The rooted maximum likelihood (ML) tree shows the evolutionary relationships between the seven ancestral eukaryotic orthologous gene families, cathepsins B, C, X, L, H and F, and the 26/29 kDa peptidase. The ML tree represents bootstrap consensus following 1000 replicates. Sequences were obtained from the GenBank database. Species names and protein accession numbers are displayed.*

Pore-forming toxins form pores in cell membranes and 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 (sponges, ctenophores and cnidarians) we discovered unexpectedly large diversity and many novel domain architectures of aerolysin superfamily. We analysed the origin, diversity and domain architecture of aerolysin superfamily in basal metazoans as well as in diverse invertebrates (crustaceans and molluscs) and deuterostomes. Our analysis demonstrates that the aerolysin superfamily is incredibly diverse in metazoans where it plays important roles from host defence to the innate immunity (D. Kordiš, in preparation).

In 2022, the first draft assembly of the huge cave salamander (*Proteus anguinus*) genome was made at the Beijing Genomics Institute. With 34 gigabase (Gb), it is among the largest genomes ever sequenced, more than 10 times larger than the human genome. In the scope of the ARIS project J1-2469, led by our colleagues at the Biotechnical Faculty UL (BF/UL), we continued with the analysis of genomic and transcriptomic data of the cave salamander. Besides the analysis of transposable elements (TEs) we investigated diverse cave adaptations, such as the chemosensory system (olfactory receptors, vomeronasal receptors and taste receptors), its G-protein-coupled receptors repertoire as well as the genome defence systems against TEs (APOBEC, SCAN-ZNF and KRAB ZNF genes) of this cave animal.

Other subjects

In 2023, we also participated in different projects outside the thematic scope of our department that were funded by ARIS or other funders. Only the projects for which publications have been published or are in preparation are listed below.

In the scope of the ARIS project J1-2482 (leading institution: BF/UL), we have been determining the impact of environmentally relevant nano- and microplastics on terrestrial vertebrates by mass spectroscopy. We also performed the proteomic analysis of the haemolymph of the terrestrial crustacean *Porcellio scaber* and revealed components of its innate immunity under baseline conditions (A. Jemec Kokalj et al., *Biochimie* 213 (2023), 12–21).

As partners on the ARIS project J2-3040 on magnetically controllable nanocarriers that mimic endogenous lipid particles to improve drug/nanoparticle delivery, we participated at analysing the effects of barium-hexaferrite nanoplatelets in low-frequency magnetic field on cancer cells (T. Goršak et al., *Journal of Colloid and Interface Science*, in press).

We also collaborated informally 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 (I. Sondi et al., in preparation).

In the study led by colleagues from the Faculty of Electrical Engineering UL, we analysed the protein corona composition of different types of nanoparticles using a proteomic approach to specify the serum protein binding (L. Peternel et al., in preparation).

We came to the aid to our colleagues from the Medical Faculty UL performing the confocal microscopic analysis for the functional validation of an α-FREM2 nanobody as a molecular tool for targeting specifically glioblastoma stem cells (N. Šamec et al., *FEBS Journal*, submitted).

We joined our colleagues from the Department of Gaseous Electronics (F6) at the Jožef Stefan Institute (JSI) to study the reduction of antigenicity of common ragweed pollen and its primary allergen Amb a 1 with cold atmospheric pressure air plasma (N. Hojnik et al., *Journal of Hazardous Materials*, submitted).

In the collaboration, led by our colleagues from the Department for Nanostructured Materials (K7), JSI, we investigated the potential of vesicles from red blood cell membranes as a safe and efficient delivery system for therapeutic nucleic acids (G. Della Pelle et al., in preparation).

Some outstanding publications in 2023

1. Dobaja-Borak, M., Grenc, D., Reberšek, K., Podgornik, H., Leonardi, A., Kurtović, T., Halassy, B., Križaj, I. and Brvar, M.: Reversible and transient thrombocytopenia of functional platelets induced by nose-horned viper venom. Thrombosis Research, 229 (2023), 152–154
2. Stazi, M., Megighian, A., D'Este, G., Negro, S., Ivanušec, A., Lonati, D., Pirazzini, M., Križaj, I. and Montecucco, C.: An agonist of CXCR4 induces a rapid recovery from the neurotoxic effects of *Vipera ammodytes* and *Vipera aspis* venoms. Journal of Neurochemistry (2023), doi: 10.1111/jnc.15803
3. Jarc Jovičić, E., Pucer Janež, A., Eichmann, T.O., Koren, Š., Brglez, V., Jordan, P.M., Gerstmeier, J., Lainšček, D., Golob-Urbanc, A., Jerala, R., Lambeau, G., Werz, O., Zimmermann, R. and Petan, T.: Lipid droplets control mitogenic lipid mediator production in human cancer cells. Molecular Metabolism, 76 (2023), 101791
4. Žun, G., Doberšek, K. and Petrovič, U.: Construction and evaluation of gRNA arrays for multiplex CRISPR-Cas9. Yeast, 40 (2023), 32–41
5. Kordiš, D. and Turk, V.: Origin and early diversification of the papain family of cysteine peptidases. International Journal of Molecular Sciences, 24 (2023), 11761