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.

Secreted phospholipases A_2

One of the major research topic of the department are secreted phospholipases A_2 ($sPLA_2$s) originating from animal toxins as well as those found in humans. We are studying the molecular mechanisms of action of the toxic $sPLA_2$s, particularly those endowed with presynaptic neurotoxicity, and the role of endogenous $sPLA_2$s in pathological and physiological processes in mammals.

We have been invited to prepare a review for a monograph entitled “Genetic Manipulation of DNA and Protein – Examples from Current Research” (T. Petan et al., *InTech Open Access*, (2013), 107–132). In this overview, our successful protein engineering approach in the structure-function studies of $sPLA_2$s from viperid venoms were presented.

By developing innovative procedures to renature recombinant $sPLA_2$s we tried to obtain enzymatically inactive form of ammodytoxin A (AtxA), a neurotoxic $sPLA_2$ from the venom of the nose-horned viper (*Vipera ammodytes ammodytes*). The results are encouraging, and we expect that we will be able to produce a sufficient amount of the correctly folded protein in the next year. This will enable an accelerated characterization of novel $sPLA_2$ receptors, an advance in the study of the translocation mechanisms of these molecules through plasma membrane and between different compartments in the cell, as well as intracellular trafficking of the $sPLA_2$ in the real time. We study the molecular mechanism of action of presynaptically neurotoxic $sPLA_2$s also using OS_2 from the venom of the Australian taipan (*Oxyuranus scutellatus scutellatus*). AtxA and OS_2 are structurally different $sPLA_2$s. The first belongs to the group II, and the second to the group I $sPLA_2$s. As model molecules they are therefore complementary. Identification of an $sPLA_2$ receptor in the presynaptic membrane of a motoneuron (N-$sPLA_2$R), which is crucial for the expression of neurotoxicity, has been a large research challenge already for many years. OS_2 binds to this receptor with an affinity 1000-fold higher than that of AtxA, therefore it represents an ideal ligand for the study of N-$sPLA_2$R. Following the preparation of large quantities of the recombinant wild-type OS_2 and its chimera with similar, but non-toxic, OS_1, from the venom of the same snake, we continued in 2013 the collaboration with a research group from the Institute of Molecular and Cellular Pharmacology of the National Centre for Scientific Research (CNRS), Valbonne, France, in the scope of an international bilateral project Proteus, by developing a procedure for preparation of the photo-reactive derivatives of these $sPLA_2$s. The derivatives are currently being characterized.

$sPLA_2$s represent physiologically very important family of multifunctional proteins, whose effects do not depend solely on their enzymatic activity but, in some cases, also with their ability to bind to other molecules. We have been searching for new $sPLA_2$-binding molecules using immuno-affinity chromatography. In this way, we identified in the venom of the nose-horned viper an Atx-binding protein, which inhibited the activity of chymotrypsin (ChI), structurally belonging to the Kunitz-type proteinase inhibitors. Interestingly, the first results show that the toxicity of AtxA is increased in the presence of ChI. ChI-like molecules are present in mammals. It will be interesting to analyse the affinity of these molecules to $sPLA_2$s and physiological consequences of their interaction with $sPLA_2$ (M. Brgles et al., *Analytical and Bioanalytical Chemistry*, in press).

Following demonstrations that $sPLA_2$s can act also inside cells, we continue to study intracellular activities of these molecules using different cellular models. In 2013 we tested the hypothesis of the molecular mechanism of AtxA action, based on our results obtained on the yeast *Saccharomyces cerevisiae*, by which AtxA inhibits endocytosis, on a mammalian cellular model, the isolated mouse neuromuscular junction. By characterising an enzymatically active mutant of the catalytically inactive, non-neurotoxic ammodytin L (AtnL), AtnL-LW, we confirmed the validity of the model also in mammalian cells. Restoration of the enzymatic activity conferred to AtnL both the ability to inhibit endocytosis in yeast and to act as a presynaptically neurotoxic $sPLA_2$ at the mammalian neuromuscular junction (N. Vardjan et al., *Communicative and Integrative Biology*, 6 (2013), e23600).

We studied intracellular action of Atx also on mammalian cell lines, murine NSC34 and rat PC12. We performed a confocal-microscopy study of the translocation dynamics of Atx into PC12 cells. Using the same technique, we determined the level of collocalization between Atx and mitochondria, as well as Atx and several intracellular proteins, following internalisation of Atx into PC12 cells. The results are prepared for publication.

We published a paper clearly demonstrating that ammodytoxins efficiently release arachidonic acid (AA) and induce apoptosis in a motoneuronal cell line in an enzymatic activity-dependent manner (Z. Jenko-Pražnikar et al., *NeuroToxicology*, 35 (2013), 91–100). The role of $sPLA_2$ enzymatic activity, including AA release, in the induction of motoneuronal apoptosis has been studied by AtxA and homologous recombinant $sPLA_2$s with different enzymatic properties. We analysed the effects of an AtxA(V31W) mutant with very high enzymatic activity, enzymatically inactive S49-$sPLA_2$ (AtnL), its mutant with restored enzymatic activity (AtnL-LW), and non-toxic, enzymatically active $sPLA_2$ ($AtnI_2$). Addition of AA, AtxA, AtxA(V31W) and AtnL-LW, but not AtnL and $AtnI_2$, to motoneuronal cells resulted in caspase-3 activation, DNA fragmentation and disruption of mitochondrial membrane potential, leading to a significant and rapid decrease in motoneuronal cell viability that was not observed in (control) mouse myoblast and human embryonic kidney cells. AtxA, AtxA(V31W) and AtnL-LW, but not AtnL and $AtnI_2$, also liberated large amounts of AA specifically from motoneuronal cells, and this ability correlated well with the ability to induce apoptotic changes and decrease cell viability. The enzymatic activity of AtxA and similar $sPLA_2$s is thus necessary, but not sufficient, for inducing motoneuronal apoptosis. These results suggests that specific binding to the motoneuronal cell surface, followed by internalisation and enzymatic activity-dependent induction of apoptosis, possibly as a consequence of both extensive extra- and intracellular AA release, is necessary for Atx-induced motoneuronal cell death.

In 2013 the postdoctoral research project was concluded, in which scope the detailed structural analysis of the interaction between Atx and calmodulin (CaM) has been studied by protein NMR spectroscopy. CaM is a regulatory protein in the cell cytosol, presumably very important for intracellular activity of Atx and homologous mammalian $sPLA_2$s. Understanding on the atomic level of its interaction with $sPLA_2$s, as well as of the interaction of the $sPLA_2$–CaM complexes with the phospholipid membrane, is very important for designing the regulation of these interactions. In collaboration with two partner groups, the Bijvoet Centre from the Utrecht University, the Netherlands, and the NMR centre from the National Institute of Chemistry in Ljubljana, we finished with the planned experimental work. Data processing and preparation of publications are underway.

Aiming to dynamically observe the interaction between Atx and CaM in cells using a FRET method, we continued development of fluorescent derivatives of both proteins.

In an attempt to formulate a protocol for preparation of an effective antiserum against the nose-horned viper venom, we discovered that the quantity of Atx in the venom positively correlates with the level of venom immunogenicity. Rapid and accurate method for the quantification of Atx in the venom is therefore one of the key steps in preparing the antisera of a high quality. Together with the colleagues from the Institute for Chemical Technologies and

Analytics, Vienna University of Technology, we made another step forward in efficient quantification of Atx in the venom. We developed an original method that is able to separate, in a single step, all three highly similar Atx isoforms (V.U. Weiss et al., *Electrophoresis*, in press).

It has been shown that nine active $sPLA_2$ enzymes known in humans display different tissue expression patterns and specific enzymatic preferences for binding to different types of phospholipid membranes, suggesting distinct biological roles for each $sPLA_2$. The multitude of cellular effects of the released free fatty acids (FFAs) and lysophospholipids, and of their numerous bioactive metabolites, further explain their involvement in a variety of physiological processes and diseases, including lipid digestion and remodelling, acute and chronic inflammatory diseases, cardiovascular diseases, reproduction and host defence against infections. Recent studies have confirmed that various $sPLA_2$s also play a significant role in cancer and metabolic disorders. For example, a few years ago, it was demonstrated that the human group X (hGX) $sPLA_2$ stimulates colon cancer cell proliferation by a mechanism dependent on the released FFAs and lysophospholipids, but not on its potent stimulation of prostaglandin E_2 synthesis. The underlying mechanisms of the action of hGX $sPLA_2$ and other $sPLA_2$ enzymes in different cancers have not been known and confirmation of their functional contribution to tumorigenesis have been waiting for additional studies. In 2013, we successfully completed and published an extensive study, using multiple breast cancer cellular models, analysing the effects of hGX $sPLA_2$ on breast cancer cell growth and survival in details, with an aim to better understand its mechanism of action (Pucer et al., *Molecular Cancer*, 12 (2013), e111). We were able to show for the first time that hGX $sPLA_2$ induces lipid droplet (LD) formation in the highly tumorigenic MDA-MB-231 breast cancer cells (Figure 1) in an enzyme activity-dependent manner, thereby stimulating cell proliferation and significantly prolonging cell survival under serum deprivation-induced stress.

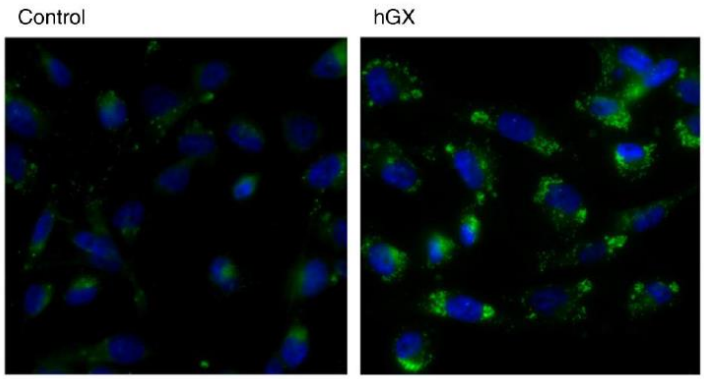

Figure 1: Human group X $sPLA_2$ induces triacylglycerol synthesis and lipid droplet (LD) formation in MDA-MB-231 cells in an enzymatic activity-dependent manner. The cells were grown in complete culture medium in the presence of 1 nM hGX for 48 h, fixed, stained with Nile red to visualise LDs (green) and DAPI to visualise nuclei (blue). Note a significant increase in the amount of LDs in hGX-treated cells in comparison with a non-treated control. The figure is reproduced from A. Pucer et al., *Molecular Cancer*, 12 (2013), e111.

Our results suggested that FFAs, in particular oleic acid, released from membrane phospholipids by the action of hGX $sPLA_2$, are substantially responsible for LD biogenesis and cell survival. We also demonstrated that the mechanism of hGX-induced cell survival and lipid accumulation is associated with alterations in the expression of key lipogenic and β-oxidation enzymes, and modulation of AMP-activated protein kinase (AMPK) and protein B/Akt kinase signalling pathways. The pro-tumorigenic effects induced by hGX $sPLA_2$ were abolished by etomoxir, suggesting a critical role for β-oxidation in hGX-induced LD formation and cell survival in breast cancer cells. The ability of hGX $sPLA_2$ to act as a modulator of basic lipid metabolism and cancer cell survival is thus well established. This could have important implications in elucidating the role of hGX and other $sPLA_2$s, such as hGV and hGIII, in cancer and human pathophysiology in general.

Experience of our group in the field of $sPLA_2$ is evidently well known also to the editors of the *Protein and Peptide Letters* as they invited us to prepare a review article on the role of these molecules in the mammalian immune system (I. Križaj, *Protein and Peptide Letters*, in press).

Other pharmacologically active components from natural toxins

In 2013 we continued to systematically analyse the components of the nose-horned venom that affect the blood coagulation process – haemostasis. We succeeded to publish a description of one of the most haemorrhagic molecules from the venom, homodimeric metalloproteinase (SVMP) VaH3 (T. Sajevic et al., *Biochimie*, 95 (2013), 1158–1170). We concluded also with the experimental work on a heterodimeric, haemorrhagic SVMP, VaH4, and published also these results (A. Leonardi et al., *Toxicon*, 77 (2014), 141–155). A very important conclusion stems from our analysis: in P-III class of SVMPs, dimers can be formed by a covalent inter-subunit crosslinking of either two Cys132 or two Cys174 residues (Figure 2).

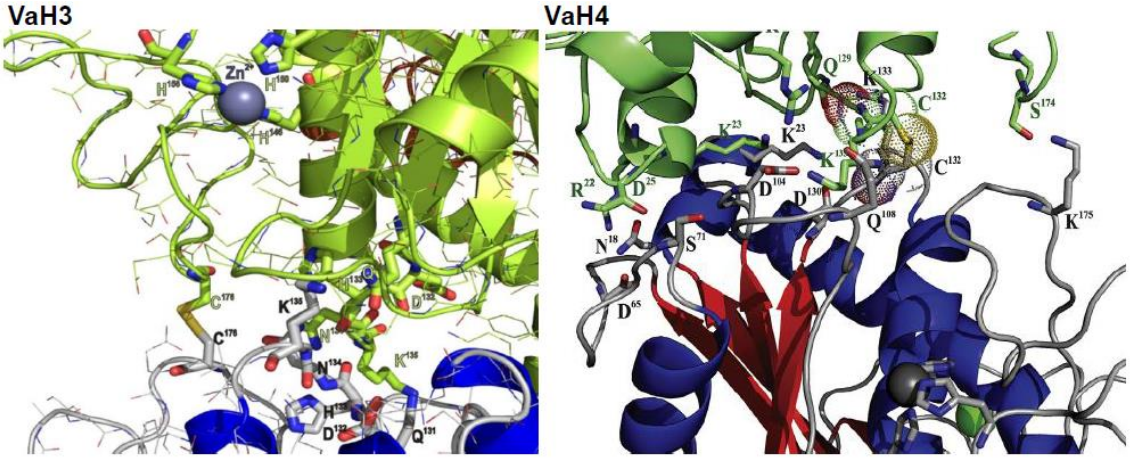

Figure 2: Comparative display of interacting areas between subunits in VaH3 and VaH4. In the case of VaH3, a disulphide bond between the subunits forms between two Cys176 residues. In the case of VaH4, an intra-subunit disulphide bond is formed, however, between two Cys132 residues. Therefore, P-IIIc SVMP can be divided into two groups, one possessing a Cys132–Cys132 and the other a Cys174–Cys174 intra-subunit disulphide bond connection. The figures are reproduced from T. Sajevic et al., *Biochimie*, 95 (2013), 1158–1170 in A. Leonardi et al., *Toxicon*, 77 (2014), 141–155.

Due to our achievements in the field of haemostatically-active components from snake venoms and connected pathologies we have been invited to submit a review article (T. Sajevic et al., *Toxin Reviews*, in press). In collaboration with our colleagues from the Institute of Immunology in Zagreb, Croatia, and the Vienna University of Technology we described another very interesting molecule from the venom of the nose-horned viper, a serine proteinase VaSP1 with the unconventional active site structure (T. Kurtović et al., *Toxicon*, 77 (2014), 93–104). Regarding the substrate specificity of this enzyme and the fact that it prolongs the prothrombin and activated partial thromboplastin times, it is very likely that it acts as anticoagulant.

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

Neonicotinoid insecticides were rather notorious in 2013 because of their proposed toxicity for bees and other non-target organisms, on the basis of which they were banned in the EU in April 2013. Using chemogenomics analysis in the yeast model we have determined side effects of neonicotinoid insecticides, and especially of additives from insecticide formulations. We have

shown examples where additives are even more toxic than neonicotinoids themselves (M. Mattiazzi Ušaj et al., *Chemosphere*, in press).

In the field of genetics, the last years have seen a rapid development of techniques and methods for polygenic traits analysis, which have been spurred by recent developments in genomics. In our group we have been developing new experimental approaches and computational tools that will enable transfer of combinations of genes (*i.e.*, genetic modules) in industrial microorganisms (Figure 3), which will revolutionise the field of metabolic engineering and industrial biotechnology.

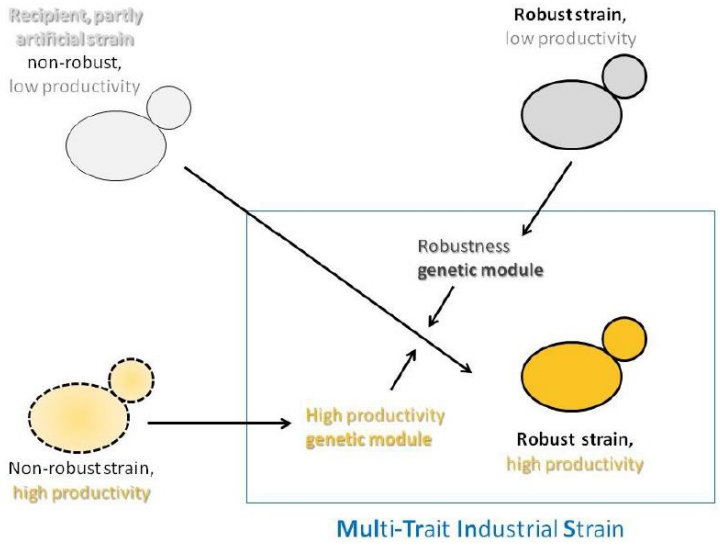

Figure 3: Metabolic engineering and industrial biotechnology are expected to gain a lot using the transfer of genetic modules into industrial microorganisms.

Regulation of cellular processes through internal metabolic intermediates is one of the most exciting areas of molecular biology, which should contribute to new treatments for cancer, type 2 diabetes and neurodegenerative diseases. Acetyl-CoA is the key metabolite that broadly affects cellular processes. In 2013 we have finished our multiyear study on the regulation of cellular metabolism through a peroxisomal protein Pex11. We established that Pex11 regulates the cytosolic concentration of acetyl-CoA, which makes Pex11 an interesting novel drug target.

Our colleague from the department, currently a postdoc at the University of Toronto, Canada, took part in preparation of a review article about the contribution of the functional genomics and high-throughput methods to the studies of the cell polarity in yeast (E. Styles et al., *Philosophical Transactions of the Royal Society B Biological Sciences*, 368 (2013), 20130118).

Evolutionary genomics and study of retrotransposons

Vertebrates, especially mammals, possess numerous single-copy domesticated genes (DGs) that have originated from the intronless multicopy transposable elements. However, the origin and evolution of the retroelement-derived DGs (RDDGs) that originated from Metaviridae has only been partially elucidated, due to the absence of genome data or limited analysis of a single family of DGs. We traced the genesis and regulatory wiring of the Metaviridae-derived DGs through phylogenomic analysis, using whole-genome information from more than 90 chordate genomes (J. Kokošar and D. Kordiš, *Molecular Biology and Evolution*, 30 (2013), 1015–1031). Phylogenomic analysis of these DGs in chordate genomes provided direct evidence that major diversification has occurred in the ancestor of placental mammals. Mammalian RDDGs have

been shown to originate in several steps by independent domestication events and to diversify later by gene duplications. Analysis of syntenic loci has shown that diverse RDDGs and their chromosomal positions were fully established in the ancestor of placental mammals. By analysis of active Metaviridae lineages in amniotes, we have demonstrated that RDDGs originated from retroelement remains. The chromosomal gene movements of RDDGs were highly dynamic only in the ancestor of placental mammals. During the domestication process, *de novo* acquisition of regulatory regions is shown to be a prerequisite for the survival of the DGs (Figure 4). The origin and evolution of *de novo* acquired promoters (Figure 5) and untranslated regions in diverse mammalian RDDGs have been explained by comparative analysis of orthologous gene loci.

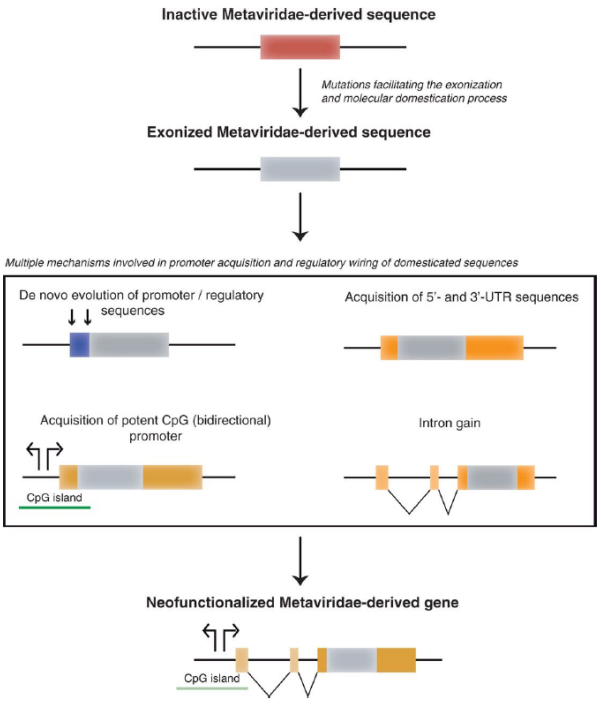

Figure 4: Mechanisms involved in the process of retroelement-derived domesticated gene (RDDG) neofunctionalisation. In the transition phase from retroelement remains to the first RDDGs, many nucleotide changes were necessary for the neofunctionalisation. One of the crucial steps in the process of neofunctionalisation was the exonisation of retroelement domains (Gag, protease, and integrase), which produced ready-to-use modules. Retroelement remains in mammalian genomes will normally turn into pseudogenes, due to lack of a promoter, and they can survive as a functional gene only if they recruit a new promoter sequence. To become expressed at a significant level and in the tissues where it can exert a selectively beneficial function, a new gene needs to acquire a core promoter and other structural elements that regulate its expression. Exons and introns are shown as orange (5'- and 3'-untranslated regions - UTRs) or grey (coding part of the exons) boxes and connecting lines. A *de novo* acquired promoter is shown in blue. The figure is reproduced from J. Kokošar in D. Kordiš, *Molecular Biology and Evolution*, 30 (2013), 1015–1031.

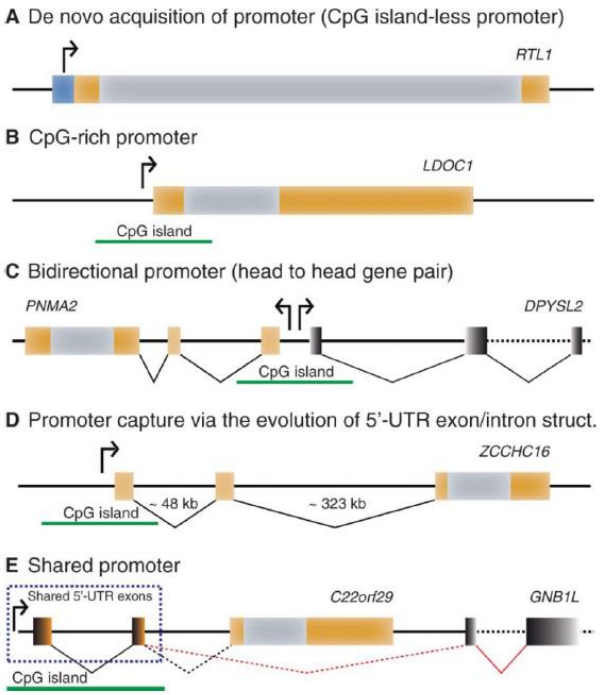

Figure 5: Diverse sources of the retroelement-derived domesticated gene (RDDG) promoters. Various scenarios that lead to the transcription of RDDG copies are illustrated. (A) Recruitment of proto-promoters from the CpG island-less region. (B) Recruitment of proto-promoters from the CpG-rich island. (C) Recruitment of a bidirectional (CpG-enriched) promoter from neighboring gene in the vicinity of the RDDG. (D) Recruitment of distant promoters in the genomic neighbourhood by the acquisition of a new 5'-untranslated (UTR) exon–intron structure. (E) Sharing of the unidirectional (CpG-enriched) promoter from a neighboring gene in the vicinity of the RDDG. Exons and introns are represented by orange and grey (RDDGs) or black (neighbouring genes in the case of bidirectional promoters) boxes and connecting lines. Distances between exons are not to scale. The figure is reproduced from J. Kokošar in D. Kordiš, *Molecular Biology and Evolution*, 30 (2013), 1015–1031.

The findings of this study thus provide a new view on the origin and evolution of the *de novo* acquired promoters, 5'- and 3'-UTRs in diverse mammalian RDDGs. The regulatory wiring of DGs and their rapid fixation in the ancestor of placental mammals have played an important role in the origin of their innovations and adaptations, such as placenta and newly evolved brain functions. DGs could thus constitute an excellent system on which to analyse the mechanisms of regulatory evolution in placental mammals.

In 2013 we participated in the study led by our colleagues from the Faculty of Chemistry and Chemical Technology, University of Ljubljana (UL), about the way in which the APOBEC3 proteins inhibit multiplication of the L2-retrotransposon. Clarification of the mechanism of action of APOBEC3 proteins is very important as these proteins inhibit multiplication of numerous retrotransposons and retroviruses, among them also the HIV virus (N. Lindič et al., *Retrovirology*, 10 (2013), e156).

Other subjects

In 2013 we also worked on several projects out of the thematic scope of our department.

We collaborated intensively with the colleagues from the Department of Biology, the Biotechnical faculty (BF), UL, at the determination of the mode of action of lipid membrane-forming proteins from the mushroom *Pleurotus ostreatus*. With structural identification we participated at the conclusion that for the formation of the pore in membranes, rich in cholesterol and sphingomyelin, pleurotolysin B requires the presence of another protein, ostreolysin A (K. Ota et al., *Biochimie*, 95 (2013), 1855–1864). During the isolation of ostreolysin A from the mushroom an additional protein co-eluted. Structural analysis of this protein revealed the first example of a protein consisting of hemopexin repeats in yeast (K. Ota et al., *Biochim. Biophys. Acta - Proteins and Proteomics*, 1834 (2013), 1468–1473). Together with the same group we also prepared a review paper on the use of pore-forming toxins for the sensing and labelling of membrane microdomains (M. Skočaj et al., *Current Medicinal Chemistry,* 20 (2013), 491–501). Very important joint project with the colleagues from the BF was dedicated to developing of an original approach against bacterial infections. In evolvement of the resistance of bacteria against antibiotics their SOS system is of a crucial importance. The key role in the bacterial SOS response is played by a complex formed between a single-stranded DNA (ssDNA) and two bacterial proteins, RecA and LexA. Based on experimental data we build a tridimensional model of the complex ssDNA–RecA–LexA (Figure 6), which will enable targeted design of substances to prevent development of bacterial resistance to antibiotics. We succeeded to publish our results in a very prominent journal (L. Kovačič et al., *Nucleic Acids Research*, 41 (2013), 9901–9910).

Together with our partners from the University Medical Centre Ljubljana, the Department of Rheumatology, we improved the isolation protocol of two proteins from human serum that are vital for diagnostics of antiphospholipid syndrome (A. Artenjak et al., *Clinical and Developmental Immunology*, in press).

With colleagues from the Institute of Biochemistry, the Medical Faculty, UL, we demonstrated that the recombinant human eritropoetin (EPO) modulates expression of some genes and stimulates the proliferation of the MCF-7 breast cancer cells. We did not, however, observe a correlation between the level of expression of different EPO receptor isoforms and invasiveness of the breast cancer cells (N. Trošt et al., *Radiology and Oncology*, 47 (2013), 382–389).

We helped the colleagues from the National Institute of Chemistry and the Centre of Excellence for Polymer Materials and Technologies (CE PoliMaT) with microbiological testing of antibacterial activity of macroporous polyurethane hybrid material with a high content of zinc and showed that it is highly bactericidal (G. Ambrožič et al., *Materials Research Bulletin*, 48 (2013), 1428–1434).

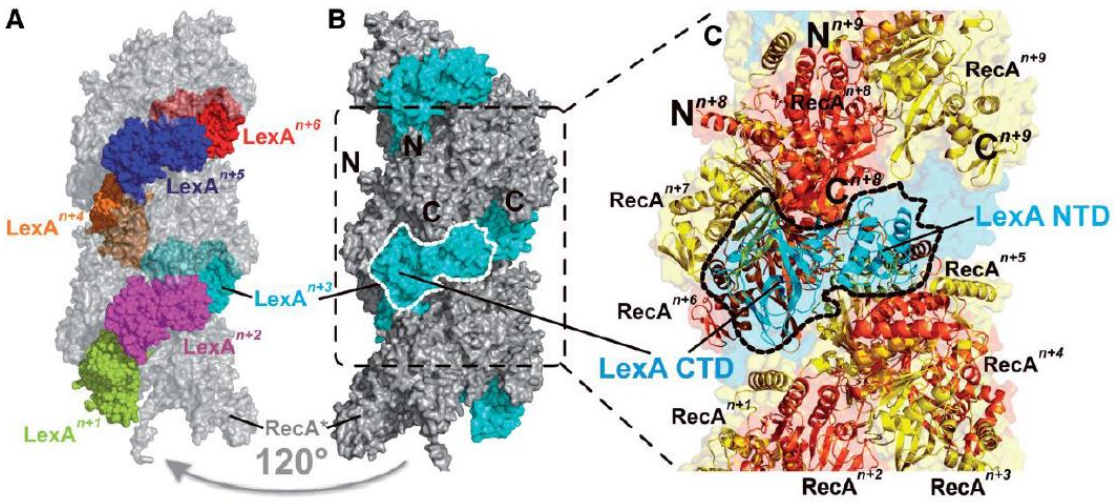

Figure 6: Model of the LexA–RecA* three-dimensional structure. (A) Six intact LexA monomers (spherical representation, each in a different colour) are docked on two turns of the RecA* (shown as grey transparent surface). (B) LexA–RecA* complex rotated by 120^{o} around vertical axis relative to the view in (A). LexA monomers are presented in cyan and RecA in grey. The N- and C-termini of the two RecA monomers are marked. One of the LexA monomers is encircled by a broken line. (C) Detailed view of the LexA–RecA* complex. The same LexA monomer as in (B) is encircled. LexA C- and N-terminal domains (CTD and NTD) are indicated. Nine successive RecA monomers (presented in yellow and orange) surround one monomer of LexA. Seven RecA protomers out of nine constitute the LexA-interaction interface. The figure is reproduced from L. Kovačič et al., *Nucleic Acids Research*, 41 (2013), 9901–9910.

The most important publications in 2013

1. Kokošar, J. and Kordiš, D. (2013): Genesis and regulatory wiring of retroelement-derived domesticated genes: a phylogenomic perspective. Mol. Biol. Evol. 30, 1015–1031
2. Kovačič, L., Paulič, N., Leonardi, A., Hodnik, V., Anderluh, G., Podlesek, Z., Žgur-Bertok, D., Križaj, I. and Butala, M. (2013): Structural insight into LexA-RecA* interaction. Nucleic Acids Res. 41, 9901–9910
3. Pucer, A., Brglez, V., Payre, C., Pungerčar, J., Lambeau, G. and Petan, T. (2013): Group X secreted phospholipase A_2 induces lipid droplet formation and prolongs breast cancer cell survival. Mol. Cancer 12, e111
4. Sajevic, T., Leonardi, A., Kovačič, L., Lang Balija, M., Kurtović, T., Pungerčar, J., Halassy, B., Trampuš-Bakija, A. and Križaj, I. (2013): VaH3, one of the principal hemorrhage-inducing factors in *Vipera ammodytes ammodytes* venom, is a homodimeric P-IIIc metalloproteinase. Biochimie 95, 1158–1170
5. Vardjan, N., Mattiazzi, M., Rowan, E.G., Križaj, I., Petrovič, U. and Petan, T. (2013): Neurotoxic phospholipase A_2 toxicity model – an insight from mammalian cells. Commun. Integr. Biol. 6, e23600