Description of the main research directions investigated by the institute

Epigenetics - E. Bártová and A. Kovařík:

We established the center of epigenetics, which is a leading scientific epigenetic cluster in the Czech Republic. Many scientists are interested in the functional regulation of DNA methylation, histone post-translational modifications, and methylation of RNAs. From the view of anticancer therapy, we study the effect of epi-drugs, also or in combination with cytostatic drugs, to know the impact of these drugs on cell proliferation and induction of apoptosis. Epigenetic processes are also studied during DNA damage repair. With this regard, we are working in the Strategy of the Czech Academy of Sciences, program Qualitas, Centre for epigenetics.

Electrochemistry- M. Fojta:

Electrochemistry of nucleic acids (NAs) has been established at the IBP as a new research area by Emil Paleček. This field of science was established in the second half of the 1950s. More recently, electrochemical studies of other biomacromolecules, namely proteins, carbohydrates, and glycoproteins, have been introduced. Electrochemical methods proved to be potent tools not only for the detection of low concentrations of the biomacromolecules or their components but also for studies of their structure and dynamics in solution and at electrically charged surfaces, chemical modifications and interactions with other macromolecules or small molecules such as drugs, toxins, etc. Nowadays, electrochemical research at the IBP is oriented towards the following main directions: (a) the development of novel methods and detection systems, including new electrode materials and their surface micro/nanostructures; (b) catalytic hydrogen evolution processes providing highly sensitive analysis of natural NAs, proteins, aminosugars, and chemically modified biopolymers; (c) development of sensitive and specific detection of rare and/or unnatural components of the NAs; and (d) studies of oligosaccharides and glycoproteins as targets for chemical modification/labeling with electrochemically active oxoosmium complexes to develop new tools for the distinction of glycan isomers. Prospective applications of the gained knowledge include the design of new methods for contemporary research in the areas of, e.g., chemical and synthetic biology or epigenetics, for diagnostics that are based on selected biomarkers, or for environmental monitoring.

CD spectroscopy, as the biophysical method - D. Renčiuk:

We continue our long-standing research of nucleic acid structure using circular dichroism spectroscopy, recently extended by studies of interactions with proteins or small molecule ligands. Due to its sensitivity, versatility, and cost-effectiveness, the CD spectroscopy is extremely useful in such studies, and IBP is significantly engaged in the respective field, which is supported by a number of original results, reviews, and book chapters in prestigious journals. We apply the CD spectroscopy on structural studies of the model or native sequences of nucleic acids, namely telomere DNA, gene promoter regions, or parts of viral genomes, with special focus on unusual conformations, such as guanine quadruplexes and cytosine i-motifs. CD spectroscopy ideally complements the methodological scope of the Institute, for example, molecular dynamic calculations or electrochemical analyses of nucleic acid structures. The CD methods range was recently supplemented by studies of fast

transition kinetics using a stopped-flow accessory. For subsequent data analyses, we employed advanced statistical methods, including singular value decomposition or principal component analysis, to investigate in detail CD spectral data sets reflecting structural transitions induced by the environment.

Biology of telomeres - J. Fajkus:

Because of the common G/C asymmetry in the telomere DNA strands, telomeres represent an ideal substrate for the existence of guanine quadruplexes and cytosine i-motifs. We study the conformational and thermodynamic properties of these structures in telomere DNA of various species, with special respect to the effect of base oxidative damage.

Cancer cell biology - K. Souček a V. Brabec:

One of our long-term interests in cancer cell biology is to bring more understanding to the mechanisms of deregulation of key signaling pathways that control cancer cell fate and discover new approaches for potential cancer therapy (Ah receptor signaling, nuclear receptors, transforming growth factor-β, death receptors, Wnt). Several of these pathways were also investigated in relation to the toxic, endocrine-disrupting, and carcinogenic action of dietary toxicants and environmental pollutants. Importantly, our field of interest also included the later tumorigenesis stages of tumor promotion and tumor progression. Here, principal attention was paid to the process of metastasis, dissemination of cancer cells to distant body sites, which represents a massive clinical challenge in cancer research. Regarding cancer therapy, one of the approaches that have come into focus in the last period is the concept of synthetic lethality, where the required phenotype is caused by a synergic modulation of two or more biological processes. Here we mostly focused on Checkpoint kinase 1 (CHK1) as a master regulator of cell division, which is involved in all defined cell cycle checkpoints, and described several novel mechanisms of potentiation of CHK1 inhibition effects in combination with chemotherapy drugs.
Also, the main research objective is to increase knowledge and understanding of the mechanisms of anticancer action of metallodrugs and to use this enhanced knowledge to develop new classes of metallodrugs with truly novel mechanisms of action and novel spectra of biomedical activity. There was a gap in the theoretical background needed for the design of new, more efficient metallodrugs consisting of the lack of information on the molecular and cellular mechanism underlying biological effects of existing or new metal-based compounds exhibiting antitumor effects. Our primary task is to investigate mechanisms of action of new antitumor platinum and other transition metal-based drugs to make it possible to design new, more effective metal-based drugs on a more rational basis. Research has been focused on the mechanism of action of metal-based compounds (including nanoparticles), which had a significant antineoplastic effect deriving from their interaction with cellular components. We introduced a number of new methods of molecular and cellular pharmacology, which make it possible to extend our studies on the molecular level to those on the cellular level, including studies on the level of cancer stem cells and 3D spheroids, which are much better at replicating in vivo environment than traditional two-dimensional (2D) cultures.

Computer simulations - J. Šponer:

The group is using a wide range of computational and theoretical methods to investigate RNA and DNA molecules and their interactions with proteins and ligands. The research is mainly directed into the following areas. The first topic is prebiotic chemistry, where the investigations aim to understand the spontaneous creation of first RNA molecules on the early Earth from simple inorganic precursors under geochemically relevant scenarios. The second topic includes studies of guanine quadruplex (G4) molecules, with the main aim to understand the basic principles of folding of G4 molecules and interactions of G4 molecules with ligands. The third topic is studies of protein-RNA and protein-protein complexes, with emphasis on characterization of the role of structural dynamics, substates, and partial disorder in molecular recognition processes. The fourth research direction is focused on the folding and catalysis of small RNA molecules, such as ribozymes and riboswitches. Finally, the group is developing methods for advanced molecular modeling of nucleic acids. The methodological research includes a parameterization of molecular mechanical force fields for classical atomistic simulations, refinement of methods for enhanced-sampling simulations as well as the development of protocols for application of modern quantum-chemical methods to nucleic acids. All research areas are mutually interrelated, while a substantial part of the research is done in extensive collaborations with established experimental laboratories.

Radiation biology - M. Falk:

This department has crucially contributed to radiobiological research in the Czech Republic and currently impersonates one of few institutes holding this research tradition, now developed at the cellular and molecular level. We explore the influence of different ionizing radiation types on normal and tumor cells and biological systems. As ionizing radiation is an important inducer of cancer and, at the same time, one of the most efficient tools for cancer treatment, the research is focused both on (radiation) carcinogenesis and the development of more efficient cancer (radio)therapy. Microscale and nanoscale studies of radiation DNA damage and repair in the context of chromatin architecture are the main concern. Having access to top-tech particle accelerators, special attention is paid to high-LET ions, which are studied in the context of future radiotherapy and protection of space flight crews. Ionizing radiation is also used as a unique tool to study the relationship between chromatin architecture and basic life processes in the cell nucleus, especially to reveal how chromatin works in healthy or pathologically altered cells.

Research activity and characterization of the main scientific results

Within the evaluating period, the Department of Molecular Cytology and Cytometry was able to publish over 20 research articles in peer-reviewed journals. Here, we provide 12 papers with the first and corresponding author exclusively publishing for the Institute of Biophysics (IBP), and one paper arising from prestigious international collaboration. *The first author with exclusive IBP affiliation; #corresponding author with only IBP affiliation.

1. Bartova E*#, Legartova S, Dundr M, Suchankova J. 2019. A role of the 53BP1 protein in genome protection: structural and functional characteristics of 53BP1-dependent DNA repair. Aging-US (Albany NY) 11: 2488-2511. doi: 10.18632/aging.101917.
A function of the 53BP1 protein is linked to a specific histone signature, including phosphorylation of histone H2AX (γH2AX) or methylation of histone H4 at the lysine 20 position (H4K20me); therefore, we discuss an epigenetic landscape of 53BP1-positive DNA lesions.
The contribution of the department is 90 %. The first author (Bartova E.) and corresponding author (Bartova E.) has an exclusive Institute of Biophysics of the Czech Academy of Sciences affiliation. Bartova E. wrote the manuscript.
IF = 4.83, Q2 in Cell Biology
2. Svobodova Kovarikova A*, Legartova S, Krejci J, Bartova E#. 2018. H3K9me3 and H4K20me3 represent the epigenetic landscape for 53BP1 binding to DNA lesions. Aging-US (Albany NY) 10: 2585-2605. doi: 10.18632/aging.101572.20.
The H3K9me3 and H4K20me3 represent epigenetic markers that are important for the function of the 53BP1 protein in non-homologous end joining (NHEJ) repair. The very late S phase represents the cell cycle breakpoint when a DDR function of the H4K20me3-53BP1 complex is abrogated due to the recruitment of the PCNA protein and other DNA repair factors of homologous recombination to DNA lesions.
The contribution of the department is 100 %. The first author (Svobodova Kovarikova A.) and corresponding author (Bartova E.) have an exclusive Institute of Biophysics of the Czech Academy of Sciences affiliation. Bartova E. designed the experiments and wrote the manuscript.
IF = 4.83, Q2 in Cell Biology

3. Legartova S*, Lochmanova G, Zdrahal Z, Kozubek S, Sponer J, Krepl M, Pokorna P, Bartova E#. 2019. DNA Damage Changes Distribution Pattern and Levels of HP1 Protein Isoforms in the Nucleolus and Increases Phosphorylation of HP1beta-Ser88. Cells 8. doi: 10.3390/cells8091097.
Our data show that DNA damage changed the morphology, levels, and interaction properties of HP1 isoforms. Also, γ-irradiation-induced hyperphosphorylation of the HP1β protein; thus, HP1β-S88ph could be considered as an important marker of DNA damage.
The contribution of the department is 75 %. The first author (Legartova S.) and corresponding author (Bartova E.) have an exclusive Institute of Biophysics of the Czech Academy of Sciences affiliation. Bartova E. designed the experiments and wrote the manuscript.
IF =4.37, Q2 in Cell Biology

4. Bartova E*#, Lochmanova G, Legartova S, Suchankova J, Fedr R, Krejci J, Zdrahal Z. 2018. Irradiation by gamma-rays reduces the level of H3S10 phosphorylation and weakens the G2 phase-dependent interaction between H3S10 phosphorylation and gammaH2AX. Biochimie 154: 86-98. doi: 10.1016/j.biochi.2018.07.029.

Together, our data show that even though H3S10ph is not directly involved in DNA repair, a decrease in H3S10 phosphorylation and weakened interaction between H3S10ph and γH2AX is a result of radiation-induced damage of the genome. In this case, γ-irradiation

decreased the number of cells in the G1 phase, characterized by no interaction between H3S10ph and γH2AX.

The contribution of the department is 85 %. The first author (Bartova E.) and corresponding author (Bartova E.) has an exclusive Institute of Biophysics of the Czech Academy of Sciences affiliation. Bartova E. designed the experiments and wrote the manuscript.
IF = 3.36, Q2 in Biochemistry and Molecular biology

5. Arcidiacono OA*, Krejci J, Suchankova J, Bartova E#. 2018. Deacetylation of Histone H4 Accompanying Cardiomyogenesis is Weakened in HDAC1-Depleted ES Cells. *Int J Mol Sci* 19. doi: 10.3390/ijms19082425.
The most important finding was differentiation-specific H4 deacetylation observed during cardiomyocyte differentiation of wt mESCs, while H4 deacetylation was weakened in HDAC1-depleted cells induced to the cardiac pathway. Additionally, explanted embryonic hearts (e15) responded to treatment with HDACi. This observation shows that explanted tissue can be maintained in a hyperacetylation state several hours after excision, which appears to be useful information from the view of transplantation strategy.

The contribution of the department is 100 %. The first author (Arcidiacono OA.) and corresponding author (Bartova E.) have an exclusive Institute of Biophysics of the Czech Academy of Sciences affiliation. Bartova E. designed the experiments and wrote the manuscript.
IF = 4.18, Q1 in Biochemistry and Molecular biology

6. Harikumar A, Edupuganti RR, Sorek M, Azad GK, Markoulaki S, Sehnalova P, Legartova S, Bartova E, Farkash-Amar S, Jaenisch R, et al. 2017. An Endogenously Tagged Fluorescent Fusion Protein Library in Mouse Embryonic Stem Cells. Stem Cell Rep 9: 1304-1314. doi: 10.1016/j.stemcr.2017.08.022.
The established library can be used in a variety of different directions, either exploiting the fluorescent tag for imaging-based techniques or utilizing the fluorescent fusion protein for biochemical pull-down assays, including immunoprecipitation, co-immunoprecipitation, chromatin immunoprecipitation, and more.
The article was done within an international collaboration, and the Department contribution is 25 %. We have done DNA repair experiments and provided data.
IF = 6.53, Q1 in Cell Biology

7. Suchankova J*, Legartova S, Ruckova E, Vojtesek B, Kozubek S, Bartova E#. 2017. Mutations in the TP53 gene affected recruitment of 53BP1 protein to DNA lesions, but the level of 53BP1 was stable after gamma-irradiation that depleted MDC1 protein in specific TP53 mutants. Histochem Cell Biol 148: 239-255. doi: 10.1007/s00418-017-1567-3.
Together, the kinetics of 53BP1 accumulation at UV-induced DNA lesions is different in various TP53 mutant cells. After γ-irradiation, despite changes in a number and a volume of 53BP1-positive foci, levels of 53BP1 protein were relatively stable. Here, we showed a link between the status of the MDC1 protein and the TP53 gene, in which specific mutations caused radiation-induced MDC1 down-regulation. This observation is significant, especially concerning radiotherapy of tumors with the abrogated function of the TP53 gene.
The contribution of the department is 85 %. The first author (Suchankova J.) and corresponding author (Bartova E.) have an exclusive Institute of Biophysics of the Czech Academy of Sciences affiliation. Bartova E. designed the experiments and wrote the manuscript.
IF = 2.16, Q1 in Microscopy

8. Vecera J, Bartova E*#, Krejci J, Legartova S, Komurkova D, Ruda-Kucerova J, Stark T, Drazanova E, Kasparek T, Sulcova A, et al. 2018. HDAC1 and HDAC3 underlie dynamic

H3K9 acetylation during embryonic neurogenesis and in schizophrenia-like animals. J Cell Physiol 233: 530-548. doi: 10.1002/jcp.25914.
Together, the results indicate that the co-regulation of H3K9ac by HDAC1 and HDAC3 is essential to both embryonic brain development and neuro-differentiation, as well as the pathophysiology of a schizophrenia-like phenotype.
The contribution of the department is 75 %. Vecera J. and Bartova E. are the Co-first authors. The corresponding author (Bartova E.) has an exclusive Institute of Biophysics of the Czech Academy of Sciences affiliation. Bartova E. designed the experiments and wrote the manuscript.
IF=4.52, Q1 in Physiology

9. Krejci J*, Legartova S, Bartova E#. 2017. Neural Differentiation in HDAC1-Depleted Cells Is Accompanied by Coilin Downregulation and the Accumulation of Cajal Bodies in Nucleoli. Stem Cells Int 2017: 1021240. doi: 10.1155/2017/1021240.

In summary, we observed that neural differentiation and HDAC1 deficiency lead to coilin depletion and coilin accumulation in body-like structures inside the nucleoli.
The contribution of the department is 100 %. The first author (Krejci J.) and corresponding author (Bartova E.) have an exclusive Institute of Biophysics of the Czech Academy of Sciences affiliation. Bartova E. designed the experiments and wrote the manuscript.
IF = 3.98, Q2 in Cell and Tissue engineering

10. Franek M*, Suchankova J, Sehnalova P, Krejci J, Legartova S, Kozubek S, Vecera J, Sorokin DV, Bartova E#. 2016. Advanced Image Acquisition and Analytical Techniques for Studies of Living Cells and Tissue Sections. *Microsc Microanal* 22: 326-341. doi: 10.1017/S1431927616000052.
Cornerstone methods for the biophysical research of living cells, such as fluorescence recovery after photobleaching and fluorescence resonance energy transfer, are discussed, as are studies on the effects of radiation at the individual cellular level.
The contribution of the department is 85 %. The first author (Franek M.) and corresponding author (Bartova E.) have an exclusive Institute of Biophysics of the Czech Academy of Sciences affiliation. Bartova E. wrote the manuscript.
IF=2.67, Q1 in Microscopy

11. Bartova E*#, Vecera J, Krejci J, Legartova S, Pachernik J, Kozubek S. 2016. The level and distribution pattern of HP1beta in the embryonic brain correspond to those of H3K9me1/me2 but not of H3K9me3. Histochem Cell Biol 145: 447-461. doi: 10.1007/s00418-015-1402-7.
We studied the histone signature of embryonic and adult brains to strengthen existing evidence of the importance of the histone code in mouse brain development. The results revealed differences in the epigenome of the embryonic and adult mouse brain and showed that specific epigenetic marks colonize the adult hippocampus, the granular layer of the adult olfactory bulb, and the ventricular ependyma of the embryonic brain.
The contribution of the department is 90 %. The first author (Bartova E.) and corresponding author (Bartova E.) has an exclusive Institute of Biophysics of the Czech Academy of Sciences affiliation. Bartova E. designed the experiments and wrote the manuscript.
IF = 3.45, Q2 in Microscopy

12. Suchankova J*, Kozubek S, Legartova S, Sehnalova P, Kuntziger T, Bartova E#. 2015. Distinct kinetics of DNA repair protein accumulation at DNA lesions and cell cycle-dependent formation of gammaH2AX- and NBS1-positive repair foci. Biol Cell 107: 440-454. doi:10.1111/boc.201500050.
We showed that the kinetics of the accumulation of selected DNA repair-related proteins is protein-specific at locally induced DNA lesions and that the formation of γH2AX- and NBS1-positive foci, but not 53BP1-positive NBs, is cell cycle-dependent in HeLa cells. Moreover,

γH2AX is the most striking protein present not only at DNA lesions but also spreading out in their vicinity. Our conclusions highlight the significant role of the spatiotemporal dynamics of DNA repair-related proteins and their specific assembly/disassembly at DNA lesions, which can be cell type- and cell cycle-dependent.
The contribution of the department is 90 %. The first author (Suchankova J.) and corresponding author (Bartova E.) have an exclusive Institute of Biophysics of the Czech Academy of Sciences affiliation. Bartova E. designed the experiments and wrote the manuscript. The publication was done within a Czech-Norwegian Research Programme CZ09 (7F14369). The project partners were the Institute of Basic Medical Sciences, University of Oslo, and the Norwegian Center for Stem Cell Research.
IF = 2.55, Q2 in Cell Biology

The most significant results are the following:

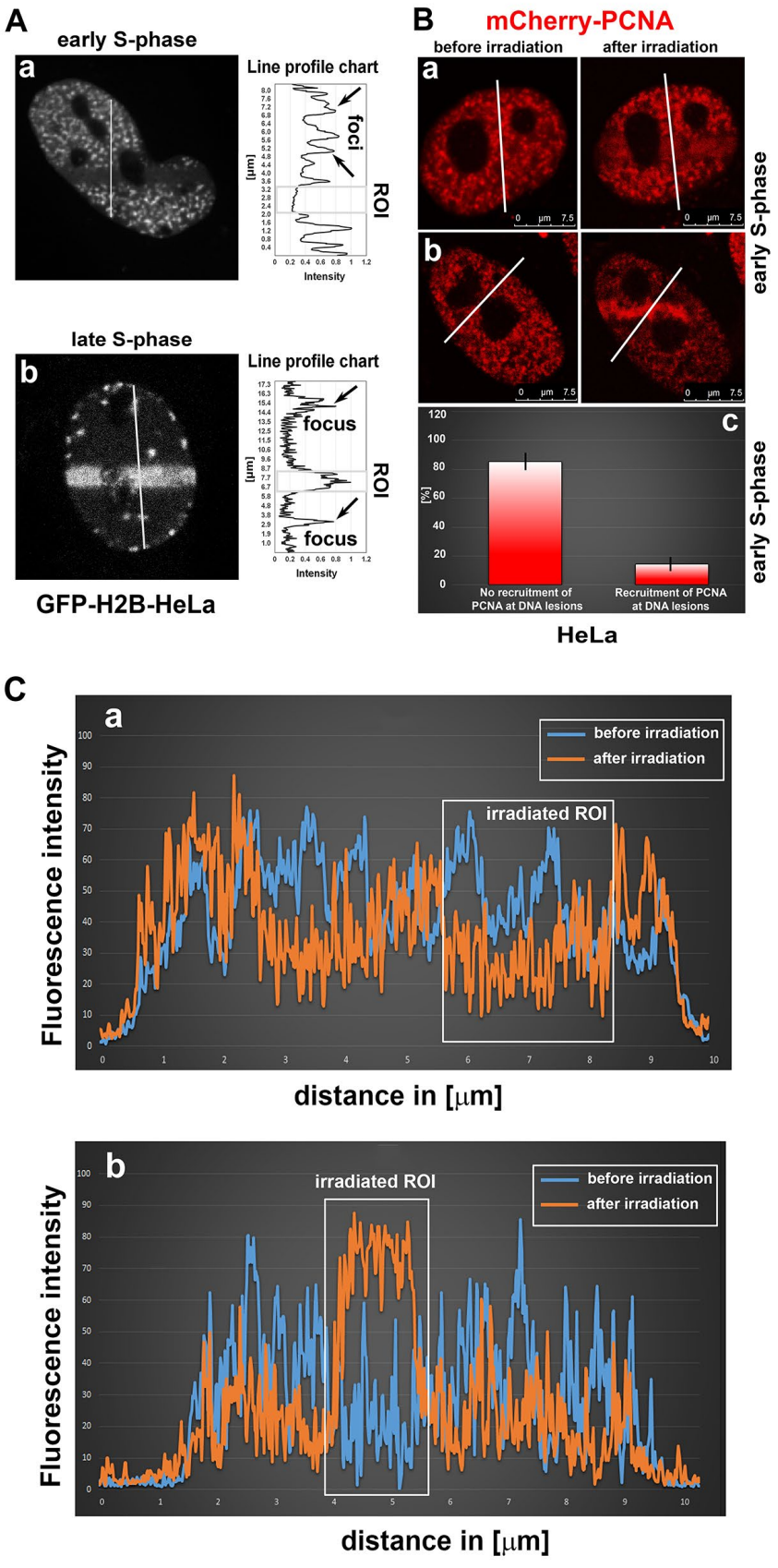
Result 1. The function of PCNA during DNA repair

DNA repair is a complex process that prevents genomic instability. Many proteins play fundamental roles in regulating the optimal repair of DNA lesions. Proliferating cell nuclear antigen (PCNA) is a key factor that initiates recombination-associated DNA synthesis after injury. Here, in the very early S-phase, we show that the fluorescence intensity of mCherry-tagged PCNA after local micro-irradiation was less than the fluorescence intensity of non-irradiated mCherry-PCNA-positive replication foci. However, PCNA protein accumulated at locally irradiated chromatin in the very late S-phase of the cell cycle, and this effect was more pronounced in the following G2 phase. In comparison to the dispersed form of PCNA, a reduced mobile fraction appeared in PCNA-positive replication foci during S-phase, and we observed a similar recovery time after photobleaching at locally induced DNA lesions. This diffusion of mCherry-PCNA in micro-irradiated regions was not affected by cell cycle phases. We also studied the link between the function of PCNA and A-type lamins in the late S-phase. We found that the accumulation of PCNA at micro-irradiated chromatin is identical in wild-type and A-type lamin-deficient cells. Only micro-irradiation of the nuclear interior, and thus the irradiation of internal A-type lamins, caused the fluorescence intensity of mCherry-tagged PCNA to increase. In summary, we showed that PCNA begins to play a role in DNA repair in the late S-phase and that PCNA function in repair is maintained during the G2 phase of the cell cycle. However, PCNA mobility is reduced after local micro-irradiation regardless of the cell cycle phase.

The mCherry-tagged PCNA protein shows pronounced accumulation at DNA lesions in the late S and G2 phases of the cell cycle, but mCherry-PCNA-positive replication foci are not recovered after local UVA micro-irradiation in the early S-phase.
In this study, we determined the distribution pattern of mCherry-tagged PCNA in locally induced DNA lesions during S-phase (Supplementary Fig. 1a-c and Fig. 1Aa, b). The phases of S-phase (S1-S4) were determined according to Smirnov et al. (2011; 2014; 2016) and the description available at http://www.cardoso-lab.org/pages/research.htm. We observed pronounced accumulation of mCherry-PCNA at DNA lesions in non-S-phase and late S-phase cells (Supplementary Fig. 1a, c and Fig. 1Ab). However, in the S1 phase of S-phase, which is characterized by the accumulation of several PCNA foci, these foci were not recovered after UVA-irradiation, and the level of PCNA at locally irradiated chromatin achieved only the level of PCNA protein in the nucleoplasm (Supplementary Fig. 1b and Fig. 1Aa, see a line graph). We observed similar nuclear distribution patterns after local micro-irradiation in both HeLa cells stably expressing GFP-H2B (Supplementary Fig. 1a-c) and wt mouse embryonic fibroblasts (Fig. 1Aa and Ab). In the S1 phase of S-phase, mCherry-PCNA failed to accumulate at lesions 0-30 min after micro-irradiation, and a very low level of PCNA

protein was observed at DNA lesions in approximately 85% of S1 cells (Fig. 1Aa, Ba, Bc, Ca). However, we observed pronounced recruitment of mCherry-PCNA to DNA lesions in approximately 15% of the S1 cells (early S-phase). In this case, PCNA accumulated at DNA lesions at the expense of the surrounding genome. This was evidenced by a reduced level of mCherry-PCNA outside the micro-irradiated region of interest (ROI) (Fig. 1Bb; see quantification in Fig. 2Bc, Cb).

Figure 1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No recruitment of PCNA at DNA lesions
Recruitment of PCNA at DNA lesions
early S-phase
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irradiated ROI
before irradiation
after irradiation
Fluorescence intensity
distance in [µm]

Result 2. Localized movement of nucleoli after gamma irradiation.

Sorokin et al., Nucleus (2015): We studied the appearance of irradiation-induced foci in cells after exposure to γ-radiation or UV light. We analyzed DNA repair foci inside and around nucleoli because nucleoli represent an important stress sensor of the nucleus. We also inhibited the transcription of ribosomal genes by treating the cells with actinomycin D (ACT-D), which is also considered a DNA damaging agent. Also, we confirmed that ACT-D treatment and selected radiation treatments induce not only DNA damage but also subsequent apoptotic events. Thus, we characterized the morphology of apoptotic cells by image analysis methods designed for actual experimental data (Fig. 2A-C). Then, we used irradiation conditions that did not induce apoptosis to continue our studies on the localized movement and morphology of GFP-UBF1-positive nucleolar regions in G1 and G2 cell cycle phases.

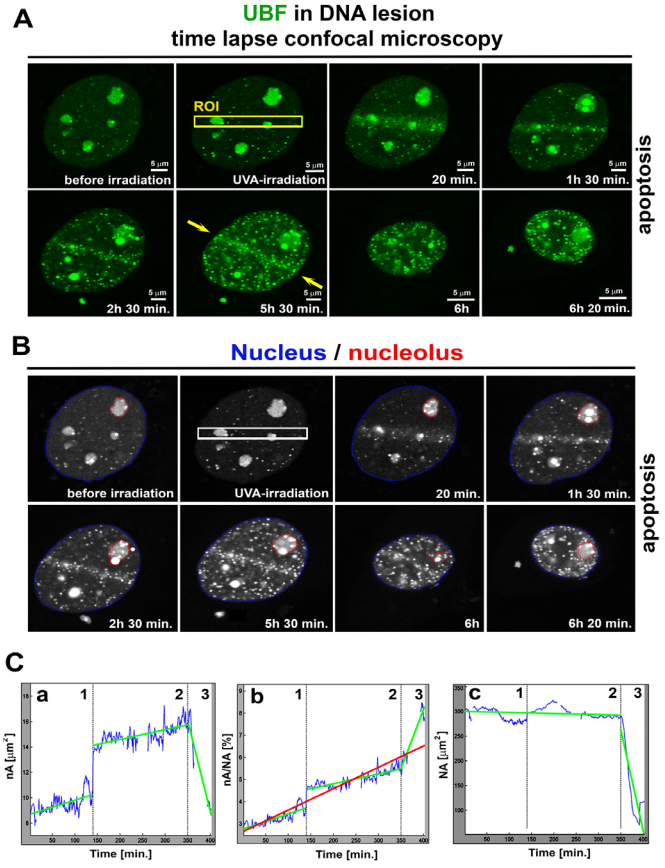


***Figure 2**. Time-lapse microscopy of apoptotic UVA-irradiated cells. (**A**) Monitoring of MEFs exposed to local micro-irradiation by UVA laser (355 nm wavelength and BrdU pre-sensitization) (~6 h of observation). The UVA-irradiated region is indicated by the yellow frame (ROI), and yellow arrows indicate the maintenance of accumulated GFP-tagged UBF1 protein at UVA-irradiated ROI during the observation time. Scale bars are 5 μm. (**B**) Contours of the cell nucleus (blue) and selected nucleolus (red) are shown for images from panel C. (**C**) Cell morphology parameters: (a) changes in nA (blue curve), (b) nA/NA, and (c) NA over time. The interruptions in blue curves correspond to unreliable portions of the image sequence where the cell was out of focus or out of the imaging plane. The data were estimated by linear regression analysis for three separate cellular stages [(1) adherent state, (2) cell during apoptosis-related detachment, and (3) terminal apoptotic shrinkage]. Green lines represent the regression line for individual stages 1-3, and the red line in panel b is the regression line for the entire cellular event when calculating nA/NA.*

Next, we showed that genome injury induced by radiation or by a DNA damaging agent changed many morphological parameters of nucleoli, including nucleoli area and a number of UBF1-positive foci (Sorokin et al., 2015). However, local nucleoli motions in G1 and G2 non-irradiated and γ-irradiated cells were nearly identical, but with pronounced variability in the G2 phase. Irradiation by γ-rays additionally induced re-location of the UBF-positive region of nucleoli (Fig. 3, white arrows).

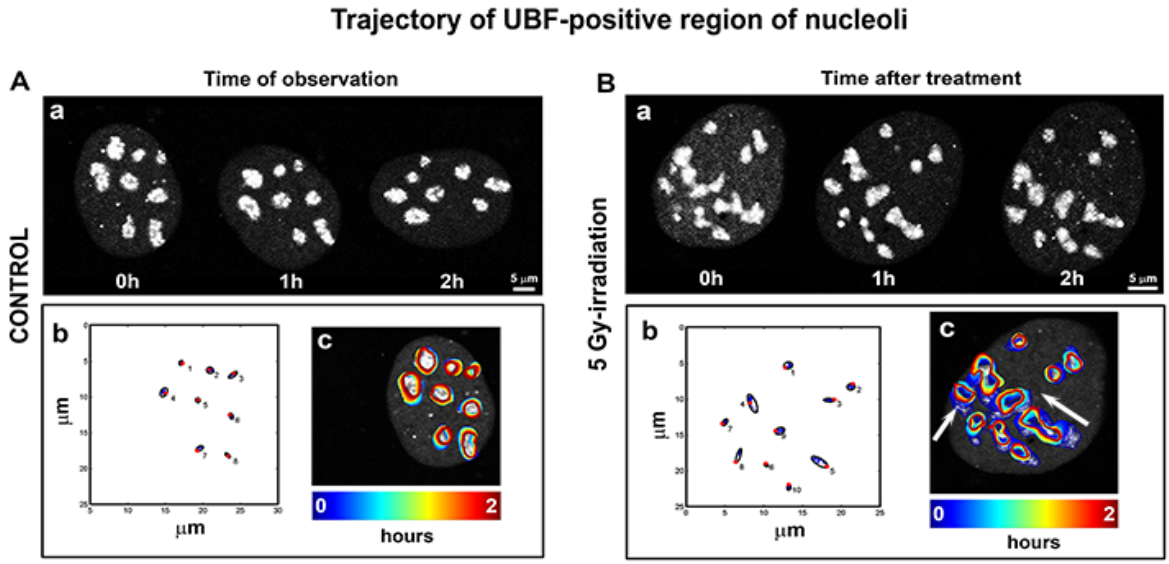


Figure 3. *Single-particle tracking analysis shows the localized movement of the GFP-UBF1-positive nucleolar compartment in iMEFs. Tracking of individual nucleoli (panels) was visualized as the trajectories of the centroids of UBF1-positive regions of nucleoli and their minimal enclosing ellipses (b panels). We constructed the evolution of contours (panel c) of UBF1-positive regions of nucleoli with time overlaid over the first frame. Blue contours correspond to the start of scanning, and red contours correspond to the end of scanning (panels labeled as for c). The analysis was performed for (**A**) control non-irradiated and untreated cells, (**B**) γ-irradiated cells treated with 5 Gy of γ-irradiation (white arrows show changes in the contour overlays over time. GFP-UBF1-positive regions were monitored every 15 s for 2 h. Scale bars are 5 µm. Five nuclei for each event (control and each treatment) were analyzed.*

Morphological changes observed in this paper, likely reflecting rearrangement of ribosomal genes, preferentially appeared in the G2 phase of the cell cycle (Sorokin et al., 2015). These conclusions imply that DNA lesions in ribosomal genes activate specific cell-cycle dependent DNA repair mechanisms. Disorder in these mechanisms can contribute to uncontrolled cell proliferation and pathophysiological processes. Our studies also show that damage and repair of ribosomal genes can be followed by changes in the morphology of nucleoli in a cell cycle-specific manner. Our data suggest the G2 phase as a key cell cycle stage for the repair of ribosomal genes and imply that the nucleolus is less stable in the G2 phase, thus most sensitive to ionizing radiation.

Result 3. Distinct kinetics of protein accumulation at DNA lesions.
Suchankova et al. Biology of the Cell (2015). We have studied the nuclear patterns of γH2AX, 53BP1, BMI1, NBS1, MDC1, BRCA1, PCNA, coilin, and cyclobutane pyrimidine dimers (CPDs) in spontaneous and γ-radiation-induced foci. In addition, we have analyzed how these proteins are distributed within UVA laser-irradiated regions of interest (ROIs), and we studied protein accumulation kinetics at DNA lesions by time-lapse confocal microscopy and fluorescence recovery after photobleaching (FRAP). Our aim was to show differences in the protein kinetics since spatiotemporal dynamics of proteins involved in DNA damage response seem to be very important in the general context of orchestrated nuclear events underlying optimal DNA repair and thus genome stability.

We observed striking differences in the kinetics of protein accumulation at DNA lesions (Fig. 4).

As an example, we show that the maximum fluorescence intensity for mCherry-tagged PCNA in DNA lesions is 8 minutes after local micro-irradiation. For mCherry-tagged 53BP1, it was 15-23 minutes after local micro-irradiation by UVA laser (Fig. 4A, B).

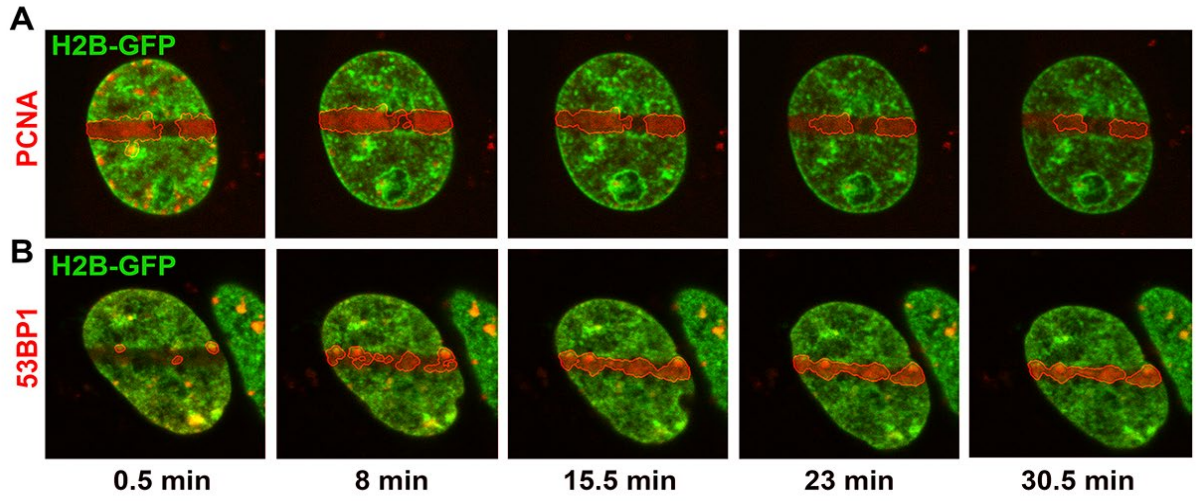
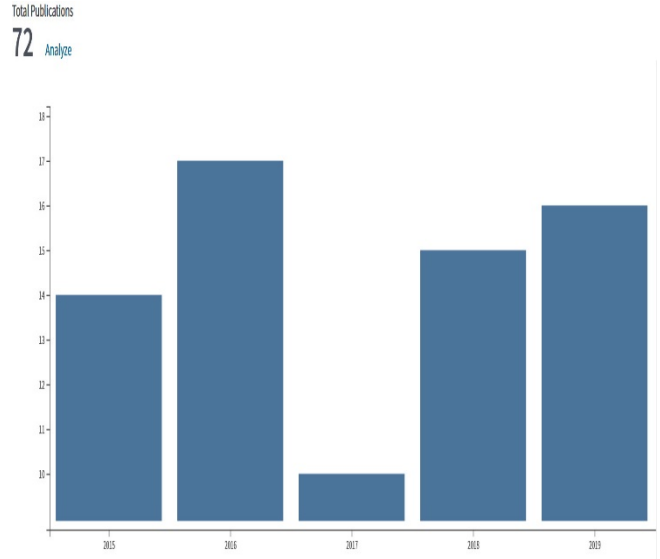


Figure 4. *The plot demonstrates the evolution of accumulated (**A**) RFP-tagged PCNA (red) and (**B**) mCherry-tagged 53BP1 (red) proteins around a UVA-irradiated region (stripe). The contour line shows the irradiated area. An example of this software analysis is documented in HeLa cells stably expressing GFP-tagged histone H2B (green). The observations were performed 0-35 min after UVA irradiation of a defined region of interest. Selected images are shown.*

Research activity and characterisation of the main scientific results

Research at the Department has been focused on **interactions between DNA and** compounds containing **platinum, ruthenium, osmium, iridium, and other metals,** which had a significant antineoplastic effect deriving from their interaction with DNA. The scope of research has also been extended to encompass processes of **DNA damage recognition and repair**.

Publication activity:
The methods we had available in the last period enabled us to obtain **a more complex view of the mechanism of action** of new metallodrugs based on a **combination of** information obtained on **molecular and cellular levels**.

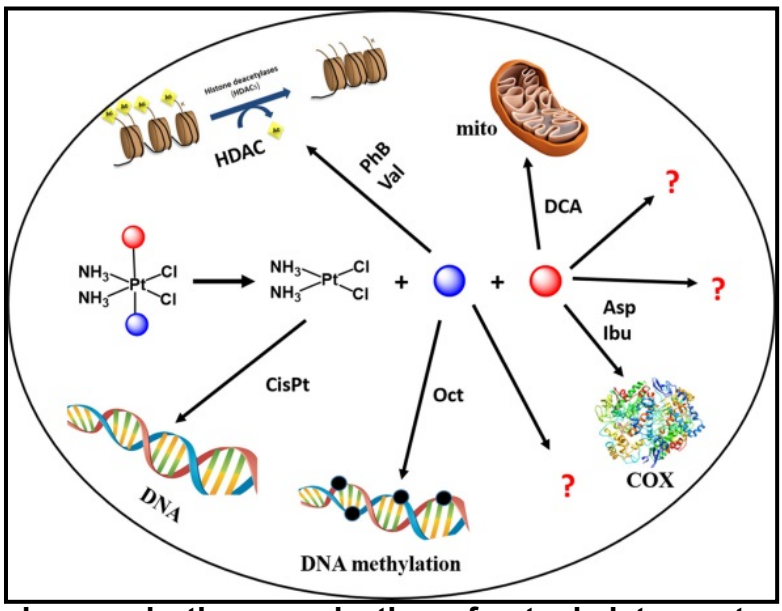


With the aid of these methods, the members of the Department published from 2015 to 2019 more than 70 full-length papers in renowned international journals (**mean IF** (2019) **= 5.340, the highest IF was 13.476**). **Contribution** to these studies by the authors **from the Department was dominant** (mostly the first and corresponding authors from the Department).
WoS: Total full-length publications published during the last 5 years (2015-2019): **72**

The main scientific results achieved by the team during 2015-2019

*The first author with exclusive IBP affiliation; #corresponding author with IBP affiliation; **IF = IF$_{2019}$**, D = journal decile, Q = journal quartile, TS = team's share

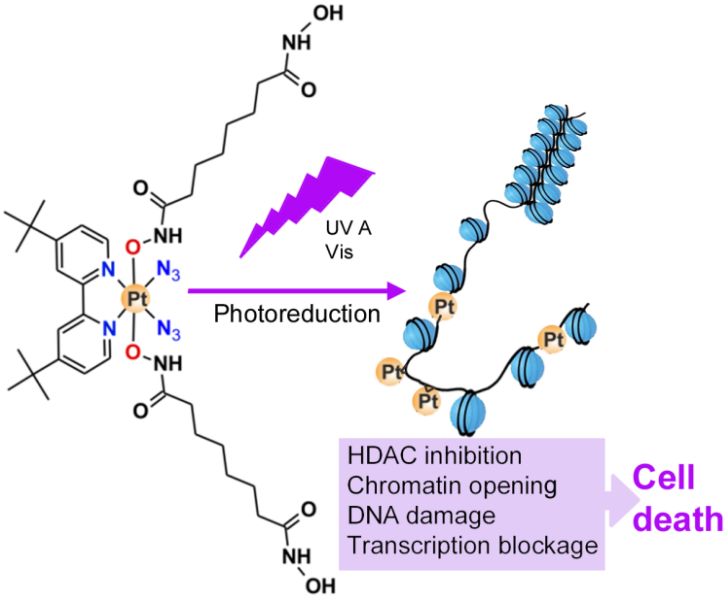
DISCOVERY OF NEW PLATINUM ANTITUMOR AGENTS THAT KILL CELLS BY A MULTIMODAL MECHANISM OF ACTION



We show that the **Pt(IV) derivatives of cisplatin, oxaliplatin, and other anticancer Pt(II) complexes with various biologically active axial ligands act by the multimodal mechanism of action (MoA)**. This MoA results in the global biological effects, that is, the Pt(IV) derivatives **damage nuclear DNA, reduce the mitochondrial membrane potential, induce the epigenetic processes**, and last but not least, the data provide evidence that **changes in the organization of cytoskeleton networks** are functionally important for some Pt(IV) derivatives, in contrast to clinically used platinum cytostatics, to kill cancer cells.

Kostrhunova, H.*; Zajac, J.; Novohradsky, V.; Kasparkova, J.; Malina, J.; Aldrich-Wright, J.
R.; Petruzzella, E.; Sirota, R.; Gibson, D.; Brabec, V.# A subset of new platinum antitumor agents kills cells by a multimodal mechanism of action also involving changes in the organization of the microtubule cytoskeleton. *J. Med. Chem.* 2019, 62, 5176-5190. **IF= 6.205**, D1, TS = 85%.
Kostrhunova, H.*; Petruzzella, E.; Gibson, D.; Kasparkova, J.; Brabec, V.# A new anticancer Pt(IV) prodrug that acts by mechanisms involving DNA damage and different epigenetic effects. *Chem. Eur. J.* 2019, 25, 5235 – 5245. **IF=4.857**, Q1, TS = 85%.
Novohradsky, V.*; Zanellato, I.; Marzano, C.; Pracharova, J.; Kasparkova, J.; Gibson, D.; Gandin, V.; Osella, D.; Brabec, V.# Epigenetic and antitumor effects of platinum(IV)-octanoato conjugates. *Sci. Rep.* 2017, 7, 3751. **IF=3.998**, Q1, TS = 65 %.
Novohradsky, V.*; Zerzankova, L.; Stepankova, J.; Vrana, O.; Raveendran, R.; Gibson, D.; Kasparkova, J.; Brabec, V.# New insights into the molecular and epigenetic effects of antitumor Pt(IV)-valproic acid conjugates in human ovarian cancer cells. *Biochem. Pharmacol.* 2015, *95*, 133-144. **IF=4.960**, D1, TS = 90%.

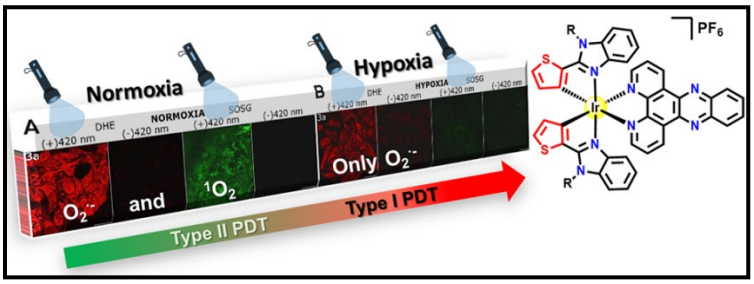
A PHOTOACTIVATABLE PLATINUM(IV) COMPLEX TARGETING GENOMIC DNA AND HISTONE DEACETYLASES



We reported the toxic effects of **photoactivatable platinum(IV) complex** conjugated with **suberoyl-bis-hydroxamic acid** in tumor cells. The conjugate exerts, after photoactivation, the two functions: activity of both **platinum(II) anticancer drug and histone deacetylase (HDAC) inhibition** in cancer cells. The novelty of this approach resides in the use of a Pt(IV) pro-drug, acting by two independent mechanisms of biological action in a cooperative manner, which **can be selectively photoactivated to cytotoxic species in and around a tumor,** thereby increasing selectivity towards cancer cells. The results suggest that this new strategy is a valuable route to design new platinum agents with higher efficacy for photodynamic anticancer chemotherapy. *Recent advances in laser and fiber-optic technologies make it possible to irradiate also internal organs with the light of highly defined intensity and wavelength.*

Kasparkova, J.*,#; Kostrhunova, H.; Novakova, O.; Křikavová, R.; Vančo, J.; Trávníček, Z.; Brabec, V. A photoactivatable platinum(IV) complex targeting genomic DNA and histone deacetylases. *Angew. Chem. Int. Ed.* 2015, 54, 14478-14482**.** **IF=12.959**, D1, TS = 75%.

THE ANTICANCER ACTIVITY OF THE NEW PHOSPHORESCENT IRIDIUM(III) COMPLEXES SUITABLE FOR PHOTODYNAMIC THERAPY ACTING AS PROTEOSYNTHESIS INHIBITORS

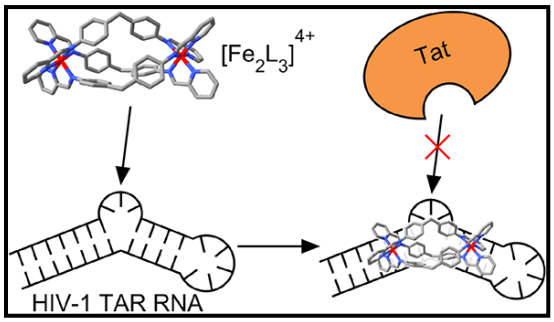


The **phototoxicity in cancer cells of three series of octahedral Ir(III) complexes** of general formula $[Ir(C^N)_2(N^N)][PF_6]$, where the N^N ligand is dipyrido[3,2-*a*:2',3'-*c*]phenazine (*dppz*) and the C^N ligands are deprotonated 2-phenyl-, 2-(naphthalen-2-yl)- or 2-(thiophen-2-yl)- benzimidazole derivatives (**series 1, 2** and **3**, respectively) with different substituents (H, methyl or 4-(trifluoromethyl)benzyl) on the imidazole units, has been studied. The compounds were found to be photoactive in model human cervical cancer HeLa cells (IC_{50} about 20 nM under irradiation conditions, λ_{exc} = 420 nm), inducing a substantial formation of apoptotic bodies as shown by flow cytometry. Some of these compounds showed **high phototoxic indexes**. Notably, the antiproliferative activity of the photoactivated Ir(III) compounds was also significant under **hypoxic conditions** (2 % O_2). Further

investigations on series **3** compounds have shown that molecular superoxide radical ($O_2^{-\bullet}$) generation is the main responsible for the oxidative stress induced by irradiation of the cells treated with the 2-(thiophen-2-yl)benzimidazole derivatives **3a-c**. The photopotentiation of compounds of series **3 also involves the formation of singlet oxygen ($^1O_2$)**. The results also showed that in tumor HeLa cells, the **generation of superoxide radicals** competed with singlet oxygen production in cells in normoxia but became dominant under hypoxia.

Novohradsky, V.*; Vigueras, G.; Pracharova, J.; Cutillas, N.; Janiak, C.; Kostrhunova, H.; Brabec, V.; Ruiz, J.; Kasparkova, J.# Molecular superoxide radical photogeneration in cancer cells by dipyridophenazine iridium(III) complexes. *Inorg. Chem. Front.* 2019, 6, 2500-2513. **IF=5.973**, D1, TS = 70%.

IRON(II) SUPRAMOLECULAR HELICATES INTERFERE WITH THE HIV-1 Tat−TAR RNA INTERACTION CRITICAL FOR VIRAL REPLICATION

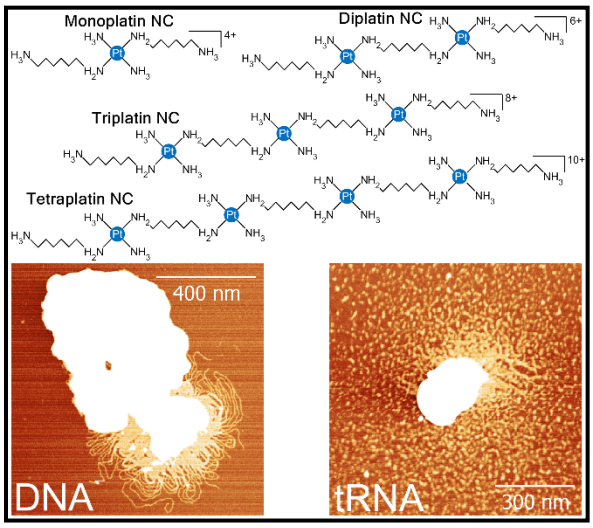


The binding of **the viral trans-activator protein (Tat) to the TAR RNA** is an essential step in the HIV-1 replication cycle. Therefore, **the blockage of the Tat-TAR interaction is a potential route for AIDS chemotherapy**. Compounds that bind to TAR RNA, and prevent binding by Tat, could disrupt processive transcription and thereby inhibit viral growth.

The interaction between the HIV-1 transactivator protein Tat and TAR (transactivation responsive region) RNA plays a critical role in HIV-1 transcription. **Iron(II) supramolecular helicates were evaluated for their *in vitro* activity to inhibit Tat–TAR RNA interaction** using UV melting studies, electrophoretic mobility shift assay, and RNase A footprinting. The results demonstrate that **iron(II) supramolecular helicates inhibit Tat-TAR interaction at nanomolar concentrations by binding to TAR RNA with high affinity**. Thus, **iron(II) supramolecular helicates inhibit the HIV-1 Tat-TAR interaction at notably lower concentrations than many inhibitors of Tat-TAR binding so far tested**. These studies provide new insight into the biological potential of metallosupramolecular helicates.

Malina, J.*; Hannon, M. J.; Brabec, V.# Iron(II) supramolecular helicates interfere with the HIV-1 Tat–TAR RNA interaction critical for viral replication. *Sci. Rep.* 2016, 6, 29674; doi: 10.1038/srep29674. **IF=3.998**, Q1, TS = 95%.

SUBSTITUTION-INERT POLYNUCLEAR PLATINUM COMPLEXES ACT AS VERY POTENT INDUCERS OF CONDENSATION/AGGREGATION OF DNA AND RNA AND INHIBITORS OF ACTIVITY OF DNA POLYMERASE IN TRIPLEX-FORMING TEMPLATES. POTENTIAL IN GENE THERAPY, BIOTECHNOLOGY, AND BIONANOTECHNOLOGY



The **substitution-inert polynuclear platinum complexes (SI-PPCs) represent a unique group of platinum-based anticancer agents** that exhibit a high affinity towards nucleic acids. We investigated the effects of SI-PPCs containing dangling amine groups in place of NH_3 as ligands to increase the length of the molecule and, therefore, overall charge and its distribution. The results obtained with the aid of **biophysical techniques, such as total intensity light scattering, gel electrophoresis, and atomic force microscopy,** show that the addition of dangling amine groups considerably **augments the ability of SI-PPCs to condense/aggregate nucleic acids**. Moreover, this **enhanced capability of SI-PPCs correlates with their heightened efficiency to inhibit DNA-related enzymatic activities**, such as those connected with DNA transcription, catalysis of DNA relaxation by DNA topoisomerase I, and DNA synthesis catalyzed by Taq DNA polymerase. Thus, the structures of SI-PPCs, which differ so markedly from the derivatives of cisplatin used in the clinic, appear to contribute to the overall biological activity of these molecules.

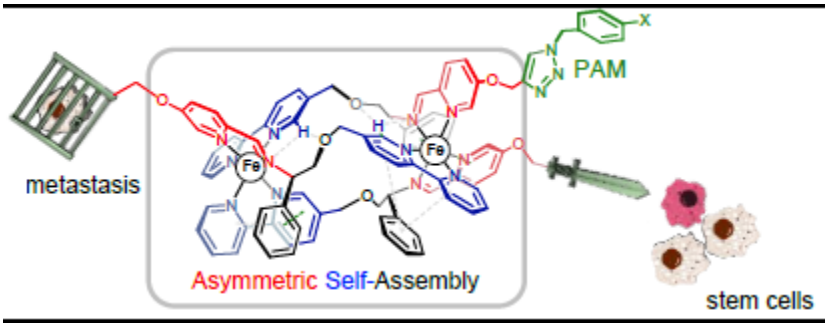
Additionally, we have found that **SI-PPCs are very efficient stabilizers of triple-stranded DNA** that might exert their action by stabilizing higher-order structures such as triple-helical DNA.

Malina, J.*; Farrell, N. P.; Brabec, V.# Substitution-inert polynuclear platinum complexes act as potent inducers of condensation/aggregation of short single- and double-stranded DNA and RNA oligonucleotides. *Chem. Eur. J.* 2019, 25, 2995–2999 **IF=4.857**, Q1, TS = 95%.

Malina, J.*; Čechová, K.; Farrell, N. P.; Brabec, V.# Substitution-inert polynuclear platinum complexes with dangling amines: Condensation/aggregation of nucleic acids and inhibition of DNA-related enzymatic activities. *Inorg. Chem.* 2019, 58, 6804-6810. **IF=4.825**, D1, TS = 95%.

Malina, J.*; Farrell, N. P.; Brabec, V.# Substitution-inert polynuclear platinum complexes that inhibit the activity of DNA polymerase in triplex-forming templates. *Angew. Chem. Int. Ed.* 2018, 57, 8535 –8539. **IF=12.959**, D1, TS = 95%.

DISCOVERY OF SELECTIVE, ANTI-METASTATIC AND ANTI-CANCER STEM CELL METALLOHELICES



Lehn envisaged, in his original report that helicates – self-assembling multi-metallic coordination compounds – may find uses in biochemistry. **Helicates** and related metallo-foldamers, synthesized by dynamic self-assembly, represent an area of chemical space inaccessible by traditional organic synthesis and yet with the potential for discovery of new classes of drug. We reported that water-soluble, optically pure Fe(II)- and even Zn(II)-based triplex metallohelices are an excellent platform for post-assembly click reactions. By these means, **the *in vitro* anticancer activity and, most notably, the selectivity of a triplex metallohelix Fe(II) system is dramatically improved**. For one compound, a remarkable array of mechanistic and pharmacological behaviors is discovered**: inhibition of Na^+/K^+ ATPase** with a potency comparable to the drug ouabain, **antimetastatic properties** (including inhibition of cell migration, re-adhesion, and invasion), **cancer stem cell targeting**, and finally **colonosphere inhibition** competitive with the drug salinomycin.
Additionally, the metallohelices were shown to bind to abasic (AP) sites in DNA in a shape-selective manner inhibiting the activity of human AP endonuclease 1. This enzyme is a valid anticancer drug target indicating the potential of utilizing well-defined metallohelices for cancer chemotherapy.

Song, H.; Rogers, N. J.; Allison, S. S.; Brabec, V.[#]; Bridgewater, H.; Kostrhunova, H.; Markova, L.; Phillips, R. M.; Pinder, E.; Shepherd, S.; Young, L.; Zajac, J.; Scott, P. Discovery of selective, antimetastatic and anti-cancer stem cell metallohelices via post-assembly modification. *Chem. Sci.* 2019, 10, 8547-8557. **IF=9.346**, Q1, TS = 40%.
Malina, J.[*]; Scott, P.; Brabec, V.[#] Shape-selective recognition of DNA abasic sites by metallohelices: inhibition of human AP endonuclease 1. *Nucleic Acids Res.* **2015,** *43*, 5297-5306. **IF=11.502,** D1, TS = 95%.

REVIEWS FOCUSED ON THE MECHANISM OF ACTION OF ANTITUMOR PLATINUM AND RUTHENIUM COORDINATION COMPOUNDS
In these reviews, we discuss how **various classes of platinum(II) complexes, which can interact with DNA** by coordination, intercalation, and other noncovalent modes of binding or by a combination of these DNA binding modes, can alter the cellular response induced by conventional antitumor platinum drugs. Moreover, we also discuss major **DNA binding modes** of hitherto identified **for ruthenium complexes of biological or biomedical significance.**

Brabec, V.[*,#]; Kasparkova, J. Ruthenium coordination compounds of biological and biomedical significance. DNA binding agents. *Coord. Chem. Rev.* **2018,** *376*, 75-94. **IF=15.367**, D1, TS = 100%..
Brabec, V.[*,#]; Hrabina, O.; Kasparkova, J. Cytotoxic platinum coordination compounds. DNA binding agents. *Coord. Chem. Rev.* **2017,** *351*, 2-31. **IF=15.367**, D1, TS = 100%.

In the last five years, we also introduced a number of new methods that make it possible to extend our studies on the molecular level to those on a cellular level.

Major methods:

Molecular biophysics, biochemistry, and molecular pharmacology:

1. DNA binding studies, polarography, FAAS, absorption spectrophotometry.
2. Sequence specificity of DNA binding.
    a) Binding to DNA with different G+C content and synthetic DNAs.
    b) HPLC analysis of DNA enzymatically hydrolyzed to nucleosides.
    c) Replication and transcription mapping.
3. EtBr, a fluorescent probe for cross-linking, interstrand cross-linking assays.
5. Conformational analysis of DNA modified by metal complexes.
    a) DNA melting, microcalorimetry (DSC, ITC), thermophoresis.
    b) CD, LD spectroscopy, Raman spectrophotometry, spectrofluorimetry, AFM.
    c) Supercoiled plasmid DNA unwinding by gel electrophoresis.
    d) Chemical probes of DNA conformation.
    e) DNA bending and unwinding by single, site-specific metal adducts.
6. Translesion DNA and RNA synthesis and replication bypass.
7. Recognition of DNA modified by metal complexes by specific proteins.
8. Hydroxyl radical, DNase I footprinting.
9. Nucleotide excision repair of DNA, DNA repair synthesis.

Cellular pharmacology assays:

Cytotoxicity in cell cultures (including **cancer stem cells and 3D spheroids**) and single-cell gel electrophoresis (comet) assay.
Flow cytometric analysis of the cell cycle.
Synthesis of DNA, RNA, and proteins in cells.
Apoptosis, necrosis, and autophagy induction.
Peptide mass fingerprinting, Western blotting.
Mutation-assays.
Real-time quantitative PCR.
Cellular drug uptake by FAAS and ICP-MS.
Localization studies by confocal laser microscopy.
Real-time cell electronic sensing.
Photoxicity testing.
Determination of reactive oxygen species.
Measurements of mitochondrial transmembrane potential, invasion, migration, and readhesion in tumor cells.

Research activity and characterisation of the main scientific results

The scientific activity of the department in the evaluated period was focused, in accordance with the above defined goals, especially on the study of: 1) biological effects of accelerated heavy ions with the aim of developing more effective antitumor radiotherapy and evaluate the health risks for crews of planned interplanetary space missions, 2) selective sensitization of tumor cells to current radiotherapy, and 3) radioprotection of normal (non-tumor) cells. At the same time, we attempted to 4) reveal the causes of radioresistance of head and neck tumors and 5) gain completely new insights into the mechanisms of repair of double-stranded DNA breaks (DSB) induced by various types of ionizing radiation at the nanoscale level. For this research, in the frame of intensive international cooperation (described in the corresponding chapter), we took advantage of a combination of world-unique technologies - particle accelerators and super-resolution SMLM (Single Molecule Localization Microscopy) microscopy, while further developing applications of the latter method in radiobiology. For instance, we were the first to use SMLM for visualization of DNA damage and repair after high-LET irradiation, described later (Laboratory of Cell Nucleus Damage and Repair). The main current aim of The Laboratory of Molecular DNA Complexes is the identification of components of plant telomerases. This includes not only identification of the two core telomerase subunits, telomerase RNA (TR) and telomerase reverse transcriptase (TERT) but also their interacting factors that control telomerase assembly, intracellular trafficking and activity. The main achievements in the evaluated period included characterisation of unusual telomere DNA repeats in a number of plant species, and identification of telomerase RNA subunits across the whole land plant phylogeny, including important crop plants. This opens a possibility of biotechnology and breeding applications of plant telomere biology.

Specifically, in the period 2015–2019, DCBR competed and dealt with the solution of 16 national and 16 international projects (as listed in the particular form). The results obtained during the evaluated period, examples of which are presented in more detail below, were published as **55 articles in impacted scientific journals**, several book chapters and articles in peer-reviewed Czech scientific periodicals. At the same time (see the relevant chapters of this form), the results were presented at prestigious scientific conferences (a total of > 100 contributions), including 5 plenary / keynote and 14 other invited lectures, and used for teaching students. The Czech media also showed interest in certain results (see the last chapter) and, in addition to theoretical knowledge, practically relevant results were also achieved. For instance, software for the analysis of DNA damage was developed, based on artificial neural networks and so-called deep-learning (now freely accessible to those interested in scientific, clinical and other communities), and a microchip for the analysis of the expression of approx. 350 genes participating in cellular response to irradiation. In the frame of our activity in the Society for Radiobiology and Crisis Planning of the Czech Medical Society of J. E. Purkyně, the book "Clinical Radiobiology" (GRADA) was published, which had been missing on the Czech market for a long time.

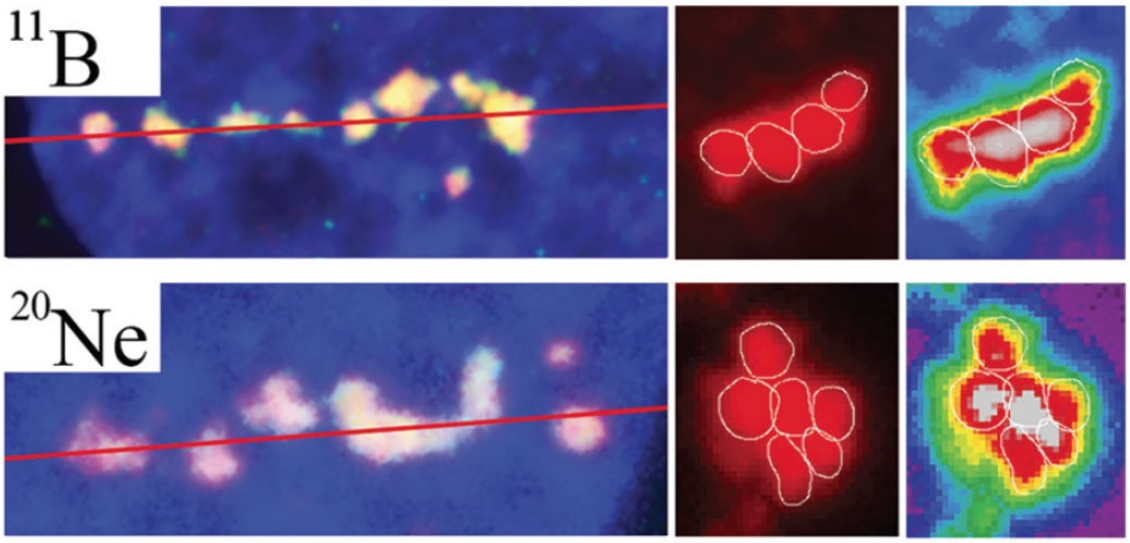
DESCRIPTION OF SELECTED RESULTS

RESULT 1. Ionizing radiations of similar high LET (linear energy transfer) cause DNA damage with different microdosimetric characteristics that influence DNA repair and cell survival (*Nanoscale*, 2018, IF 7.4, Q1 DOI: 10.1039/C7NR06829H).

Biological effects of high-LET (linear energy transfer) radiation have received increasing attention, particularly in the context of more efficient radiotherapy and space exploration. Efficient cell killing by high-LET radiation depends on the physical ability of accelerated particles to generate complex DNA damage, which is largely mediated by LET. However, the characteristics of DNA damage and repair upon exposure to different particles with similar LET parameters remain unexplored.
We employed high-resolution confocal microscopy to examine phosphorylated histone H2AX (γH2AX)/p53-binding protein 1 (53BP1) focus streaks at the microscale level, focusing on the complexity, spatiotemporal behaviour and repair of DNA double-strand breaks generated by boron and neon ions accelerated at similar LET values (∼135 keV μm^{-1}) and low energies (8 and 47 MeV per n, respectively). Cells were irradiated using sharp-angle geometry and were spatially (3D) fixed to maximize the resolution of these analyses. Both high-LET radiation types generated highly complex γH2AX/53BP1 focus clusters with a larger size, increased irregularity and slower elimination than low-LET γ-rays. Surprisingly, neon ions produced even more complex γH2AX/53BP1 focus clusters than boron ions, consistent with DSB repair kinetics. Although the exposure of cells to γ-rays and boron ions eliminated a vast majority of foci (94% and 74%, respectively) within 24 h, 45% of the foci persisted in cells irradiated with neon. Our calculations suggest that the complexity of DSB damage critically depends on (increases with) the particle track core diameter. Thus, different particles with similar LET and energy may generate different types of DNA damage, which should be considered in future research.
These results, achieved using a combination of world-class technologies (particle accelerators, advanced microscopy) and several experimental "tricks", can change certain biological paradigms and have been published in the prestigious journal *Nanoscale*. As already mentioned, the biological effects of accelerated heavy ions are today derived primarily from their LET. However, several radiation parameters are reflected in the LET value, such as energy, charge, particle size, etc. We were therefore interested in how the biological effects differ for different particles with different parameters but the LET. In our study, we focused on the formation and repair of double-stranded DNA breaks (DSBs), because they represent the most serious type of DNA damage generated by ionizing radiation as well as in general.
However, heavy ions with high LET form highly complex and clustered DSB lesions, which cannot be studied in sufficient detail under standard irradiation conditions and using conventional confocal microscopy. We have therefore proposed several experimental innovations. We adjusted the irradiation system so that it was possible to irradiate the cell monolayer at a sharp angle, moving the traces of particles visualized by DSB markers to a plane perpendicular to the optical axis of the microscope and improving the resolution by at least 1 order of magnitude. We then performed the DSB analysis in 3D space using a visual and sophisticated software approach, thus achieving the resolution required for the overall morphological analysis of DSB clusters.
The analyses subsequently revealed surprisingly significant differences in the micro-morphology and complexity of DSB clusters, their microdosimetric distribution in the cell nucleus, and the ability of cells to repair these DNA lesions. Calculations subsequently performed in cooperation with NPI CAS indicated that the cause of

different microdosimetric track structures of different ions with similar LET is different width of the so-called track-core, which is given by individual particle parameters and leads to the formation of DSBs of various complexities.
The ability of DSB repair correlated with post-irradiation cell survival, confirming that different accelerated ions with very similar LET can have significantly different biological effects. Our results are thus important both for finding the most therapeutically suitable ions for developing hadron radiotherapy (which promises a much greater antitumor effect and at the same time much greater protection of surrounding, in some cases vital tissues), optimization of radiotherapy, but also for estimating health risks to crews of planned interplanetary flights from exposure to mixed fields of ionizing radiation.
The work was carried out in extensive cooperation with JINR Dubna (Russia) and NPI CAS under the leadership of DCBR IBP CAS. Currently, the topic is further developed using super-resolution microscopic techniques in an extended cooperation with KIP Heidelberg (Germany). The contribution of DCBR IBP is dominant: JINR was responsible for cell preparation and irradiation, experiments and analyses were subsequently performed on DCBR IBP where also the manuscript was prepared.

Illustration: Accelerated ^{20}Ne ions generate more complex and less reparable DNA double strand breaks (DSB) than ^{11}B ions despite of having very similar LET (132-171 vs. 138-148 keV/μm). Left panel: nuclei of human fibroblasts stained with DAPI (blue), green (γH2AX) and red (53BP1) signals are the markers of DSB. Middle panel: microscopic image demonstrating the complexity of DSB clusters (53BP1, red). Right panel: quantification of 53BP1 fluorescence maximum peaks (corresponding to individual foci) in DSB clusters using CellProfiler software.



Main relevant grant projects:
- **Czech Science Foundation (GAČR) P302/10/1022**: Chromatin dynamics during DNA repair (2010-15)
- **Project of the Czech Plenipotentiary** (with JINR Dubna, Russia) 2015
- **Project 3-PLUS-3** (with JINR Dubna, Russia) 2019 – 2021

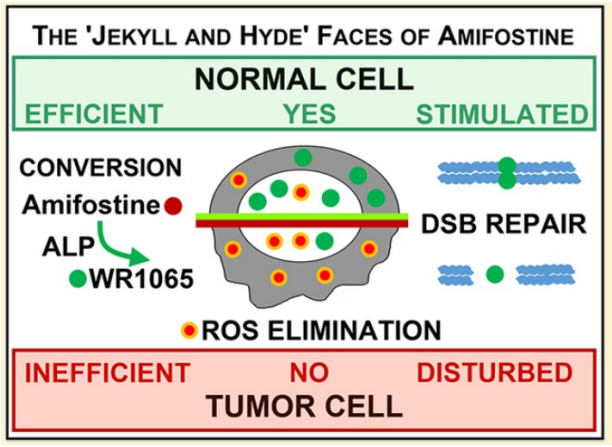
RESULT 2. Two New Faces of Amifostine: Protector from DNA Damage in Normal Cells and Inhibitor of DNA Repair in Cancer Cells (*J Med Chem*, 2016, IF= 6.054. Q1/decile 1).

This result concerns the search for more effective radio-protectants for clinical practice and radiation protection and the study of the mechanisms of their effects. In this study, we focused on the only clinically approved radioprotectant to date - amifostine. Amifostine protects normal cells from DNA damage induction by ionizing radiation or chemotherapeutics, whereas cancer cells typically remain uninfluenced. While confirming this phenomenon, we have revealed by comet assay and currently the most sensitive method of DNA double strand break (DSB) quantification (based on γH2AX/53BP1 high-resolution immunofluorescence microscopy) that amifostine treatment supports DSB repair in γ-irradiated normal NHDF fibroblasts but alters it in MCF7 carcinoma cells. These effects follow from the significantly lower activity of alkaline phosphatase measured in MCF7 cells and their supernatants as compared with NHDF fibroblasts. Liquid chromatography−mass spectrometry confirmed that the amifostine conversion to WR-1065 was significantly more intensive in normal NHDF cells than in tumor MCF cells. In conclusion, due to common differences between normal and cancer cells in their abilities to convert amifostine to its active metabolite WR-1065, amifostine may not only protect in multiple ways normal cells from radiation-induced DNA damage but also make cancer cells suffer from DSB repair alteration.

Based on our results, we have postulated three hypotheses that may explain “the opposite” effect of amifostin on normal and (some) tumor cells, respectively. In cancer cells, amifostine is almost not converted to WR-1065 (because of low levels of ALP and acidic pH) and behaves as “Bad”. While amifostine was considered as biologically inactive in previous works, some authors show that this pro-drug *per se* is rather toxic, with direct and/or indirect negative effects on DSB repair and cell survival. On the other hand, amifostine in normal cells is converted to WR-1065, its “Good” active metabolite. WR-1065 primarily ensures protection of normal cells against immediate cytoplasmic and DNA radiation-induced damage by scavenging free radicals (ROS). However, as also shown here, it supports the repair of DSBs too, directly by (physicochemical) interactions with damaged DNA and/or indirectly by modifying gene expression and biochemical cell regulatory pathways. Alternatively, the situation could be similar as described but the negative effect on cancer cells may be exerted by WR-1065 (instead of amifostine). Low amounts of WR-1065 in cancer cells cannot protect these cells from DSB induction but may be sufficient to negatively influence their DSB repair (and potentially other functions). The opposite effects of WR-1065 on DSB repair in normal and cancer cells follow from different WR-1065 levels and/or genetic backgrounds of these cells. WR-1065 thus only shows its “Mr. Hyde” face in cancer cells but “Mr. Jekyll” face in normal cells. It is also possible that varying mixtures of amifostine, WR-1065 and their metabolites are produced in normal and cancer cells, respectively; these mixtures interact with processes in normal and cancer cells in specific ways.

The result, dominantly obtained under the leadership of the DCBR IBP, opens up many new questions, but also the door to a rational search for other radio-protectants. It is clinically important that amifostine not only protects normal cells but also increases the effectiveness of radiotherapy against cancer cells. Unfortunately, the wider use of amifostine is hindered by its toxicity. A better understanding of its efficacy may therefore be essential for its targeted modifications to reduce this toxicity.

Illustration: Normal cells have the capacity to convert the pro-drug amifostine to its active metabolites (WR1065). These metabolites are subsequently responsible for the radioprotection of normal cells due to the sequestration of reactive free radicals (ROS) formed in the cells by radiolysis of water. Amifostine metabolites may also have other protective effects on normal cells, such as promoting DNA repair (upper half of the figure). In contrast, in tumor cells (lower half of the figure), the capacity to convert amifostine is insufficient due to their altered pH. Unconverted amifostine in combination with the absence of its "good" metabolites and the possible contribution of harmful metabolites not only does not protect tumor cells from radiation, but also impairs their ability to repair radiation lesions.



Main relevant grant projects:

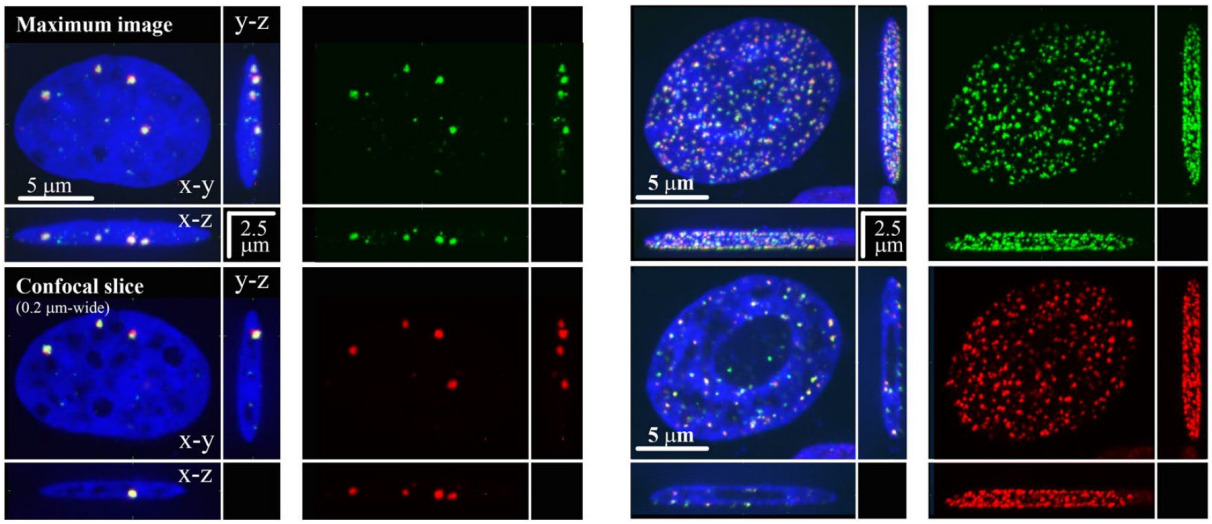
- **Project of the Czech Plenipotentiary** (with JINR Dubna, Russia) 2015
- **Project 3-PLUS-3** (with JINR Dubna, Russia) 2019 – 2021

RESULT 3. Chromatin architecture changes and DNA replication fork collapse are critical features in cryopreserved cells that are differentially controlled by cryoprotectants (*Sci Rep, Nature Publishing, 2018*, IF= 4.259. Q1/decile 1), *Langmuir. 2018*, IF= 4.122, Q1)

This result concerns the study of non-radiation DNA / chromatin damage, which could possibly be used to enhance the effect of radiotherapy of some tumors. At the same time, the result potentially explains why there have been conflicting views in the literature on the formation of DNA breaks during cell freezing. The result, which was obtained in cooperation with the Institute of Physics of CAS, was published in the journal Scientific Reports (Nature Publishing), aroused media interest (2 x radio interview), and was awarded 1st place at the cryobiological conference (see chapter AWARDS).
In this work, we shed new light on the highly debated issue of chromatin fragmentation in cryopreserved cells. Moreover, for the first time, we describe replicating cell-specific DNA damage and higher-order chromatin alterations after freezing and thawing. We identified DNA structural changes associated with the freeze-thaw process and correlated them with the viability of frozen and thawed cells. We simultaneously evaluated DNA defects and the higher-order chromatin structure of frozen and thawed cells with and without cryoprotectant treatment. We found that in replicating (S phase) cells, DNA was preferentially damaged by replication fork collapse, potentially leading to DNA double strand breaks (DSBs), which represent an important source of both

genome instability and defects in epigenome maintenance. This induction of DNA defects by the freeze-thaw process was not prevented by any cryoprotectant studied. Both in replicating and non-replicating cells, freezing and thawing altered the chromatin structure in a cryoprotectant-dependent manner. Interestingly, cells with condensed chromatin, which was strongly stimulated by dimethyl sulfoxide (DMSO) prior to freezing had the highest rate of survival after thawing.
Our results will facilitate the future design of compounds and procedures to decrease injury to cryopreserved cells. Since tumor cells in principle show higher replication activity than normal cells, and replicating cells are also significantly more sensitive to freezing due to the risk of replication fork collapse, cryoablation (used to treat some tumors) can also be considered in a synergistic context with radiotherapy.
The Institute of Physics of CAS performed physical measurements and calculations while DCBR IBP CAS performed all biological experiments and analyses.

Illustration: While non-replicating cells show only few DNA double strand breaks (DSBs) upon freezing/thawing (left), we have revealed a new class of cells highly affected by DSBs; these cells have been identified as just-replicating (S-phase) cells with collapsed replication forks (right). Blue – chromatin (TOPRO3), green – γH2AX + red – 53BP1 = DSB markers.



Main relevant grant projects:

- **MEYS of CR (LC535):** The Project of Excellence: Dynamics and Organization of Chromosomes in the Cell Cycle and during Differentiation under Normal and Pathological Conditions (2012-2018)

RESULT 4. Challenges and Contradictions of Metal Nano-Particle Applications for Radio-Sensitivity Enhancement in Cancer Therapy (*J Nanobiotechnol, 2016*, IF= 6.518. Q1/decile 1, *IJMS. 2018*, IF= 4.122, Q1)

Tumor targeting by radiotherapy represents a great challenge. Treatment of radiosensitive tumors would require escalation of the radiation dose, while this escalation is also hindered by the risk of damage to the surrounding vital tissue. The addition of multimodal nanoparticles, such as 3 nm gadolinium-based nanoparticles (GdBNs), has been proposed as a promising strategy to amplify the effects of radiation

in tumors and improve diagnostics using the same agents, while preserving the surrounding vital tissue. This ability and singular property named theranostics is a unique advantage of GdBNs.
This is due to the fact that nanoparticles are preferentially accumulated by tumor cells and, after irradiation, can selectively increase their radiation damage. It has been established that the amplification of radiation effects by metal nanoparticles appears due to fast electronic processes (i.e., emission of electron showers that locally, at microscale, amplify the absorbed dose). However, the influence of these nanoparticles on cells is not yet understood. In particular, it remains dubious how nanoparticles activated by ionizing radiation interact with cells and their constituents. A crucial question remains open of whether damage to the nucleus is necessary for the radiosensitization exerted by GdBNs (and other nanoparticles).
We studied the effect of various metal nanoparticles on the induction and repair of DNA double-strand breaks (DSBs) in the nuclear DNA of various normal and tumor cell types irradiated with γ-rays. For this purpose, we used currently the most sensitive method of DSBs detection based on high-resolution confocal fluorescence microscopy coupled with immunodetection of two independent DSBs markers.
We show that, in the conditions where nanoparticles amplify radiation effects, they remain localized in the cytoplasm, i.e. do not penetrate into the nucleus. In addition, the presence of GdBNs in the cytoplasm neither increases induction of DSBs by γ-rays in the nuclear DNA nor affects their consequent repair. Our results thus suggest that the radiosensitization mediated by metal nanoparticles is a cytoplasmic event that is independent of the nuclear DNA breakage, a phenomenon commonly accepted as the explanation of biological radiation effects. Considering our earlier recognized colocalization of metal nanoparticles with the lysosomes and endosomes, we revolutionary hypothesize here about these organelles as potential targets for (some types of) nanoparticles. If confirmed, this finding of cytoplasmically determined radiosensitization opens new perspectives of using nano-radioenhancers to improve radiotherapy without escalating the risk of pathologies related to genetic damage Nevertheless, interestingly, some changes in DNA damage upon NP-application were observed by super-resolution (Single Molecule Localization) Microscopy (SMLM), leaving the precise mechanism of NP-mediated radiosensitization open.

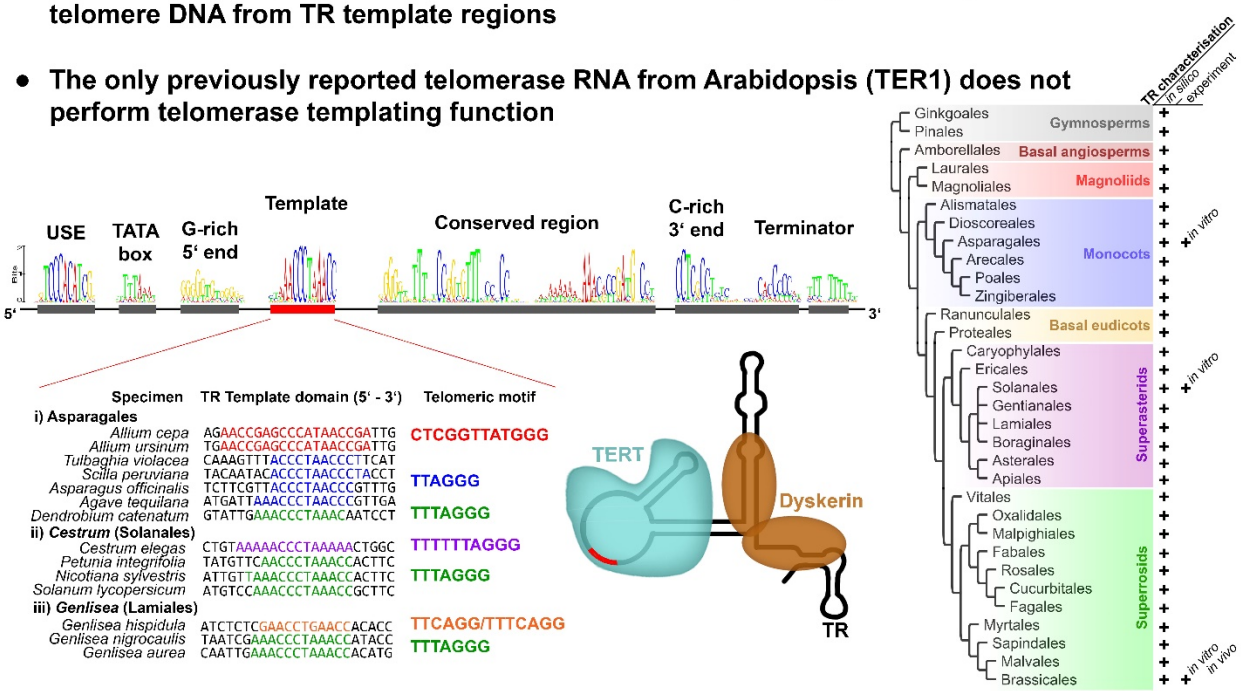
Main relevant grant projects:
- **DAAD-19-03 Projects of the Czech Academy of Sciences + DAAD** 'Nano-enhancers for future radiotherapy: New insights into the mechanism of nanoparticle-mediated tumor cell radiosensitization (NanoCancer)' – Principal Investigator (CR): Falk Martin, Principal Investigator (Germany): Hausmann Michael (KIP, Heidelberg)

RESULT 5. Telomerase RNAs in land plants. Nucleic Acids Res 47(18), 2019. doi: 10.1093/nar/gkz695

To elucidate the molecular nature of evolutionary changes of telomeres in the plant order Asparagales, we aimed to characterize telomerase RNA subunits (TRs) in these plants. The unusually long telomere repeat unit inAllium plants (12 nt) allowed us to identify TRs in transcriptomic data of representative species of the Allium genus. Orthologous TRs were then identified in Asparagales plants harbouring telomere DNA composed of TTAGGG (human type) or TTTAGGG (Arabidopsis-type) repeats. Further, we identified TRs across the land plant phylogeny, including common model plants, crop plants, and plants with unusual telomeres. Several lines of functional

testing demonstrate the templating telomerase function of the identified TRs and disprove a functionality of the only previously reported plant telomerase RNA in Arabidopsis thaliana. In contrast to animal or yeast TRs which are transcribed with RNA Polymerase II, plant TRs are Pol III-dependent lncRNAs, and their transcription is dependent on SNAPc transcription factor. Importantly, our results change the existing paradigm in plant telomere biology which has been based on the existence of a relatively conserved telomerase reverse transcriptase subunit (TERT) associating with highly divergent TRs even between closely related plant taxa. Our finding of a monophyletic origin of genuine TRs across land plants opens the possibility to identify TRs directly in transcriptomic or genomic data and/or predict telomere sequences synthesized according to the respective TR template region.
Our results thus allow for potential biotechnology applications and enrichment of telomerase research with numerous new model systems which (i) differ in lifespan and developmental strategies; (ii) must efficiently cope with environmental changes and (iii) could be easily regenerated from their totipotent cells.
(see the figure below used for public outreach activities).

Figure: The *bona fide* telomerase RNA subunits (TR) were identified across land plant phylogeny



Reference: Fajkus P., Peška V. et al. Telomerase RNAs in land plants. Nucleic Acids Res 47(18), 2019. doi: 10.1093/nar/gkz695

Main relevant grant projects:

- **Czech Science Foundation (GAČR) GA13-06943S:** Structural and Functional Components of Plant Telomeres (2013 – 2017)
- **Czech Science Foundation (18-07027S)** – Telomerase involvement in the cellular interactome – 2018 – 2020

PRACTICAL RESULTS

RESULT 6. DeepFoci: Deep Learning-Based Algorithm for Fast Automatic Analysis of DNA Double Strand Break Ionizing Radiation-Induced Foci. Computational and Structural Biotechnology Journal, IF = 6.018 (in revision)

In this result, we introduced DeepFoci - a deep learning-based fully-automatic method for counting and its morphometric analysis Ionizing Radiation-Induced (Repair) Foci (IRIF). IRIFs are repair complexes that form at sites of DNA double strand breaks, the most serious DNA lesions, dangerous to human health. IRIF quantification based on confocal microscopy represents the most sensitive and gold standard method in radiation biodosimetry and allows research of DSB induction and repair at the molecular and a single cell level.

DeepFoci is designed to work with 3D multichannel data (trained for 53BP1 and γH2AX) and uses U-Net for the nucleus segmentation and IRIF detection, together with maximally stable extremal region-based IRIF segmentation.

The proposed method was trained and tested on challenging datasets consisting of mixtures of non-irradiated and irradiated cells of different types and IRIF characteristics - permanent cell lines (NHDF, U-87) and cell primary cultures prepared from tumors and adjacent normal tissues of head and neck cancer patients. The cells were dosed with 1-4 Gy gamma-rays and fixed at multiple (0-24 h) post-irradiation times. Upon all circumstances, DeepFoci was able to quantify the number of IRIF foci with the highest accuracy among current advanced algorithms. Moreover, while the detection error of DeepFoci remained comparable to the variability between two experienced experts, the software kept its sensitivity and fidelity across dramatically different IRIF counts per nucleus. In addition, information was extracted on IRIF 3D morphometric features and repair protein colocalization within IRIFs. This allowed multiparameter IRIF categorization, thereby refining the analysis of DSB repair processes and classification of patient tumors with a potential to identify specific cell subclones.

The developed software improves IRIF quantification for various practical applications (radiotherapy monitoring, biodosimetry, etc.) and opens the door to an advanced DSB focus analysis and, in turn, a better understanding of (radiation) DNA damaging and repair.

Main relevant grant projects:

- **MEYS of CR (AZV 16-29835A)** – Molecular-genetic markers for prediction of radiotherapy response in head and neck cancer, 2016-2019

RESULT 7. New RNA chip for expression analysis of genes associated with DNA repair and genes possibly useful as biomarkers of (tumor) cell radio-resistance:

Motivated by the need to compare complex DNA damage response to irradiation among different tumor cell primary cultures derived from patents' tumors, we have extended our microscopy studies for OMICS experiments. For this purpose, we have designed (and later let commercially synthesize on the custom basis) the RNA chip for

simultaneous expression analysis of expression of about 350 genes associated with DNA damage and repair (with focus on DSBs, double strand breaks that are the most dangerous type of DNA damage), cell cycle regulation, apoptosis, drug resistance etc. An important advantage of the chip is that it can be repeatedly used for several times.

Main relevant grant projects:

- **Czech Science Foundation (GAČR) 16-12454S** – Characterizing & modifying complex response of head & neck tumor cells to different radiations - a step forward to combined personalized radiotherapy. 2016-2018
- **EU COST Action CA16113 – CliniMARK**: 'good biomarker practice' to increase the number of clinically validated biomarkers. 2017 – 2021 (M. Falk – Management Committee delegate for CR)

Research activity and characterisation of the main scientific results

(In the lists of publication examples, authors affiliated to the DBCMO are typed in **bold).**

(1) Development of novel electroanalytical tools

a. Electrodes decorated with silver amalgam micro/nanoparticles

A considerable part of electrochemical studies summarized below relies on application of mercury containing electrodes, particularly in cases when hydrogen evolution catalyzed by natural or modified biomacromolecules is exploited. Consumption of mercury in modern electroanalytical devices is minimum and risk of contamination under laboratory conditions very small, which makes the classical mercury electrode fully acceptable for basic studies. Nevertheless, there are reasons to seek alternative tools retaining unique properties of mercury electrodes but being non-toxic, mechanically more robust and applicable in integrated devices such as miniaturized flowing or separation systems, spectroelectrochemical cells etc. Amalgam (particularly silver amalgam) electrodes of various designs have been developed as potent surrogates of the hanging mercury drop electrode (HMDE) exhibiting very similar properties in the negative potential region, where analytically useful reduction or catalytic signals of biomacromolecules occur (see below).

To further decrease consumption of mercury and exploit advantages of particle-modified electrodes, we have developed technology of decoration of various solid conductive substrates with silver amalgam nano- or microparticles (AgAP). Controlled electrodeposition from solutions containing Ag^{+} and Hg^{2+} ions in different ratios allows one to “tune” and optimize their properties, which has been demonstrated for indium-tin oxide (ITO) [1], thin gold film [2] and more recently (published in 2020) pyrolytic graphite substrates. Optimized AgAP-modified substrates have been successfully tested using model analytes related to research pursued at the DBCMO, such as unmodified and osmium-labelled DNA, nitro compounds and proteins. Moreover, in collaboration with prof. T. Šikola group from CEITEC-Brno University of Technology *we have demonstrated [3] for the first time unique plasmonic properties of the AgAPs*, namely an extremely wide spectral range covered by the silver amalgam localized plasmonic resonances, ranging from ultraviolet all the way to the mid-infrared wavelengths. Our results establish silver amalgam as a suitable material for introduction of plasmonic functionalities into photochemical and spectroelectrochemical systems, where the plasmonic enhancement of electromagnetic fields and light emission processes could synergistically meet with the superior electrochemical characteristics of mercury in biomacromolecules research.

b. Carbon electrodes

Carbon materials are probably the most popular among all materials used to construct electrodes for electroanalysis, particularly in methods based on analyte oxidation due to their wide positive potential window. It was generally accepted that cathodic potential window in aqueous media is limited by relatively low overpotential of hydrogen evolution and does not encompass region where reduction of NA bases takes place. Corresponding processes and electrochemical signals were thus accessible only with mercury containing electrodes. Nevertheless, in 2017 we reported [4] on *an innovative approach to label-free voltammetric analysis of DNA at a pyrolytic graphite electrode*

(PGE) within a broad range of potentials (from -2.0 to + 1.6 V). We demonstrated not only anodic oxidation, but for the first time also cathodic reduction of nucleobases at the PGE. In addition, products of irreversible oxidation/reduction of the parent bases were shown to yield analytically useful, base-specific cathodic/anodic signals, making it possible to distinguish between the canonical bases (adenine, cytosine, guanine and thymine), uracil (U) and 5-methylcytosine (mC) in DNA. Selective electrochemical "switching off" of the redox signals specific to certain nucleobases was presented as a way to resolve overlapping signals. The performance of this approach has been further demonstrated in a report dealing with electrochemical reduction and oxidation of six natural 2 '-deoxynucleosides [5] and more recently in work devoted to unnatural nucleosides designed for synthetic biology to expand genetic code (published in 2020).

Further we have been focused on surface pretreatments of carbon electrodes to create surface micro- or nanostructures with specific properties for bioelectroanalysis. Decoration of electrode surfaces with different carbonaceous nanoobjects (such as graphene, graphene oxide or carbon nanotubes) is a widely applied approach to improve properties of designed electrochemical sensors. In contrast to more or less laborious procedures used in the literature we have developed a simple electrochemical *in situ* modification of graphite electrodes by graphene oxides. These species, in connection with occurrence of surface structural motifs such as graphite edges and basal planes (generated at the surface under precisely controlled conditions), facilitate oxidation of free nucleic acid bases [6], which can thus be detected in an excess of oligo- or polynucleotides. Analogous effect was observed in a study involving free tryptophan and intact proteins. In contrast, polymeric NA are strongly adsorbed onto non-anodized basal plane PGE which facilitates their electrochemical detection and allows design of simple biosensors based on DNA hybridization and/or other affinity interactions (e.g., [7]).

c. Miniaturized devices for bioanalysis

In collaboration with the team of prof. F. Foret (Institute of Analytical Chemistry of the CAS) we have been involved in the development of microfluidic separation systems [8, 9] coupled to electrochemical detectors based on principles developed at the DBCMO (see parts (2) and (3)). Miniaturized experimental arrangements were also developed in collaboration with prof. J. Barek group (Charles University, Prague) [10].

[1] **A. Danhel, F. Ligmajer,** T. Sikola, A. Walcarius, **M. Fojta**, Electrodeposition of silver amalgam particles on ITO - Towards novel electrode material, J Electroanal Chem, 821 (2018) 53-59.
[2] **P. Havranova, F. Ligmajer, A. Danhel**, Electrodeposition of Silver Amalgam on Thin Gold Film Electrodes for Voltammetric Detection of 4-Nitrophenol and DNA Labeled with Osmium Tetroxide-Bipyridine Complex, Electroanalysis, 31 (2019) 1952-1960.
[3] F. Ligmajer, M. Horak, T. Sikola, **M. Fojta, A. Danhel,** Silver Amalgam Nanoparticles and Microparticles: A Novel Plasmonic Platform for Spectroelectrochemistry, J Phys Chem C, 123 (2019) 16957-16964.
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[5] **J. Spacek, M. Fojta**, J. Wang, Electrochemical Reduction and Oxidation of Six Natural 2'-Deoxynucleosides at a Pyrolytic Graphite Electrode in the Presence or Absence of Ambient Oxygen, Electroanalysis, 31 (2019) 2057-2066.
[6] **S. Hason, M. Fojta, V. Ostatna**, Label-free electrochemical analysis of purine nucleotides and nucleobases at disposable carbon electrodes in microliter volumes, J Electroanal Chem, 847 (2019) 113252.
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[8] J. Novotny, **V. Ostatna**, F. Foret, Electrochemical Analysis of Glycoprotein Samples Prepared on a Pneumatically-controlled Microfluidic Device, Electroanalysis, 31 (2019) 1994-2000.
[9] **M. Trefulka, V. Dorcak**, J. Krenkova, F. Foret, **E. Palecek**, Electrochemical analysis of Os(VI)-modified glycoproteins and label-free glycoprotein detection eluted from lectin capillary column, Electrochim Acta, 239 (2017) 10-15.
[10] J. Gajdar, T. Gonec, J. Jampilek, **M. Brazdova, Z. Babkova, M. Fojta**, J. Barek, J. Fischer, Voltammetry of a Novel Antimycobacterial Agent 1-Hydroxy-N-(4-nitrophenyl)naphthalene-2-carboxamide in a Single Drop of a Solution, Electroanalysis, 30 (2018) 38-47.

(2) Electrochemistry of proteins

In 2015-2019, research in this field the foundation of which were laid in the preceding period, was further developed. Previously established tools, particularly thiol-coated mercury electrodes and constant current chronopotentiometric stripping (CPS) were predominantly used in the following studies: **(a)** *roles of particular amino acid residues*, namely those bearing basic side groups (arginine, lysine and histidine) in the catalytic hydrogen evolution reactions (CHER) were identified [1]. **(b)** *conditions promoting structural changes* in bovine serum albumin adsorbed at negatively charged electrode surfaces were explored using the CPS technique to identify effects of temperature, current density and time of exposure at ms scale [2]. Bovine and human serum albumins, ovalbumin, α-macroglobulin and α-synuclein, showed various profiles in dependence on different parameters. Using this approach, new methods for testing surface-attached protein stabilities in relation to the electric field effects and to other factors can be developed. **(c)** A comparative study involving human serum albumin, lysozyme, β-synuclein, H2A and H3 histones was performed [3] to find out the *association between amino acid content and its electrochemical CPS responses*. In general, presence of cysteine facilitated the CHER making the peak H appear at less negative potentials while acidic and basic proteins not containing Cys could be recognized due to their different CPS response after adsorption at the positive and negative charged interface.

Newly, the following pilot studies were focused on the effects of protein chemical modification: **(d)** *Oxidative damage to BSA* by singlet oxygen was studied [4] by square wave voltammetry at glassy carbon electrode and by constant current chronopotentiometry at the HMDE. Particularly the CPS peak H of BSA at HMDE sensitively responded to the BSA photooxidation, making the CPS analysis promising tool for simple label-free sensing of covalent damage to proteins. **(e)** For the first time, *effects of methylation and acetylation* of BSA to its native and denatured forms were

studied [5] by CPS and other methods. Acetylation of bovine serum albumin resulted in decrease of chronopotentiometric peak H due to modification of the catalytically active residues. The present results show the capability of label- and reagent-free electrochemical methods to detect post-translational modifications in proteins (including those involved in epigenetic regulations).

Another field of important novel applications of label-free protein electrochemistry encompassed detection of non-covalent association interactions. **(f)** Complexes of tumour suppressor *p53 core domain (p53CD) and various DNA recognition elements* were studied by means of the CPS [6]. Disintegration of these complexes due to the effect of the electric field was accompanied by a remarkable increase in the electrocatalytic peak H. By adjusting stripping current intensities and temperature, the transition between intact and disintegrated complex reflected differences in the stabilities of sequence-specific complexes with different recognition elements, making the CPS a potent technique for assessment of the protein-DNA binding affinity. **(g)** *Polysaccharide-protein interactions* were detected by the CPS methods for the first time [7]. BSA-sodium alginate (SA) complex formation was accompanied by the shift of the structural transition of BSA to lower stripping current intensities while another polysaccharide, dextran, did not alter the stripping current dependent structural transition of BSA. BSA-SA complex could be disturbed by an electric field effect or high ionic strength confirming the electrostatic nature of BSA-SA interaction. **(h)** Two studies were devoted to *interactions of an important cancer biomarkers, anterior gradient (AGR) proteins, with specific peptides [8] or with antibodies [9]* (Ab). Surface-attached AGR2-peptide complexes can be disintegrated as a result of their exposure to negative potentials. By controlling the exposure time and temperature the weakly bound nonspecific complexes (involving a mutant peptide) can be discriminated from tightly bound specific complexes. Specific interaction of the Ab, adsorbed at HMDE, with AGR3 protein induced a significant increase in chronopotentiometric peak H in comparison to CPS response of the Ab alone and the one observed after incubation with nonspecific proteins. These proof-of-concept results show promise as a new option for studying the dynamics of protein-protein interactions. **(i)** A study devoted to concanavalin A (ConA)-ovalbumin (Ova) interaction represents the first appearance of CPS application to a *lectin-glycoprotein interaction* [10]. Incubation of ConA with Ova resulted in an increase of the CPS peak H of the complex as compared to the CPS peaks of individual Ova and ConA proteins, providing a valuable proof-of-principle of electrochemistry application in glycoprotein research.

[1] **V. Dorcak, V. Vargova, V. Ostatna, E. Palecek**, Lysine, Arginine, and Histidine Residues in Peptide-Catalyzed Hydrogen Evolution at Mercury Electrodes, Electroanalysis, 27 (2015) 910-916.
[2] **H. Cernocka, V. Ostatna, E. Palecek**, Protein structural transition at negatively charged electrode surfaces. Effects of temperature and current density, Electrochim Acta, 174 (2015) 356-360.
[3] E. Melnikova, **N. Izadi**, M. Gal, **V. Ostatna**, Chronopotentiometric Analysis of Proteins at Charged Electrode Surfaces, Electroanalysis, 31 (2019) 1868-1872.
[4] **V. Vargova**, R.E. Gimenez, **H. Cernocka**, D.C. Trujillo, F. Tulli, V.I.P. Zanini, **E. Palecek**, C.D. Borsarelli, V. Ostatna, Label-free electrochemical detection of singlet oxygen protein damage, Electrochim Acta, 187 (2016) 662-669.

[5] **V. Ostatna, V. Kasalova**, K. Kmetova, O. Sedo, Changes of electrocatalytic response of bovine serum albumin after its methylation and acetylation, J Electroanal Chem, 821 (2018) 97-103.
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[7] **H. Cernocka, N. Izadi, V. Ostatna,** S. Strmecki, BSA-Polysaccharide Interactions at Negatively Charged Electrode Surface. Effects of Current Density, Electroanalysis, 31 (2019) 2007-2011.
[8] **V. Ostatna, V. Kasalova**, L. Sommerova, R. Hrstka, Electrochemical sensing of interaction of anterior gradient-2 protein with peptides at a charged interface, Electrochim Acta, 269 (2018) 70-75.
[9] **V. Ostatna, S. Hason, V. Kasalova**, M. Durech, R. Hrstka, Anterior gradient-3 protein-antibody interaction at charged interfaces. Label-free chronopotentiometric sensing, Electrochim Acta, 297 (2019) 974-979.
[10] **V. Vargova, R. Helma, E. Palecek, V. Ostatna**, Electrochemical sensing of concanavalin A and ovalbumin interaction in solution, Anal Chim Acta, 935 (2016) 97-103.

(3) Chemical modification and electroanalysis of glycoproteins

There is accumulating evidence of crucial roles of protein glycosylation in physiology and pathology. It is thus of great importance to develop novel and robust methods applicable in diagnostics based on glycoprotein and glycan analysis. In the previous evaluation period we have established several approaches to carbohydrate electroanalysis, including those based on hydrogen evolution catalysed by aminosugars (which is switched off by acetylation of the aminogroups) and those involving modification of vicinal diol groups in sugar moieties with osmate complexes and electrochemical detection of reaction products. This research was further developed in 2015-2019 towards proof-of-concept diagnostic applications.

The latter approach was *for the first time used to design a method for distinguishing between glycan isomers,* 2,3-sialyllactose (3-SL) and 2,6-sialyllactose (6-SL). Following the pilot work [1], a more detailed study was performed [2]. The 6-SL molecule can bind three Os(VI) moieties, while the 3-SL only two. A similar pattern of Os(VI)-modification was found for isomers of sialyl-N-acetyllactosamine and sialylgalactose. Covalent adducts of Os(VI)temed with glycans yielded three reduction voltammetric peaks. The ratio of peak I/peak II heights depended on the content of individual regioisomers in the sample. These results show a new promising method for the discrimination between glycan isomers representing cancerous and non-cancerous biomarkers.

Other analytical techniques were designed to involve *glycoprotein interaction with specific lectins and/or their separation on lectin columns*, followed by electrochemical detection. **(a)** Interactions of a sialylated biomarker, the prostate specific antigen (PSA), with two important lectins were studied in collaboration with Dr. J. Tkáč group (Slovak Academy of Sciences) [3]: Sambucus nigra agglutinin (SNA) and Maackia amurensis agglutinin (MAA). Incubation of PSA-modified electrode with specific SNA resulted in an increase of the CPS peak H as compared to the peak of PSA alone. By adjusting the stripping current and temperature, PSA-MAA interaction could be either eliminated or distinguished from the more abundant PSA-SNA complex. CPS data

were in a good agreement with the data obtained by complementary methods. **(b)** Glycosylated RNAse B was successfully distinguished from non-glycosylated RNAse A due to differences in electrocatalytic CPS peak H (being much higher in the case of RNAse B) and differences in reactivity towards Os(VI)temed (specific for RNAse B) [4]. Using the peak H, it was shown that glycosylation of RNase stabilizes its molecule adsorbed at the electrode. Peak H was also used for the detection of RNase B separated from a large excess of non-glycosylated proteins on a lectin (concanavalin A) monolithic flow-through column. **(c)** A pneumatically operated microfluidic device for isolation and purification of glycoprotein samples was designed and manufactured in collaboration with prof. F. Foret team (IACH CAS, Brno) [5]. The biochemical functionality was provided by beaded support modified by lectins which was stacked inside the micro-channel packed affinity columns. Unlabeled glycoproteins, namely fetuin, asialofetuin, and prostate-specific antigen, were voltammetrically analyzed using catalytic peak H at a silver amalgam electrode.

[1] **M. Trefulka, E. Palecek**, Distinguishing glycan isomers by voltammetry. Modification of 2,3-sialyllactose and 2,6-sialyllactose by osmium(VI) complexes, Electrochem Commun, 85 (2017) 19-22.
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[3] S. Belicky, **H. Cernocka**, T. Bertok, A. Holazova, K. Reblova, **E. Palecek**, J. Tkac, V. Ostatna, Label-free chronopotentiometric glycoprofiling of prostate specific antigen using sialic acid recognizing lectins, Bioelectrochemistry, 117 (2017) 89-94.
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[5] J. Novotny, **V. Ostatna**, F. Foret, Electrochemical Analysis of Glycoprotein Samples Prepared on a Pneumatically-controlled Microfluidic Device, Electroanalysis, 31 (2019) 1994-2000.

(4) Chemically modified nucleic acids

In the area of modified nucleic acids, we mainly continued fruitful cooperation with prof. Michal Hocek group (Institute of Organic Chemistry and Biochemistry of the ASCR, Prague) established in 2006. Within this collaboration, the essential role of Hocek group lies in organic synthesis of base-modified nucleoside triphosphates applied as building blocks to construct correspondingly modified DNAs using DNA polymerases. This biochemical part is conducted by both partners while electrochemical analysis and experiments with DNA binding p53-family proteins are pursued by the DBCMO.

Reactive DNA probes. N-(3-Azidopropyl)vinylsulfonamide was developed [1] as a new bifunctional bioconjugation reagent suitable for the cross-linking of biomolecules through copper(I)-catalyzed azide-alkyne cycloaddition and thiol Michael addition reactions under biorthogonal conditions. The reagent was clicked to an acetylene-modified DNA and then *reacted with cysteine-containing peptides or proteins (such as the core domain of p53 protein) to form covalent cross-links*. In another study [2] we introduced chloroacetamido group-functionalized DNA probes for protein crosslinking via thiol (cysteine) or imidazole (histidine) moieties. The modified DNA efficiently cross-

linked with p53 protein through alkylation of cysteine and showed potential for cross-linking with histidine (in C277H mutant of p53).

In the area of *DNA redox labeling* we designed novel applications of earlier introduced potent electroactive labels. For example, using combination of reducible nitrophenyl and benzofurazane labels, *dual redox labeling was applied in an electrochemical pull-down assay* suitable for assessment of p53 protein binding affinity to different DNA substrates [3]. Using *azidophenyl moiety newly introduced as a reducible and click-convertible DNA label*, in combination with nitrophenyl acetylene, another dual-labeling electrochemical technique for protein sites in DNA binding, based on protection of azidophenyl groups within the binding sites, was developed [4]. We continued in detailed *studies of mechanisms* behind electrochemical signals of already introduced redox labels (e.g. [5]) and chose some of them as probes for characterization of model analyte interactions with electrode surfaces [6] (see also part (1)).

Other modified DNAs developed and published in 2015-2019 include those bearing electrochemically oxidizable *phenothiazine* [7] to complete the palette of redox coding of DNA bases, and *DNAs bearing butylacrylate [8] or cis-diol [9] moieties suitable for post-synthetic attachment of well-tried redox active osmium tags* via reactions with osmium tetroxide or osmate complexes, respectively.

An important field of study introduced in DBCMO within the evaluated period includes *application of electrochemistry as a tool for synthetic biology research* based on expansion of genetic code by introduction of unnatural base pairs. Here, the potential of electrochemical analysis in determination of unnatural and/or rare components in minute amounts of DNA was demonstrated. Two pilot studies were completed and published in 2020 in collaboration with US groups of prof. F. Romesberg and prof. S. Benner.

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(5) Nucleic acids structure in solution, at interfaces and in cells

Historically, polarographic techniques were among those that brought early evidence of DNA structural polymorphism and proved their potential to detect even small changes in the DNA structure. Especially signals measured at negatively charged surfaces of mercury-based electrodes, both reduction and tensammetric, exhibit sensitivity to DNA structure. In contrast to proteins, *the ability of natural NAs to catalyze hydrogen evolution* was not exploited analytically until recently, when conditions were found under which both DNA [1] and RNA [2] yield catalytic CPS (or voltammetric) signals analogous to the “peak H”. The latter signal is influenced by changes in DNA structure and offers a high sensitivity for NA determination. In addition, *catalytic deuterium evolution* by deuterated DNA was for the first time investigated [3]. Due to differences in both intensities and positions of signals due to catalytic evolution of light hydrogen and the one of the deuterium evolution it was possible to observe DNA structure-dependent H/D exchange by an inherently simple electrochemical method which was, to our best knowledge, the first appearance of such kind of experiment. Taken together, in these contributions new electrochemical tools for studying NA structure and dynamics were introduced.

The DBCMO is involved in an integrative OP RDE project SYMBIT, taking part especially in development of novel tools for NA (and protein) analysis (see part (1)) and their applications in studies of alternative (non-canonical) DNA structures (particularly G-quadruplexes and i-motifs) and their interactions with specific ligands. A pilot study dealing with the behavior of G-quadruplex (G4) forming oligonucleotides and positively or negatively charged surfaces was published in 2015. More recently we have performed complex studies of various DNA elements forming G4 and single-stranded (ss) homooligonucleotides [4, 5]. Single stranded stretches often occur in the alternative structures as overhangs or loops. Specific electrochemical behavior of homonucleotide or repetitive single-stranded stretches and the propensity of homopyrimidine sequences to 2D condensation at a negatively charged surface [4] underline *the importance of DNA primary structure for its behavior at the electrodes.* Several papers dealing with these phenomena are prepared for submission.

The interest of the DBCMO (partially in context of the SYMBIT project) in non-canonical DNA or RNA structures has been extended, in collaboration with teams at the University of Ostrava and Mendel University in Brno, towards development of *bioinformatics tools for prediction of these structures* (G4Hunter web application [6], Palindrome Analyzer [7]) and their application to analyze sequences with

corresponding propensities in genomes (of e.g., bacteria [8], chloroplasts [9], mitochondria [10]).

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(6) DNA-protein interactions

Interactions of proteins important in cancer pathogenesis and therapy, particularly the tumor suppressor p53 family transcription factors, have been studied at the DBCMO since the middle of 1990's. In last decade this research is focused mainly on the roles of non-canonical DNA structures in the protein-DNA recognition. In the previous evaluation period these studies were oriented towards *p53 interactions with cruciform DNA extruded within inverted repeat sequences*. In the present period, another two papers focused on the inverted repeats were published (one [1] dealing with the effects of cruciform extrusion in p53 response elements on transcription activation by the p53 protein and the other [2] with the cruciform recognition by a p53 homologue, the p73 protein) but in general our interest has been shifted towards studies involving *triplex and particularly G4 structures*. T.A.T triplexes [3], as well as non-B structures adopted by the pyrimidine strand in triplet repeat sequence GAA.TTC associated with a neurodegenerative disorder Friedreich ataxia [4], were recognized by the p53 protein in vitro and in cells. The G4 structures, namely intramolecular G4 formed by G-rich strand of human telomeric repeat [5] or the one occurring in the promoter of c-myc

oncogene [6], were also identified as structural *elements recognized by the p53 protein, stabilized by the protein binding and influencing its transcription activation function*. In addition to the p53 family proteins, the abilities to bind preferentially to topologically constrained (supercoiled) DNA or to G4s were observed with BRCA1 [7] or IFI16 [8] proteins, respectively.

In general, here mentioned studies have utilized a palette of biophysical biochemical or molecular biology experimental approaches, such as gel shift or pulldown assays, western blotting, circular dichroism (in collaboration with the Department of Biophysics of Nucleic Acids at the IBP), luciferase reporter assay and chromatin immunoprecipitation for the in-cell experiments, as well as bioinformatics approaches to identify common properties of DNA structure-recognizing proteins [9, 10]. In addition, *we have developed novel DNA-protein binding assays based on electrochemistry and/or application of reactive DNA probes* (for more details see parts (2) and (4)). In these cases, we exploit our interdisciplinary expertise, particularly access to in-house production of proteins (including specifically mutated ones) and long-term experience with the p53 as a well-established model protein exhibiting different DNA binding modes.

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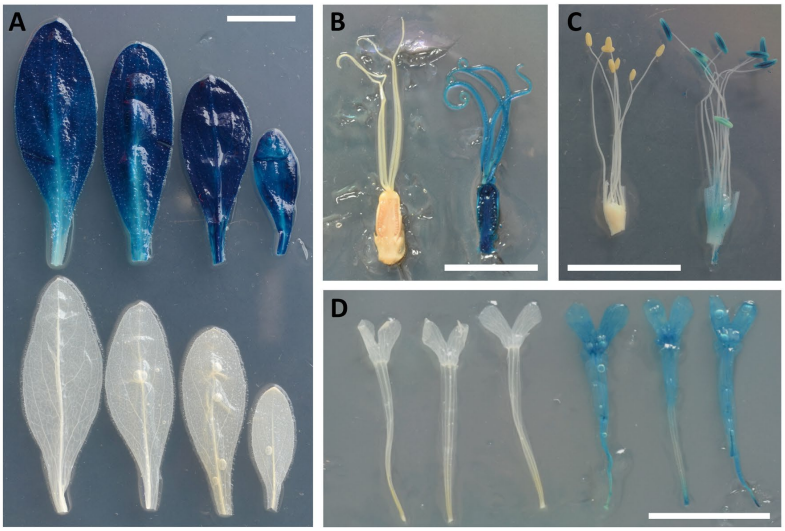
Research activity and characterisation of the main scientific results

Here, we present just brief overview of our recent results. In evaluated period we published almost 30 papers mostly in Q1 journals (e.g. in Nature Plants, Genome Biology and Evolution, Genome Biology, Plant Cell, Genome Research, Proceedings of the National Academy of Sciences of the United States of America, Plant Journal, BMC Bioinformatics, Annals of Botany, New Biotechnology, Genetics, Scientific Reports and many others). Out of all our results, we pick only several examples to show broad spectrum of our interests concerning plant structural and functional genomics.

Our efforts to analyse plant sex chromosomes including resulted in identification of genes potentially involved in sex determination and formation of unisexual flowers. The functional analyses to validate the role of these genes and discover the molecular mechanisms leading to gonochorism in plants was not feasible in our model organisms, such as *S. latifolia*. Therefore, we started a brand new research direction focused on methodology of gene delivery (Hudzieczek *et al*., 2019 – both first and corresponding authors are our Department members). *S. latifolia* exhibited strong recalcitrance to *in vitro* regeneration and *Agrobacterium tumefaciens*-mediated transformation, we had to opt for alternative ways to establish gene delivery in this species. Firstly, we screened the library of synthetic plant growth regulators to induce shoot regeneration from callus cultures. Next, we explored the possibility of transgene integration using various microbes and found out that specific strains of *Agrobacterium rhizogenes* successfully transform *S. latifolia*.

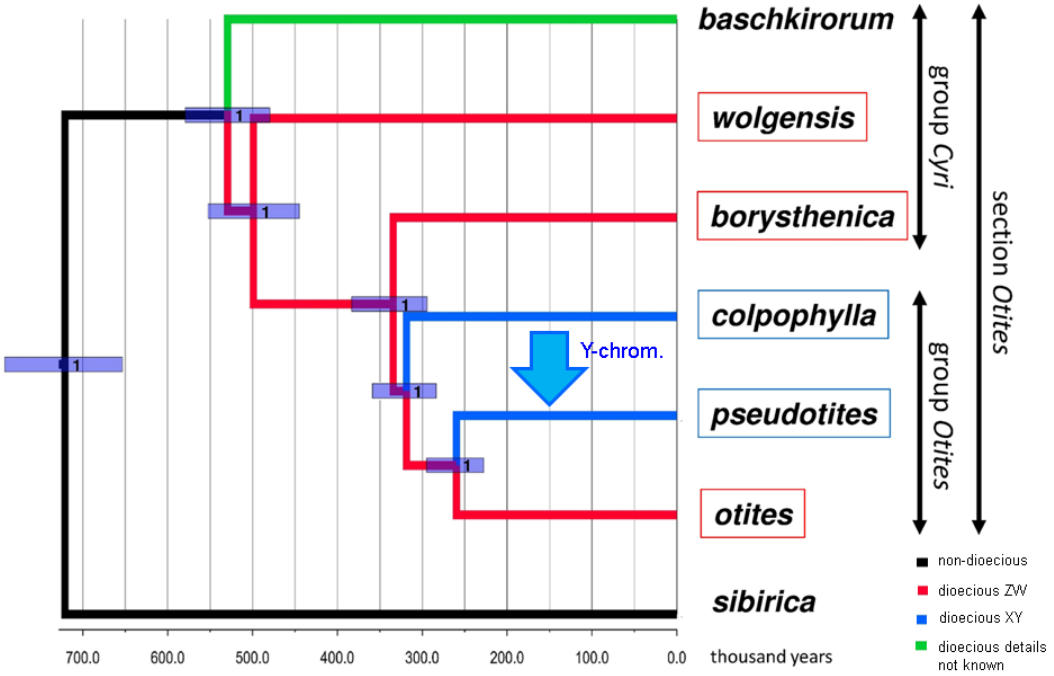


Expression of GUS reporter gene in T1 plants. Gus positive and GUS negative leaves (A) and floral organs – pistils (B), anthers (C) and petals (D) – of T1 plants (Hudzieczek et al. 2019)

In addition, the transgene was tracked into following generations and Mendelian pattern of inheritance was confirmed. Moreover, we have developed a protocol for quick transfection of protoplast – such technique is useful for cell biology applications and rapid testing of recombinant constructs. We used protoplast transfection to assess the activity of genome editing CRISPR/Cas9 and TALEN nucleases by disrupting the coding sequence of X- and Y-linked allele of *SlAP3* gene. This was the first ever demonstration of targeted genome editing on plant sex chromosomes. These findings allow us and rest of scientific community to study gene function on sex chromosomes and address evolutionary/development questions to our classical models.

We have developed a protocol for the nuclease efficiency comparison using the amplicon sequencing and bioinformatic data analysis. We used this tools in independent experiments (Čermák *et al.,*2015; Baltes *et al*., 2015; Čermák *et al*., 2017 – we have contributed by our bioinformatic expertise) to evaluate the effectivity of TALENs, CRISPR/Cas9 and multi-gRNAs expression systems.
In Čermak *et al.* (2015) we show that geminivirus vectors are efficient tools for gene targeting in tomato. Coupled with TALENs or CRISPR/Cas9 reagents, geminivirus vectors allow the targeting of virtually any sequence in a given genome, making it possible to extend this technology to other crop species to create valuable traits. This work provides a foundation for efficient genome editing of crop genomes without the random integration of foreign DNA. In Čermák *et al.* (2017) we report a comprehensive toolkit that enables targeted, specific modification of monocot and dicot genomes using a variety of genome engineering approaches. Our reagents, based on TALENs and CRISPR/Cas9 system, are systematized for fast, modular cloning and accommodate diverse regulatory sequences to drive reagent expression. In Nature Plants (Baltes *et al*. (2015) we demonstrate novel strategy for engineering resistance to geminiviruses. In conclusion, we effectively interfered with geminivirus replication and systemic movement by transferring elements of the CRISPR–Cas prokaryotic immune system to plants. Finally, with this approach it is possible to direct nucleases against multiple viruses and satellites. This represents a key advantage in defeating the mixed infections of geminivirus disease complexes.

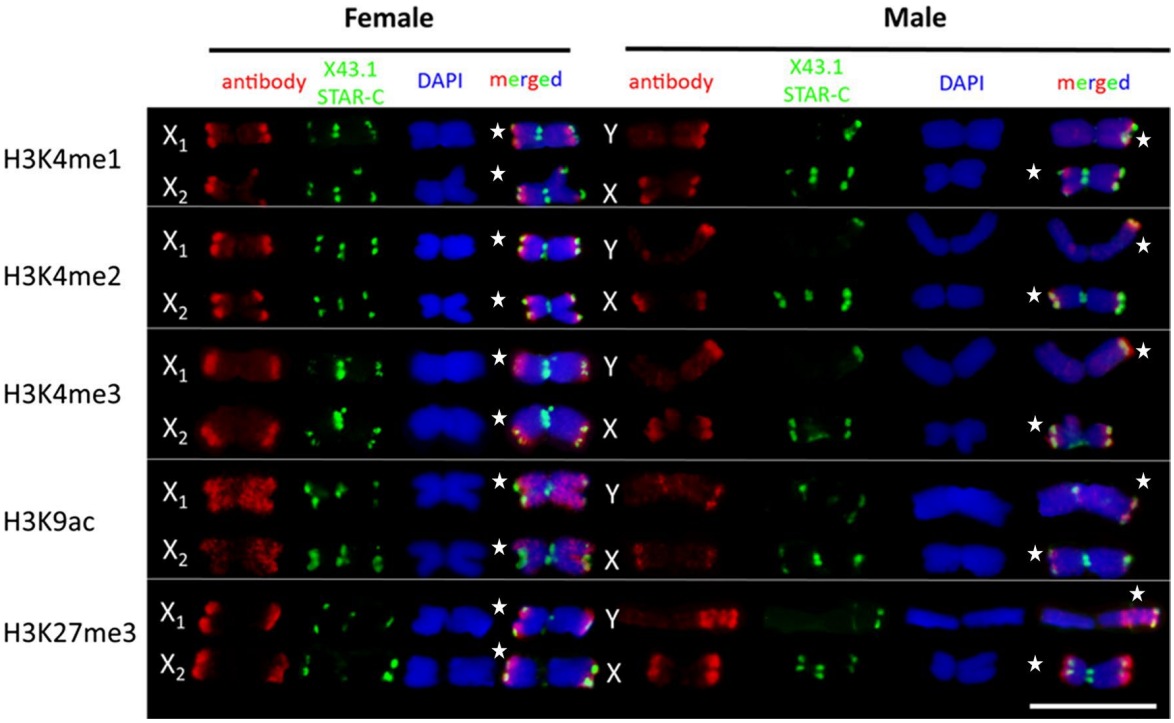
One of our main scientific focuses is to study various stages of sex chromosome evolution in the context of gene degeneration and possible dosage compensation mechanisms (*Silene latifolia, Rumex acetosa, Humulus lupulus*). In collaboration with prof. John Pannell (University of Lausanne) we investigated the evolutionary fate of *Mercurialis annua* sex chromosomes that evolved relatively recently. This project led to unravelling of basic chromosomal structure of *M. annua* sex chromosomes with two evolutionary strata being described. Sexually antagonistic selection as an ancient process was followed degeneration of Y chromosome and subsequent gene loss. Our group contributed in this project by generating transgenic *Mercurialis* plants, analysing the karyotype by fluorescent *in situ* hybridization and bioinformatic analyses.
We have recently obtained interesting results about the dynamics of the sex determining systems in plants (Balounova *et al*., 2019). Our findings are important in two aspects. First, the presented results suggest that the switch from ZW to XY sex determination occurred in the genus *Silene*. It is the only switch in heterogamety that was so far studied in detail in plants. Second, our analyses suggest a possibility that has so far not been considered, the change in heterogamety through hybridization event. In this event a male-determining chromosome from one species could introgrese into another species, and over-rides its previous sex-determining system. This work is almost entirely result of our department (first-authorship is shared by Roman Gogela and Veronika Balounova - Bohuslav Janousek is corresponding author).



Results of StarBeast phylogenetic analysis of 662 single-copy orthogroup sequences. Species with female heterogamety are indicated by red boxes, and male heterogamety by blue boxes. The values indicated at the nodes are posterior probabilities. Branch colours indicate the most probable scenario for the evolution of the sex-determining system in section Otites. Branches and nodes where female heterogamety is inferred are coloured red, while the branches with male heterogamety are in blue. The branch to S. baschkirorum is coloured green as its heterogametic state is not known. Silene sibirica is not dioecious (Balounova et al., 2019)

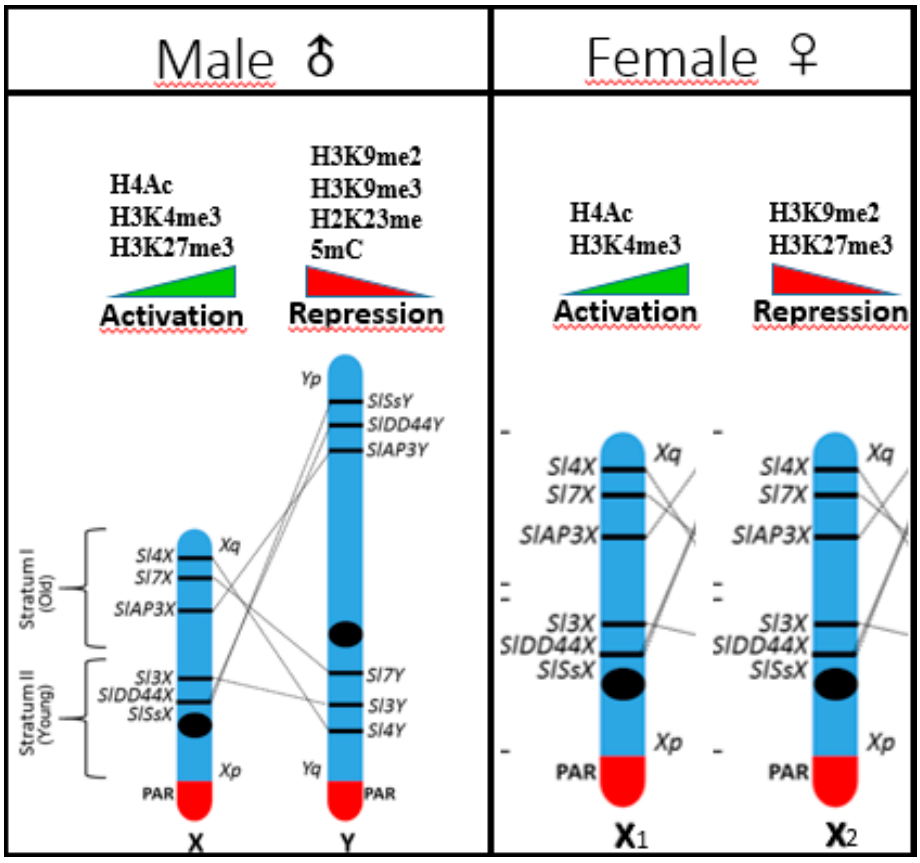
We have also participated in establishment of SEX-CHROM database, which includes crucial information concerning sex determining systems and sex chromosomes in plants. Apart of the importance of the database for the basic research, it also brings important information to those plant breeders who deal with dioecious species. This project was mainly performed in collaboration of our laboratory with the laboratory of Dr. Sònia Garcia (Institut Botànic de Barcelona, Spain) with minor participation of other institutions. One of two co-first authors (Simona Barankova) and one of the two corresponding authors (Bohuslav Janousek) are members of our Department.

In the evaluated period, our group used various cytogenetic, molecular and bioinformatic tools to understand principal mechanisms of the Y chromosome degeneration. We also focused on epigenetic regulation in the context of sex chromosome degeneration and subsequent dosage compensation evolution. We used chemical treatment influencing DNA methylation and acetylation to affect the proper flower development and RNA-seq transcriptome profiling to detect candidate genes controlling gynoecium development. More importantly, we showed that the chemical treatment caused heritable changes with the high penetrance in the third generation. Following these changes at the transcriptome level, we found that Y chromosome in *S. latifolia* is undergoing down-regulation prior to genetic degeneration, similarly as showed in *Drosophila* species. Besides, we found that the downregulation is fully reversible process, affected by the evolutionary history of both sex chromosomes. Next, we used immunolabelling to visualise active and suppressive histone marks to characterize differences between X and Y chromosome at the chromatin level in *Silene latifolia.* Using this approach, we discovered that the Y chromosome is rather suppressed, and the X is being enriched for specific histone marks, suggesting X up-regulation (Bačovský *et al.*, 2019 - both first and corresponding authors are our Department members).



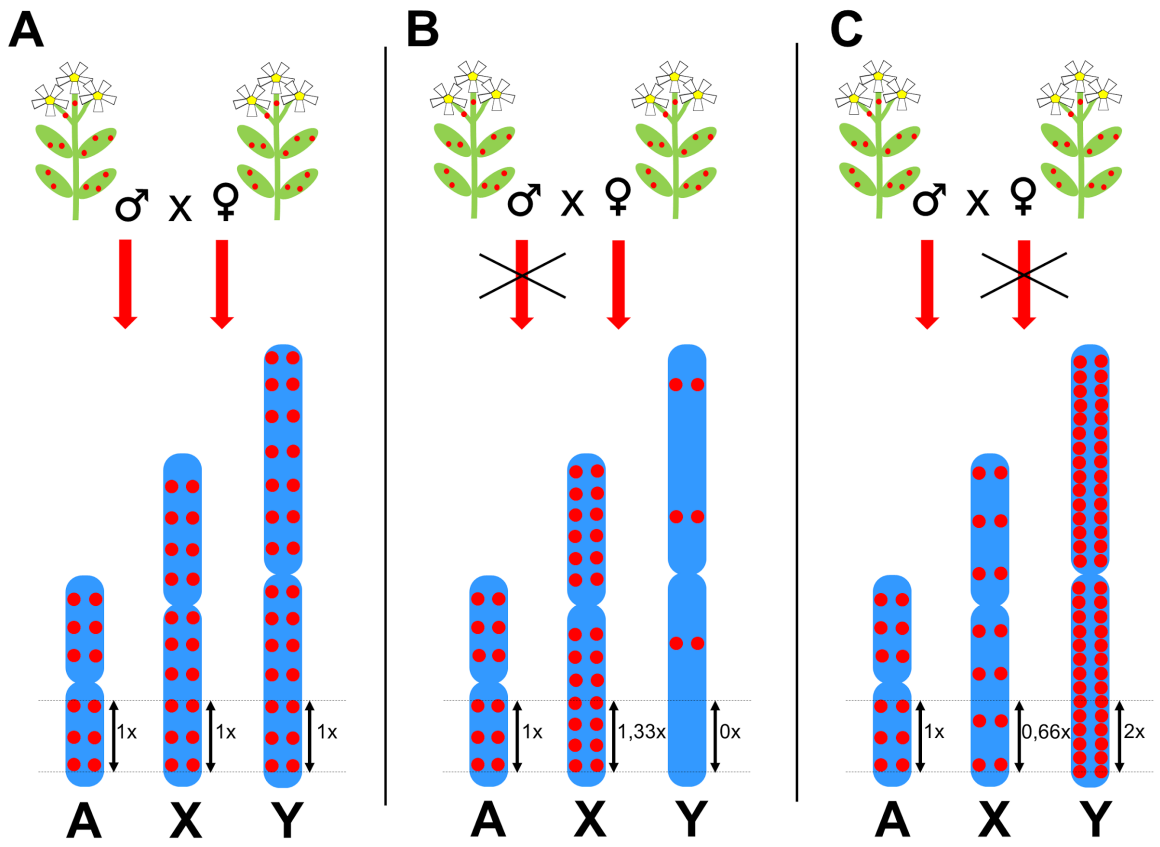
Distribution of histone modifications typical for active chromatinon on the sex chromosomes in female and male of S. latifolia. Note the higher acetylation and additional subtelomeric H3K4me2 enrichment of one of X chromosomes in females. Y chromosome is enriched on the p-arm for histone marks important for transcriptional competence, confirming the existence of gene rich region. PAR is indicated by stars. STAR-C and X43.1 satellites hybridize to centromere and PAR region. Scale bar = 10 μm (Bacovsky et al., 2019)

Tuning new protocols up, like DNA and chromatin immunoprecipitation techniques, we interrogated epigenetic status of selected sex-linked genes in *S. latifolia* (Rodriguez *et al*., 2018 - all authors are our Department members). Generally, sex chromosome formation is accompanied by stop of recombination between both X and Y chromosomes. This recombination suppression takes place in discreet events, enabling the formation of individual strata (non-recombining part of the sex chromosomes that differ from each other in level of divergence). Studied genes were selected based on their position in stratum I (older) or stratum II (younger). The alleles of Y-linked genes of stratum I were enriched for CG methylation compared to their X alleles. High level of methylation in the CHH context in most sequences indicated de novo methylation through the RdDM pathway. This suggests that the level of methylation is affected by the evolutionary history and the age of both strata. The methylation of genes is also influenced by the proximity of TEs and other repetitive sequences. We speculate that TE accumulation and not gene decay is the cause of DNA methylation in the *S. latifolia* Y chromosome affecting the process of Y suppression and down-regulation. Regarding histone post-translational modifications, we concluded that the promoters of the Y allele are associated with inactive histone marks meanwhile promoters of the X allele in males are associated with histone marks important for transcription. Moreover, both X alleles in females were found to have opposite mode transcriptional regulation. We suggested that *S. latifolia* possesses dosage compensation system similar to mammals. We have partially confirmed these findings by large scale transcriptome analysis (Muyle *et al*., 2018 – we have performed Y chromosome analysis).



Summary of the epigenetic regulation of several sex-linked genes from S. latifolia. The comparison of male and female individuals is presented. The individual epigenetic regulation is indicated for each sex chromosome (X:Y in males; X:X in females). Our results suggest similar sex chromosome epigenetic regulation as in mammals (Rodriguez et al*., 2018)*

As mentioned earlier, we study structural features, regulation and epigenetic silencing of transposable elements. We are specifically interested in whether new heritable TE insertions are equally frequent in male and female lineage. This is the question, which is purely studied in traditional hermaphroditic species for obvious reason – absence of sex specific chromatin, sex chromosomes. Our cytogenetic and bioinformatics analyses of genomic TEs distribution revealed that TEs may be sex-specifically active in dioecious plants (Puterova *et al*., 2018 - both first and corresponding authors are or have been our Department members). We also propounded a model of sex-specific proliferation of TEs in plants with sex-chromosomes, which is being tested in our laboratory (Hobza *et al*., 2017 - all authors are our Department members). Activity of TEs is regulated by epigenetic mechanisms that differ in male and female germlines in *Arabidopsis thaliana*. Therefore, we established transgenic plant lines to study transgenerational proliferation of selected TE types and effect of epigenetics on activity of these TEs.

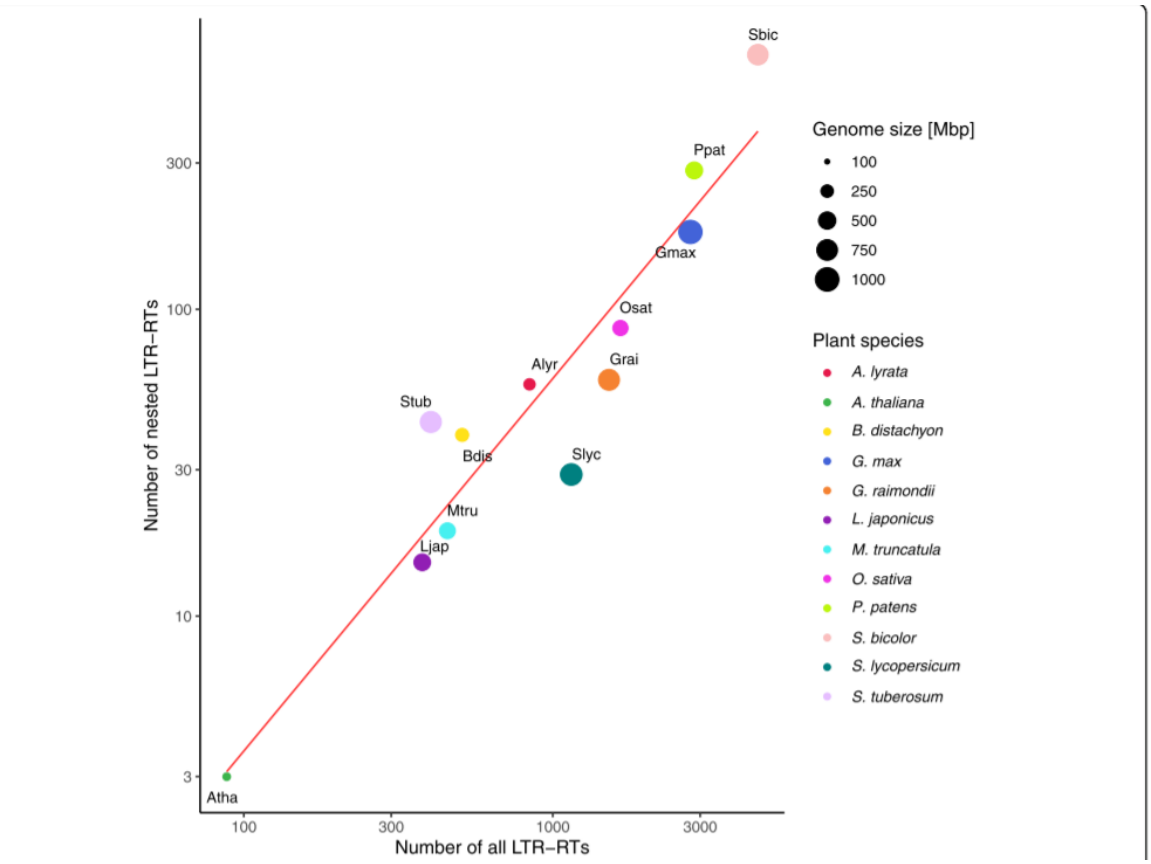


Three scenarios of transgenerational proliferation of transposable elements (TEs) in plants and their impact on chromosomal distribution of a TE. Red dots indicate TE insertions - in blue colored meiotic chromosomes, A-autosomes, X-chromosomes and Y-chromosomes. Numbers next to double arrows indicate expected density of TE insertions per one unit of length of a respective chromosome. (A) If a TE is passed down to offspring equally in males and females, TE insertion density is identical on all chromosomes. (B) If TE proliferation is disrupted in males, TE density is 1,33 times higher on the X chromosome than on autosomes, but nearly zero on the Y chromosome. (C) If TE proliferation is disrupted in females, TE insertion density is lower on the X chromosome compared to autosomes and twice higher on the Y chromosome than on autosomes. These three scenarios represent extreme cases of sex specific TE activity. Real world TEs range from TEs that are almost fully sex specifically inheritable to TEs with only slight sex-dependent inheritance (Hobza et al., 2017)

One of our dominant activities is bioinformatic analysis of sequencing data. We are not only users of widespread platforms and pipelines but we actively develop software for scientific community focused on genome analysis. We have recently developed freely accessible software LinkYX tool for identification of sex chromosome linked genes based on nucleotide polymorphism analysis of RNA-seq data (this software also automatically evaluates expression levels of analysed genes). The pipeline is suitable for all non-model plant and animal species without an existing genome reference and is available to a broad community of scientists focused on sex chromosome structure and evolution (Michalovova *et al.*, 2015 - this paper is a result of a long term effort of our laboratory to integrate genetics, genomics and bioinformatics approaches to study sex chromosome evolution. The whole study was done in our laboratory).

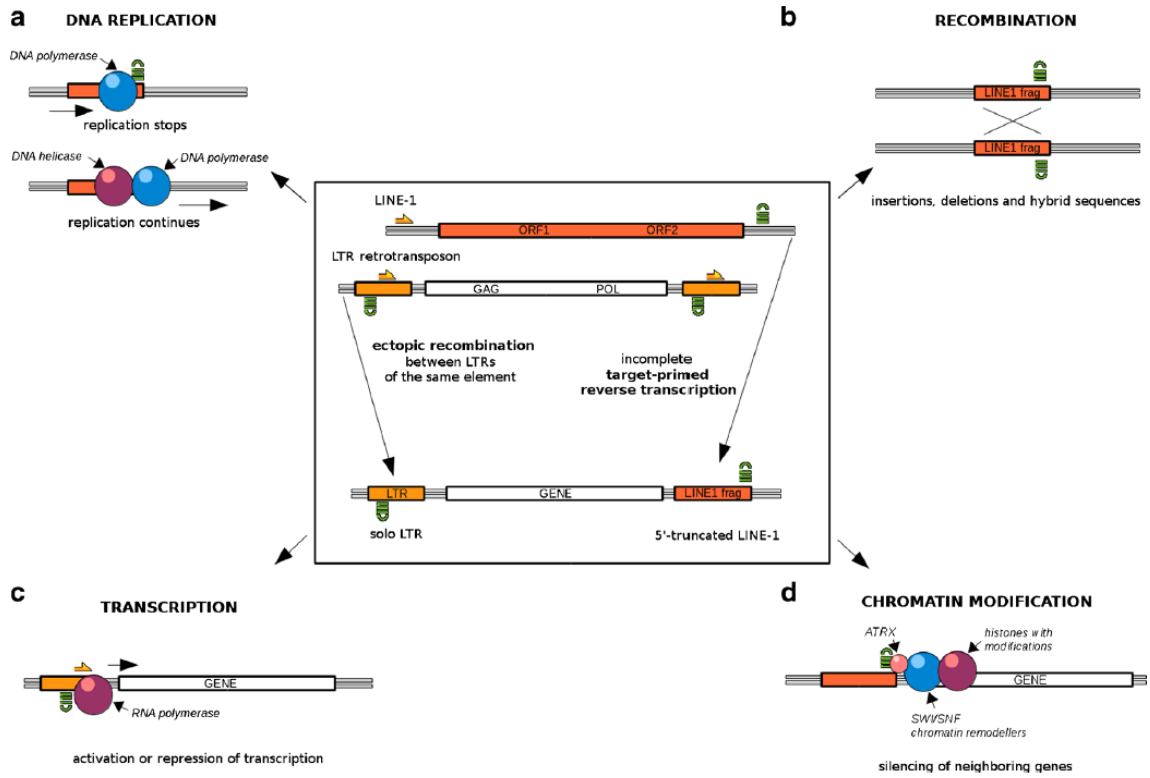
We have also developed our own software to analyse repetitive DNA in large plant genomes. Long terminal repeats retrotransposons (LTR-RTs) are mobile genetic elements constituting remarkable portions of plant genomes that significantly contribute to genome structure, size

and regulation. Due to their multiple insertions which occur during LTR-RTs bursts, retrotransposons form islands consisted of elements within elements. Therefore, in order to reconstruct the original retrotransposons from their fragments in the multiple 'nested' insertions, we developed a tool 'TE-greedy-nester'. This tool is also designed to classify reconstructed elements into their respective retrotransposon families. Using TE-greedy-nester we found that certain families showed a higher nesting frequency as well as a higher preference for older copies of the same family (Jedlička *et al.*, 2019 - three out of five authors are members of our laboratory; since they did the major portion of the workload, they are occupying the first and corresponding author positions).



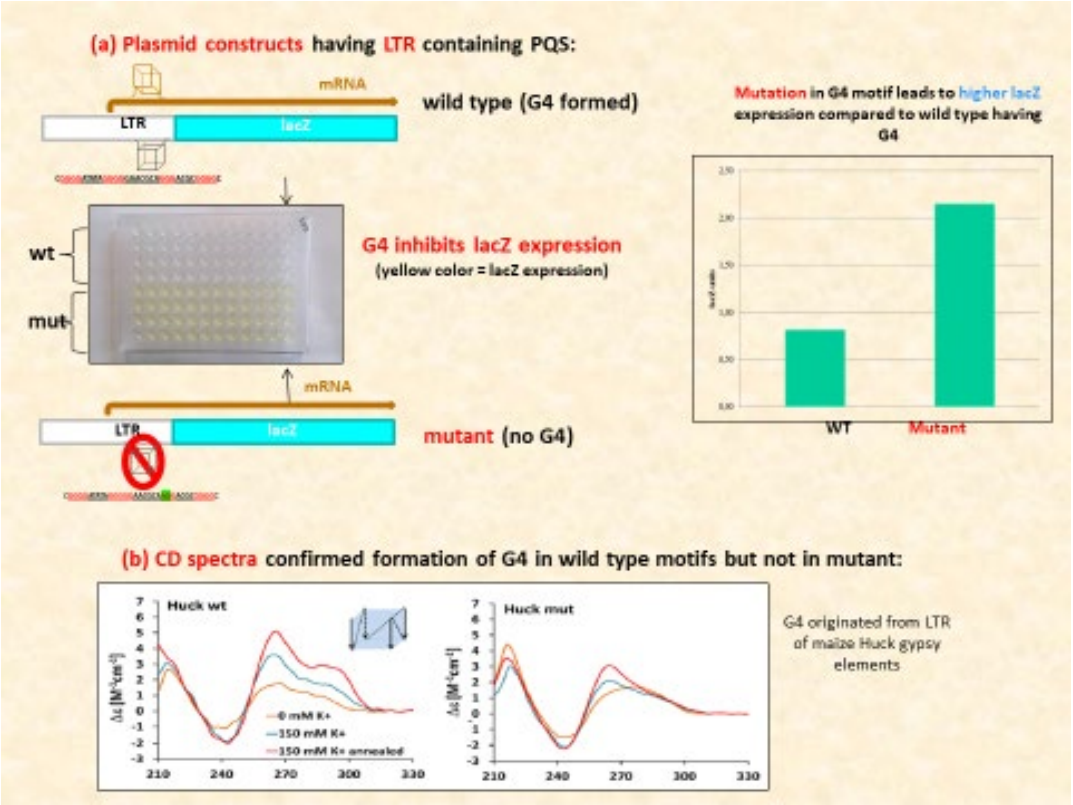
Extent of LTR retrotransposon nesting in plant genomes. Relationship between number of nested LTR retrotransposons and all LTR retrotransposons found in 12 plant genomes. Genome sizes are marked by the smaller or bigger circles, respectively (Jedlička et al., 2019)

We also study the role of secondary DNA structures on LTR retrotransposon targeting. A significant part of eukaryotic genomes is formed by transposable elements (TEs) containing not only genes but also regulatory sequences. Some of the regulatory sequences located within TEs can form secondary structures like hairpins or three-stranded (triplex DNA) and four-stranded (quadruplex DNA) conformations. We investigate potential role of these structures in the TE life cycle as well as the impact of G quadruplexes on replication, transcription, translation, chromatin status, and recombination. The non-canonical DNA structures and their conformational switches may constitute another regulatory system that, together with small and long non-coding RNA molecules and proteins, contribute to the complex cellular network resulting in the large diversity of eukaryotes (Kejnovsky *et al*., 2015 - all authors are our Department members).



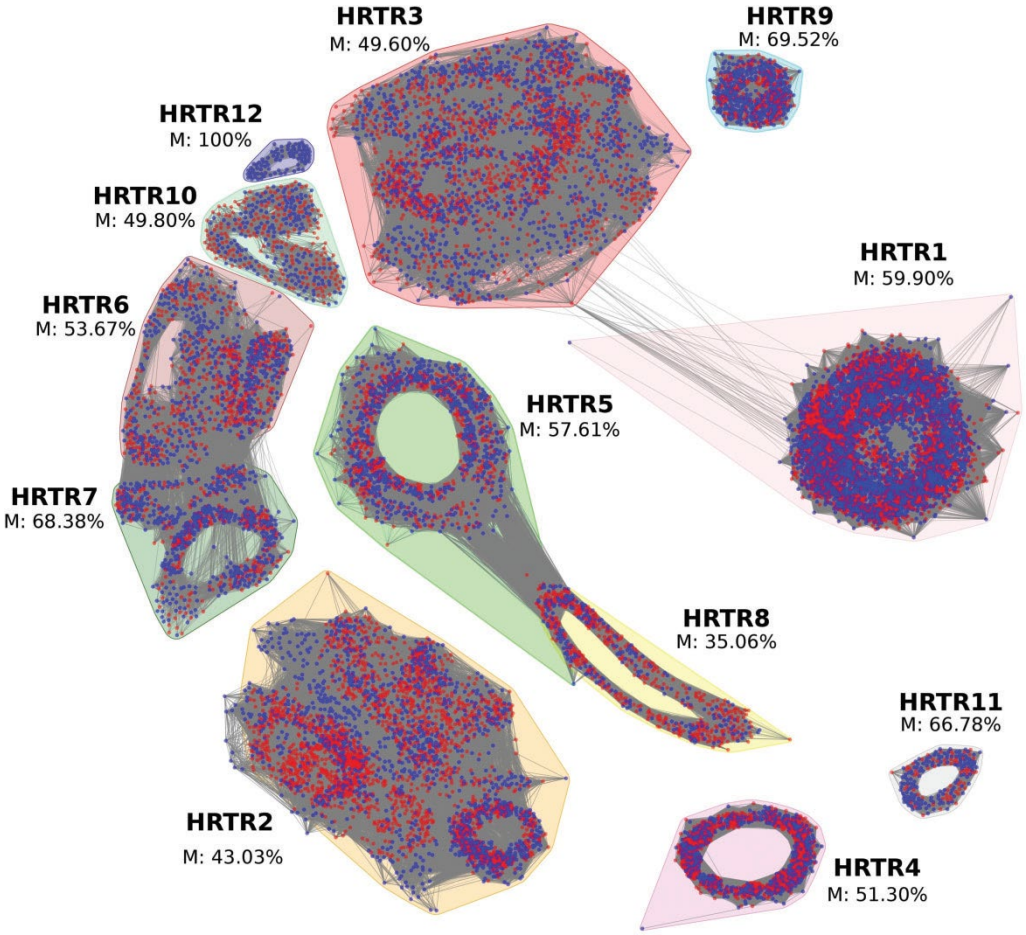
Potential roles of G-quadruplexes in genome. Formation of short variants of transposable elements containing Gquadruplexes — solo LTRs by ectopic recombination and 5' end truncated LINE-1 by non-complete target-primed reverse transcription, TPRT (middle box). Effect of G-quadruplexes on DNA replication (a), recombination (b), transcription (c), and chromatin remodeling (d) elements (Kejnovsky et al., 2015)

Many studies have shown that guanine-rich DNA sequences form quadruplex structures (G4) in vitro but there is scarce evidence of guanine quadruplexes in vivo. The majority of potential quadruplex-forming sequences (PQS) are located in transposable elements (TEs), especially close to promoters within long terminal repeats of plant LTR retrotransposons. In order to test the potential effect of G4s on retrotransposon expression, we cloned the long terminal repeats of selected maize LTR retrotransposons upstream of the lacZ reporter gene and measured its transcription and translation in yeast (Tokan *et al.*, 2018 - all authors are our Department members). We found that G4s had an inhibitory effect on translation in vivo since “mutants” (where guanines were replaced by adenines in PQS) showed higher expression levels than wild-types. In parallel, we confirmed by circular dichroism measurements that the selected sequences can indeed adopt G4 conformation in vitro. Analysis of RNA-Seq of polyA RNA in maize seedlings grown in the presence of a G4-stabilizing ligand (NMM) showed both inhibitory as well as stimulatory effects on the transcription of LTR retrotransposons. Our results demonstrate that quadruplex DNA located within long terminal repeats of LTR retrotransposons can be formed in vivo and that it plays a regulatory role in the LTR retrotransposon life-cycle, thus also affecting genome dynamics.



Quadruplex localized in LTR inhibits an expression of lacZ reporter gene in yeast (summary of results by Tokan et al. (2018))

We are also analysing structure of plant genomes and sex chromosomes using our local bioinformatics pipelines (Puterova *et al*., 2017 - both first and corresponding authors are or have been our Department members). Seabuckthorn (*Hippophae rhamnoides*) is a dioecious shrub commonly used in the pharmaceutical, cosmetic, and environmental industry as a source of oil, minerals and vitamins. We analysed the transposable elements and satellites in its genome. We carried out Illumina DNA sequencing and reconstructed the main repetitive DNA sequences. For data analysis, we developed a new bioinformatics approach for advanced satellite DNA analysis and showed that about 25% of the genome consists of satellite DNA and about 24% is formed of transposable elements, dominated by Ty3/Gypsy and Ty1/Copia LTR retrotransposons. FISH mapping revealed X chromosome-accumulated, Y chromosome-specific or both sex chromosomes-accumulated satellites but most satellites were found on autosomes. Transposable elements were located mostly in the subtelomeres of all chromosomes. The 5S rDNA and 45S rDNA were localized on one autosomal locus each. Although we demonstrated the small size of the Y chromosome of the seabuckthorn and accumulated satellite DNA, we were unable to estimate the age and the extent of the Y chromosome degeneration. Analysis of dioecious relatives such as Shepherdia would shed more light on the evolution of these sex chromosomes.



Visualization of male/female reads homogeneity in satellite families. Graph nodes correspond to sequenced reads and edges connect overlapping reads withmore than 70% of sequence identity over at least 55% read length. Distances between reads are inversely proportional to their sequence similarity. Male reads are labeled by blue and female reads by red color. Individual families are highlighted by different colors. Please note HRTR12 family that is composed of male reads only assuming to be Y-specific.families (Puterova et al., 2017)

Finally, to assess the dynamics of the Y chromosome, we studied intraspecific genome size variation and genome composition of male and female individuals in *S. latifolia (*Puterova *et al*., 2018 - both first and corresponding authors are or have been our Department members). Our results suggest that the large Y chromosome of *S. latifolia* has slowed down or stopped its expansion. Female-specific proliferation of retrotransposons, enlarging the genome with exception of the Y chromosome, was probably caused by silencing of highly active retrotransposons in males and represents an adaptive mechanism to suppress degenerative processes in the haploid stage. Sex specific silencing of transposons might be widespread in plants but hidden in traditional hermaphroditic model plants.

Research activity and characterisation of the main scientific results

Out team contributes to various fields of the genetics focusing on rDNA biology, chromosome evolution and epigenetic regulations. The most significant achievements (2015-2019) are listed below:

1. **Matyasek, R; Krumpolcova, A; Lunerova, J**; Mikulaskova, E; Rossello, JA; **Kovarik, A** (2019) Unique Epigenetic Features of Ribosomal RNA Genes (rDNA) in Early Diverging Plants (Bryophytes). Frontiers in Plant Science 10, Article Number 1066. DOI: 10.3389/fpls.2019.01066; **IF 4.4**

We explored rDNA chromatin structure in early diverging land plants from Bryophyta and Marchantiophyta. DNA methylation was investigated by methylation-sensitive Southern blot hybridization, whole genomic bisulfite sequencing and in silico analysis of available model plant methyloms. Higher order chromatin structure was analyzed using fluorescence in situ hybridization (FISH) and chromatin immunoprecipitation (CHIP) assays. In contrast to seed plants, bryophyte rDNAs were efficiently digested with methylation-sensitive enzymes indicating no or low levels of CG and CHG methylation in these loci. The rDNA methylom analyses revealed variation between species ranging from negligible (<3%, P formosum, P patens) to moderate (7 and 17% in M. polymorpha and D. scoparium, respectively) methylation levels. There were no differences between coding and noncoding parts of rDNA units and between gametophyte and sporophyte tissues. However, major satellite repeat and transposable elements were heavily methylated in P formosum and a scoparium. In P formosum rDNA, the euchromatic H3K4m3 and heterochromatic H3K9m2 histone marks were nearly balanced contrasting the angiosperms data where H3K9m2 typically dominates rDNA chromatin. In moss interphase nuclei, rDNA was localized at the nucleolar periphery and its condensation level was high. Unlike seed plants, the rRNA genes seem to escape global methylation machinery in bryophytes. Distinct epigenetic features may be related to rDNA expression and the physiology of these early diverging plants that exist in haploid state for most of their life cycles.

(At least 90% of the work on the article was done by members of the Laboratory of Molecular Epigenetics)

2. **Matyášek R, Kuderová A, Kutílková E, Kučera M, Kovařík A**. (2019) Intragenomic heterogeneity of intergenic ribosomal DNA spacers in Cucurbita moschata is determined by DNA minisatellites with variable potential to form non-canonical DNA conformations. DNA Research 26(3):273-286. DOI: 10.1093/dnares/dsz008. **IF 4.0**

To estimate the intragenomic variability in intergenic spacer (IGS) of rDNA, we employed PacBio sequencing of the Cucurbita moschata genome, in which thousands of rDNA copies are distributed across a number of loci. The rRNA coding regions are highly conserved, indicating intensive interlocus homogenization and/or high selection pressure. However, the IGS exhibits high intragenomic structural diversity. Two repeated blocks, R1 (300-1250 bp) and R2 (290-643 bp), account for most of the IGS variation. They exhibit minisatellite-like features built of multiple periodically spaced short GC-rich sequence motifs with the potential to adopt non-canonical DNA conformations, G-quadruplex-folded and left-handed Z-DNA. The mutual arrangement

of these motifs can be used to classify IGS variants into five structural families. Subtle polymorphisms exist within each family due to a variable number of repeats, suggesting the coexistence of an enormous number of IGS variants. The substantial length and structural heterogeneity of IGS minisatellites suggests that the tempo of their divergence exceeds the tempo of the homogenization of rDNA arrays. As frequently occurring among plants, we hypothesize that their instability may influence transcription regulation and/or destabilize rDNA units, possibly spreading them across the genome.

(All authors were members of the Laboratory of Molecular Epigenetics)

3. **Sochorova, J**; Garcia, S; Galvez, F; Symonova, R; **Kovarik, A** (2018) Evolutionary trends in animal ribosomal DNA loci: introduction to a new online database. Chromosoma 127(1): 141-150 DOI: 10.1007/s00412-017-0651-8**, IF 3.5**

We set up the animal rDNA database containing cytogenetic information about these loci in 1343 animal species (264 families) collected from 542 publications. The data are based on in situ hybridisation studies carried out in major groups of vertebrates and invertebrates. The database is accessible online at www. animalrdnadatabase. com. The median number of 45S and 5S sites was close to two per diploid chromosome set for both rDNAs despite large variation (1-74 for 5S and 1-54 for 45S sites). No significant correlation between the number of 5S and 45S rDNA loci was observed, suggesting that their distribution and amplification across the chromosomes follow independent evolutionary trajectories. Each group, irrespective of taxonomic classification, contained rDNA sites at any chromosome location. However, the distal and pericentromeric positions were the most prevalent (> 75% karyotypes) for 45S loci, while the position of 5S loci was more variable. We also examined potential relationships between molecular attributes of rDNA (homogenisation and expression) and cytogenetic parameters such as rDNA positions, chromosome number, and morphology. This database is being refreshed regularly to bring new data.

[Jana Sochorová (a member of IBP lab) and Sonia Garcia (co-laboaration with Botanical Institute in Barcelona) contributed equally.]

4. **Sochorova, J**; Coriton, O; **Kuderova, A; Lunerova, J**; Chevre, AM; **Kovarik, A.** (2017) Gene conversion events and variable degree of homogenization of rDNA loci in cultivars of Brassica napus. Annals of Botany 119(1): 13-26., DOI: 10.1093/aob/mcw187; **IF 4.0**

Brassica napus (AACC, 2n=38, oilseed rape) is a relatively recent allotetraploid species derived from the putative progenitor diploid species Brassica rapa (AA, 2n =20) and Brassica oleracea (CC, 2n=18). To determine the influence of intensive breeding conditions on the evolution of its genome, we analysed structure and copy number of rDNA in 21 cultivars of B. napus, representative of genetic diversity. We used next-generation sequencing genomic approaches, Southern blot hybridization, expression analysis and fluorescence in situ hybridization (FISH). Subgenome-specific sequences derived from rDNA intergenic spacers (IGS) were used as probes for identification of loci composition on chromosomes. Most B. napus cultivars (18/21, 86 %) had more A-genome than C-genome rDNA copies. Three cultivars analysed by FISH

('Darmor','Yudal' and 'Asparagus kale') harboured the same number (12 per diploid set) of loci. In B. napus 'Darmor', the A-genome-specific rDNA probe hybridized to all 12 rDNA loci (eight on the A-genome and four on the C-genome) while the C-genome-specific probe showed weak signals on the C-genome loci only. Deep sequencing revealed high homogeneity of arrays suggesting that the C-genome genes were largely overwritten by the A-genome variants in B. napus 'Darmor'. In contrast, B. napus 'Yudal' showed a lack of gene con-version evidenced by additive inheritance of progenitor rDNA variants and highly localized hybridization signals of subgenome-specific probes on chromosomes. Brassica napus 'Asparagus kale' showed an intermediate pattern to 'Darmor' and 'Yudal'. At the expression level, most cultivars (95 %) exhibited stable A-genome nucleolar domi-nance while one cultivar ('Norin 9') showed co-dominance.The B. napus cultivars differ in the degree and direction of rDNA homogenization. The prevalent di-rection of gene conversion (towards the A-genome) correlates with the direction of expression dominance indicating that gene activity may be needed for interlocus gene conversion.

(At least 80% of the work on the article was done by members of the Laboratory of Molecular Epigenetics)

5. **Lunerova, J**; Renny-Byfield, S; **Matyasek, R**; Leitch, A; **Kovarik, A**. (2017) Concerted evolution rapidly eliminates sequence variation in rDNA coding regions but not in intergenic spacers in Nicotiana tabacum allotetraploid, Plant Systematics and Evolution 303 (8): 1043-1060, DOI: 10.1007/s00606-017-1442-7; **IF=1.3**

Nicotiana tabacum (tobacco) is a natural allotetraploid that formed from two diploid progenitors (N. sylvestris-S-genome, N. tomentosiformis-T-genome) within past 0.2 million years. Previous classical studies have shown that its 35S rDNA has been largely homogenised towards T-genome-like homeologs. However, the degree of conversion at single nucleotide resolution remains unknown. Here, we analysed intragenomic variation of rDNA at high resolution in natural tobacco, synthetic tobacco and the progenitors employing genomic, molecular and cytogenetic methods. In synthetic tobacco, we identified 13 highly (>= 10% units) polymorphic sites in the 18S-5.8S-26S coding region. In contrast, only a single polymorphic site was detected in natural tobacco, indicating that gene conversion has removed most of the polymorphisms over shallow evolutionary times. However, the non-coding 26S-18S intergenic spacer (IGS) was highly polymorphic in both natural (57 polymorphic sites) and synthetic tobacco (128 polymorphic sites). In natural tobacco, most (64%) IGS polymorphisms were inherited from the N. tomentosiformis progenitor, while 36% appeared de novo indicating rapid rates of sequence divergence of IGS. FISH revealed that the T-genome-like units (harbouring N. tomentosiformis-type IGS) occurred on all four loci in tobacco variety 095-55, including those loci derived from N. sylvestris progenitor, while the variety SR-1 retained 1-2 S-genome loci unconverted and transcriptionally silenced. We discuss potential caveats associated with experimental and in silico approaches used for determination of rDNA polymorphisms. We also hypothesise that polyploidy-associated gene conversion may eliminate mutated and non-functional genes that have accumulated in progenitor genomes, thereby contributing to success of polyploidy species.

(At least 70% of the work on the article was done by members of the Laboratory of Molecular Epigenetics)

6. **Matyasek, R; Dobesova, E; Huska, D; Jezkova, I**; Soltis, PS; Soltis, DE; **Kovarik, A.** (2016) Interpopulation hybridization generates meiotically stable rDNA epigenetic variants in allotetraploid Tragopogon mirus. Plant Journal 85(3): 362-377; DOI: 10.1111/tpj.13110; **IF=6.1**

Allotetraploid Tragopogon mirus composed of Tragopogon dubius (d) and Tragopogon porrifolius (p) genomes shows highly variable ND. To examine the molecular basis of such variation, we studied the genetic and epigenetic features of rDNA homeologs in several lines derived from recently and independently formed natural populations. Inbred lines derived from T. mirus with a dominant d-rDNA homeolog transmitted this expression pattern over generations, which may explain why it is prevalent among natural populations. In contrast, lines derived from the p-rDNA dominant progenitor were meiotically unstable, frequently switching to co-dominance. Interpopulation crosses between progenitors displaying reciprocal ND resulted in d-rDNA dominance, indicating immediate suppression of p-homeologs in F-1 hybrids. Original p-rDNA dominance was not restored in later generations, even in those segregants that inherited the corresponding parental rDNA genotype, thus indicating the generation of additional p-rDNA and d-rDNA epigenetic variants. Despite preserved intergenic spacer (IGS) structure, they showed altered cytosine methylation and chromatin condensation patterns, and a correlation between expression, hypomethylation of RNA Pol I promoters and chromatin decondensation was apparent. Reversion of such epigenetic variants occurred rarely, resulting in co-dominance maintained in individuals with distinct genotypes. Generally, interpopulation crosses may generate epialleles that are not present in natural populations, underlying epigenetic dynamics in young allopolyploids. We hypothesize that highly expressed variants with distinct IGS features may induce heritable epigenetic reprogramming of the partner rDNA arrays, harmonizing the expression of thousands of genes in allopolyploids.

(At least 90% of the work on the article was done by members of the Laboratory of Molecular Epigenetics)

7. **Huska, D**; Leitch, IJ; de Carvalho, JF; Leitch, AR; Salmon, A; Ainouche, M; **Kovarik, A.** (2016) Persistence, dispersal and genetic evolution of recently formed Spartina homoploid hybrids and allopolyploids in Southern England, Biological Invasions, 18(8): 2137-2151, DOI: 10.1007/s10530-015-0956-6; **IF=3.1**

In Southampton Water, UK, the recent (c. 150 years ago) interspecific hybridisation between Spartina alterniflora (2n = 6x = 62; A-genome) and S. maritima (2n = 6x = 60; M-genome) gave rise to the homoploid hybrid (S. x townsendii, 2n = 6x = 62), and subsequently to the invasive allododecaploid species S. anglica (2n = 12x = 120-124) that has since spread worldwide. To address the question of dynamics of mixed ploidy populations involving these plants, we analysed number of Spartina populations in Southern England, UK, one of which was the presumed place of origin of the homoploid hybrid (Hythe). Using a combination of flow cytometry and ribosomal DNA (rDNA) genotyping we were able to identify the genomic composition and ploidy level of each

individual analysed. The data show that the homoploid hybrid still dominates the population at Hythe (82 % of individuals collected in that locality) since its origin in the nineteenth century. We also identified S. x townsendii for the first time on Hayling Island (66 % individuals), indicating dispersal beyond its likely origin. The fertile allododecaploid S. anglica was mainly found in populations outside the initial hybridisation site, on Hayling Island and at Eling Marchwood. Quantification of the rDNA contributions from each parental genome showed that the ratios were mostly balanced in S. x townsendii. However, two (3 %) S. anglica individuals analysed have lost nearly all M-genome homeologs, indicating extensive repeat loss. Such variation indicates that despite the presumed single allopolyploid origin of S. anglica and genetic uniformity at other loci, it has undergone substantial changes at the rDNA loci following genome duplication.

(At least 60% of the work on the article was done by members of the Laboratory of Molecular Epigenetics)

8. **Dobesova, E., Malinska, H., Matyasek, R.**, Leitch, AR., Soltis, DE., Soltis, PS.**, Kovarik, A**. (2015) Silenced rRNA genes are activated and substitute for partially eliminated active homeologs in the recently formed allotetraploid, Tragopogon mirus (Asteraceae), Heredity 114(3), 356-365, DOI: 10.1038/hdy.2014.111, **IF 3,8**

To study the relationship between uniparental rDNA silencing (nucleolar dominance) and rRNA gene dosage, we studied a recently emerged allotetraploid Tragopogon mirus, formed from the diploid progenitors T. dubius (D-genome donor) and T. porrifolius (P-genome). We used molecular, cytogenetic and genomic approaches to analyse rRNA gene activity in two sibling T. mirus plants (33A and 33B) with widely different rRNA gene dosages. Plant 33B had similar to 400 rRNA genes at the D-genome locus, which is typical for T. mirus, accounting for similar to 25% of total rDNA. We observed characteristic expression dominance of T. dubius-origin genes in all organs. Its sister plant 33A harboured a homozygous macrodeletion that reduced the number of T. dubius-origin genes to about 70 copies. It showed biparental rDNA expression in root, flower and callus, but not in leaf where D-genome rDNA dominance was maintained. There was upregulation of minor rDNA variants in some tissues. The RNA polymerase I promoters of reactivated T. porrifolius-origin rRNA genes showed reduced DNA methylation, mainly at symmetrical CG and CHG nucleotide motifs. We hypothesise that active, decondensed rDNA units are most likely to be deleted via recombination. The silenced homeologs could be used as a 'first reserve' to ameliorate mutational damage and contribute to evolutionary success of polyploids. Deletion and reactivation cycles may lead to bidirectional homogenisation of rRNA arrays in the long term.

(At least 90% of the work on the article was done by members of the Laboratory of Molecular Epigenetics)

9. Wencai Wang, Tao Wan, Hannes Becher, **Alena Kuderova**, Ilia J. Leitch, Sònia Garcia, Andrew R. Leitch and **Aleš Kovařík** (2019) Remarkable variation of ribosomal DNA organization and copy number in gnetophytes, a distinct lineage of gymnosperms. Annals of Botany 123:767–781; DOI: 10.1093/aob/mcy172; **IF=4.0**

We showed that gnetophytes are distinct from other gymnosperms and angiosperms as they display surprisingly large variability in rDNA organization and rDNA copy and locus numbers between genera, with no relationship between copy numbers and genome sizes apparent. The 5S and 35S rRNA genes were separate in Gnetum montanum, Gnetum gnemon and Welwitschia mirabilis and linked in Ephedra altissima. There was considerable variability in 5S rDNA abundance, ranging from as few as ~4000 (W. mirabilis) to >100 000 (G. montanum) copies. A similar large variation was also observed in 5S rDNA locus numbers (two to 16 sites per diploid cell). Concerted evolution of 5S rDNA units seems to have led to the amplification of 5S pseudogenes in both Gnetum montanum and Ephedra altissima. 5S rRNA pseudogenes were interspersed between functional genes forming a single unit in both species. Their copy number was comparable or even higher than that of functional 5S rRNA genes. In E. altissima internal transcribed spacers of 35S rDNA were long and intrinsically repetitive while in G. montanum and W. mirabilis they were short without the subrepeats. Evolutionary patterns of rDNA show both gymnosperm and angiosperm features underlining the diversity of the group.

(Approximately 50% of the work on the article was done by members of the Laboratory of Molecular Epigenetics)

10. Wang, WC (Wang; Ma, L; Becher, H; Garcia, S; Kovarikova, A; Leitch, IJ; Leitch, AR; **Kovarik, A.** (2016) Astonishing 35S rDNA diversity in the gymnosperm species Cycas revoluta Thunb. Chromosoma 125(4): 683-699, DOI: 10.1007/s00412-015-0556-3; **IF=3.4**

We compared 35S rDNA divergence in several seed plants using next generation sequencing and a range of molecular and cytogenetic approaches. Most species showed similar 35S rDNA homogeneity indicating concerted evolution. However, Cycas revoluta exhibits an extraordinary diversity of rDNA repeats, influencing both the coding and non-coding rDNA regions nearly equally. In contrast, its rRNA transcriptome was highly homogeneous suggesting that only a minority of genes encode functional rRNA. The most common SNPs were C > T substitutions located in symmetrical CG and CHG contexts which were also highly methylated. Both functional genes and pseudogenes appear to cluster on chromosomes. The extraordinary high levels of 35S rDNA diversity in C. revoluta, and probably other species of cycads, indicate that the frequency of repeat homogenisation has been much lower in this lineage, compared with all other land plant lineages studied. This has led to the accumulation of methylation-driven mutations and pseudogenisation. Potentially, the reduced homology between paralogs prevented their elimination by homologous recombination, resulting in long-term retention of rDNA pseudogenes in the genome.

(Approximately 40% of the work on the article was done by Aleš Kovařík, a member of the Laboratory of Molecular Epigenetics)

11. Symonova, R; Ocalewicz, K; Kirtiklis, L; Delmastro, GB; Pelikanova, S; Garcia, S; **Kovarik, A.** (2017) Higher-order organisation of extremely amplified, potentially functional and massively methylated 5S rDNA in European pikes (Esox sp.), BMC Genomics 18, Article Number: 391, DOI: 10.1186/s12864-017-3774-7, **IF=3.6**

The 5S rDNA loci occupy exclusively (peri) centromeric regions on 30-38 acrocentric chromosomes in both E. lucius and E. cisalpinus. The large number of loci is accompanied by extreme amplification of genes (>20,000 copies), which is to the best of our knowledge one of the highest copy number of rRNA genes in animals ever reported. Conserved secondary structures of predicted 5S rRNAs indicate that most of the amplified genes are potentially functional. Only few SNPs were found in genic regions indicating their high homogeneity while intergenic spacers were more heterogeneous and several families were identified. Analysis of 10-30 kb-long molecules sequenced by the PacBio technology (containing about 40% of total 5S rDNA) revealed that the vast majority (96%) of genes are organised in large several kilobase-long blocks. Dispersed genes or short tandems were less common (4%). The adjacent 5S blocks were directly linked, separated by intervening DNA and even inverted. The 5S units differing in the intergenic spacers formed both homogeneous and heterogeneous (mixed) blocks indicating variable degree of homogenisation between the loci. Both E. lucius and E. cisalpinus 5S rDNA was heavily methylated at CG dinucleotides. Extreme amplification of 5S rRNA genes in the Esox genome occurred in the absence of significant pseudogenisation suggesting its recent origin and/or intensive homogenisation processes. The dense methylation of units indicates that powerful epigenetic mechanisms have evolved in this group of fish to silence amplified genes. We discuss how the higher-order repeat structures impact on homogenisation of 5S rDNA in the genome.

(Approximately 40% of the work on the article was done by Aleš Kovařík, a member of the Laboratory of Molecular Epigenetics)

12. Herklotz, V; **Kovarik, A; Lunerova, J**; Lippitsch, S; Groth, M; Ritz, CM (2018) The fate of ribosomal RNA genes in spontaneous polyploid dogrose hybrids [Rosa L. sect. Caninae (DC.) Ser.] exhibiting non-symmetrical meiosis. Plant Journal 94 (1): 77-90 DOI: 10.1111/tpj.13843; **IF=6.1**

Dogroses represent an exceptional system for studying the effects of genome doubling and hybridization: their asymmetrical meiosis enables recombination in bi-parentally inherited chromosomes but prevents it in maternally inherited ones. We employed fluorescent insitu hybridization, genome skimming, amplicon sequencing of genomic and cDNA as well as conventional cloning of nuclear ribosomal DNA in two phylogenetically distinct pentaploid (2n=5x=35) species, Rosa canina and Rosa inodora, and their naturally occurring reciprocal hybrids, Rosa dumalis (5x) and Rosa agrestis (5x, 6x). Both progenitor species differed in composition, meiotic behaviour and expression of rDNA loci: R.canina (five 18S and 5-8 5S loci) was dominated by the Canina ribotypes, but R.inodora (four 18S loci and 7-8 5S loci) by the Rubiginosa ribotype. The co-localized 5S/18S loci occurred on either bivalent-forming (R.canina) or univalent-forming (R.inodora) chromosomes. Ribosomal DNA loci were additively inherited; however, the Canina ribotypes were dominantly expressed, even in genotypes with relatively low copy number of these genes. Moreover, we observed rDNA homogenization towards the paternally transmitted Canina ribotype in 6x R.agrestis. The here-observed variation in arrangement and composition of rDNA types between R.canina and R.inodora suggests the involvement of different genomes in bivalent formation. This results supports the hypothesis that the asymmetrical meiosis arose at least twice by independent ancient hybridization events.

The asymmetrical meiosis in dogroses transfers in addition to pairing chromosomes several non-recombining genomes exclusively via egg cells to the offspring and is presumed to be a unique product of the allopolyploid origin of these plants. Cytogenetic and genomic analyses of two distantly related dogrose species and their natural hybrids revealed that pairing chromosomes profoundly differ in rDNA composition, copy number, and expression supporting a multiple origin of the meiosis system.

(Approximately 50% of the work on the article was done by members of the Laboratory of Molecular Epigenetics)

13. Corinne Mhiri, Christian Parisod, Julien Daniel, Maud Petit, K. Yoong Lim, Francßois Dorlhac deBorne, **Ales Kovarik**, Andrew R. Leitch and Marie-Angele Grandbastien. (2019) Parental transposable element loads influence their dynamics in young Nicotiana hybrids and allotetraploids. New Phytologist 221:1619–1633; DOI: 10.1111/nph.15484; **IF=8.5**

The genomic shock hypothesis suggests that allopolyploidy is associated with genome changes driven by transposable elements, as a response to imbalances between parental insertion loads. To explore this hypothesis, we compared three allotetraploids, Nicotiana arentsii, N. rustica and N. tabacum, which arose over comparable time frames from hybridisation between increasingly divergent diploid species. We used sequence-specific amplification polymorphism (SSAP) to compare the dynamics of six transposable elements in these allopolyploids, their diploid progenitors and in correspond-ing synthetic hybrids. We show that element-specific dynamics in young Nicotiana allopolyploids reflect their dynamics in diploid progenitors. Transposable element mobilisation is not concomitant with immediate genome merger, but occurs within the first generations of allopolyploid formation. In natural allopolyploids, such mobilisations correlate with imbalances in the repeat profile of the parental species, which increases with their genetic divergence. Other restructuring leading to locus loss is immediate, nonrandom and targeted at specific subgenomes, indepen-dently of cross orientation. The correlation between transposable element mobilisation in allopolyploids and quantitative imbalances in parental transposable element loads supports the genome shock hypothesis proposed by McClintock.

(Approximately 10% of the work on the article was done by Aleš Kovařík, a member of the Laboratory of Molecular Epigenetics)

14. Garcia, S; **Kovarik, A**; Leitch, AR; Garnatje, T. (2017) Cytogenetic features of rRNA genes across land plants: analysis of the Plant rDNA database. Plant Journal 89(5): 1020-1030, DOI: 10.1111/tpj.13442; **IF=6.1**

The online resource http://www.plantrdnadatabase.com/ stores information on the number, chromosomal locations and structure of the 5S and 18S-5.8S-26S (35S) ribosomal DNAs (rDNA) in plants. This resource was exploited to study relationships between rDNA locus number, distribution, the occurrence of linked (L-type) and separated (S-type) 5S and 35S rDNA units, chromosome number, genome size and ploidy level. The analyses presented summarise current knowledge on rDNA locus numbers and distribution in plants. We analysed 2949 karyotypes, from 1791 species

and 86 plant families, and performed ancestral character state reconstructions. The ancestral karyotype (2n = 16) has two terminal 35S sites and two interstitial 5S sites, while the median (2n = 24) presents four terminal 35S sites and three interstitial 5S sites. Whilst 86.57% of karyotypes show S-type organisation (ancestral condition), the L-type arrangement has arisen independently several times during plant evolution. A non-terminal position of 35S rDNA was found in about 25% of single-locus karyotypes, suggesting that terminal locations are not essential for functionality and expression. Single-locus karyotypes are very common, even in polyploids. In this regard, polyploidy is followed by subsequent locus loss. This results in a decrease in locus number per monoploid genome, forming part of the diploidisation process returning polyploids to a diploid-like state over time.

(Approximately 30% of the work on the article was done by Aleš Kovařík, a member of the Laboratory of Molecular Epigenetics).

15. D'Ambrosio U, Alonso-Lifante MP, Barros K, **Kovarik, A**, Mas de Xaxars G, Garcia S. (2017) B-chrom: A database on B-chromosomes of plants, animals and fungi. New Phytologist 216(3):635-642, DOI: 10.1111/nph.14723, **IF=8.5**
In this article we present a new B chromosome database available on line at http://www.bchrom.csic.es/. The 5760 entries available in the database correspond to 2828 eukaryotic species which have been reported to present Bs in their genomes, of which 73.56% (2087 species) were plants (53.20% monocots and 46.80% eudicots), 25.95% (736 species) animals and 0.49% (14 species) fungi.

(Approximately 20% of the work on the article was done by Aleš Kovařík, a member of the Laboratory of Molecular Epigenetics).

Research activity and characterization of the main scientific results

The department focuses on investigation of molecular mechanisms related to immune system regulation under both physiological and pathological conditions including inflammation, infection and tissue injury. Inflammation in general is directed toward isolating and destroying invading microorganisms and injured cells and preparing the tissue for repair and regeneration. However, a variety of diseases are linked to deregulated inflammatory processes, including autoimmune, cardiovascular, or neurodegenerative diseases. We are improving our understanding of molecular mechanisms involved in the development of pathological inflammatory processes and detrimental effects of external factors. That will help to suggest new therapeutic strategies. In collaboration with medicinal chemists and pharmacological companies, we evaluate novel potential therapeutic targets and newly designed compounds to bring about improvements to therapy in the context of human disease. Whether alone or in collaboration, we are trying to take advantage of data validation *in vivo* where possible, having incorporated mouse experimental models to our repertoire, housed in our on-site animal facility at IBP.
The following topics were solved and major results were reached during the evaluated period:

Role of PMNLs in pathological inflammatory response
Our key scientific questions concern the most abundant phagocytes, the polymorphonuclear neutrophils (PMNLs), responsible for releasing mediators that trigger the inflammatory process. Upon activation, these cells, among other functional manifestations, produce reactive forms of oxygen and nitrogen during the so-called respiratory burst of PMNLs. Activated PMNLs contribute to the induction of vascular endothelial dysfunction followed by leukocyte adherence and their extravasation to the site of inflammation where they contribute to the target tissue damage.
Several specific objectives were devoted to the topic of the pathological role of PMNLs.

Myeloperoxidase affects endothelial and immune functions
We focus on an abundant PMNL enzyme myeloperoxidase (MPO) that is primarily responsible for antimicrobial defense. MPO is expressed in large amounts in PMNLs and is released upon their activation that evokes inflammatory processes in a decisive manner. Recently, with our collaborators, we have shown that MPO induces motility of PMNLs that is dependent on electrostatic interactions. Next, we focused on the interaction of highly cationic MPO with endothelial glycocalyx glycosaminoglycans. The major objective was to characterize to which extent MPO modulates the charge and three-dimensional structure of the glycocalyxs *in vitro* (Figure 1) **(Manchanda 2018)**. MPO mediated reduction in glycocalyx thickness and integrity was also observed by use of intravital microscopy *in vivo*. Similar effects were also observed in wild-type mice after a local inflammatory stimulus but not in MPO-knockout mice. MPO also induced neutrophil-mediated shedding of the endothelial glycocalyx protein syndecan-1. Since many of the glycocalyx functions are related to total glycocalyx negative charge, we concluded that the cationic MPO has major impact in this regard. Findings have significantly extended the knowledge of MPO inflammatory properties and identified previously unrecognized features of MPO as a potential pharmacological target.

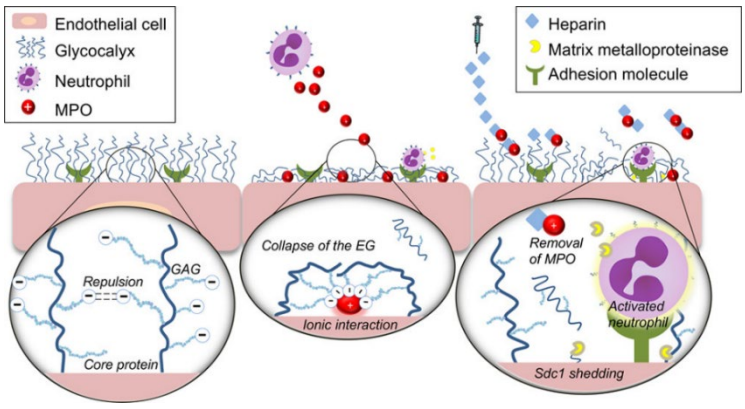


Figure. 1: MPO mediated alternation of endothelial glycocalyx

Importantly, the role of MPO in regulation of inflammatory processes is clearly dependent on the trigger and targeted tissue. In our following study we reported MPO dependent modulation of the course of acute pulmonary inflammation in mice induced by lipopolysaccharide (LPS) **(Kremserova 2016)**. In this model, we found a significance of MPO in apoptotic process of PMNLs that is important for a clearance of PMNLs from the site of inflammation. Collectively, the importance of MPO in regulation of pulmonary inflammation, independent of its putative microbicidal functions, can be potentially linked to MPO ability to modulate the life span of PMNLs and to affect accumulation of chemotactic factors at the inflammatory site.

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Note: The authors from the department are highlighted in the reference lists

Mediators released by PMNLs and prospects to modulate adverse PMNL affects

Regarding the role of PMNL in chronic inflammation, we searched for an obesity-induced nucleosome release which predicts poor cardio-metabolic health. We showed association between circulating nucleosomes and the risk of developing obesity, metabolic syndrome and/or a dysfunctional cardiovascular performance **(Lo Re 2019)**. We found that PMNLs are a bona fide source of circulating nucleosomes in an obesogenic environment and in overweight/obese patients. High nucleosome levels are associated with metabolic syndrome and cardiovascular performance, and they might represent novel candidate biomarkers for cardio-metabolic health.

While respiratory burst is important for the elimination of invading pathogens, the overproduction of reactive oxygen species (ROS) followed by the impairment of endogenous antioxidant defenses may lead to a damage of membrane lipids, DNA, proteins, and lipoproteins which results in various autoimmune and inflammatory diseases. Thus, an inhibition of this negative phagocyte activity is an important therapeutic task. We have been studying the possibilities of professional phagocyte modulation by exogenous compounds of plant origin as food constituents (polyphenols and polysaccharides) **(Georgiev 2017a, b, c and Kassab 2017)**.

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The importance of nitrated fatty acids as endogenous cardioprotective mediators and their possible therapeutic application

Two major effector systems are frequently implicated in the immune and endothelial cell alternations associated with inflammation and cardiovascular diseases: enhanced production of ROS and diminished bioavailability of nitric oxide (NO). Importantly, the oxidative milieu generated during inflammatory processes consists of a broad spectrum of oxidizing, nitrosating, and nitrating species and their products. This is also the case of nitrated unsaturated fatty acids (NO_2-FAs) that were shown to be endogenously occurring products of oxidant-induced nitration reactions. NO_2-FAs are present in the vascular compartment at nanomolar to low micromolar concentrations, enough to exert biological actions including inhibition of macrophage activation, pro-inflammatory cytokine secretion, and vascular smooth muscle cell proliferation. In light of the above described cell signaling roles of NO_2-FAs, it may be expected that there is a “threshold level” over which their physiological activities switch from signaling to pharmacological actions. The effects of NO_2-FAs are diverse; to begin with, these species serve as a potential chemical reserve of NO or can covalently modify nucleophilic protein targets to alter the structure and function of enzymes, receptors and transcription factors. Thus, it is possible that at the levels expected to be found in the vasculature during chronic inflammatory conditions, NO_2-FAs may serve not only as biomarkers or “footprints” of pathophysiological processes but also as novel protective agents, significantly counteracting pro-inflammatory effects of oxidant exposure. Thus, solid knowledge of molecular mechanisms responsible for NO_2-FAs action on cells and effects of NO_2-FAs in various animal models is needed.
In one study we found that nitro-oleic acid (NO_2-OA) regulates the functional specialization of macrophages, the so-called macrophage polarization, into specific pro-inflammatory "M1-like" or regulatory "M2-like" subsets. NO_2-OA modulated a broad range of "M1-" and "M2-like" macrophage functions. This was connected with NO_2-OA mediated inhibition of myocardial fibrosis *in vivo* **(Ambrožová 2016 FRBM).** Similarly, NO_2-OA regulates the process of macrophage differentiation, as was shown, employing isolated bone marrow and purified cells from bone marrow **(Vereščáková 2017, Macečková 2015).** We showed that NO_2-OA reduces colony formation and proliferation of differentiated macrophages via downregulation of STAT5, ERK, and PI3K activation. Additionally, NO_2-OA also attenuates activation of macrophages. Next, we focused on vascular endothelial inflammatory responses and the endothelial-mesenchymal transition as a consequence of the altered healing phase of the immune response **(Ambrožová 2016 BBA)**. NO_2-OA limited the activation of macrophages and ECs by reducing pro-inflammatory cytokine production and adhesion molecule expression through its modulation of STAT3 and 5, MAPK and NF-κB-regulated signaling. NO_2-OA also decreased endothelial-mesenchymal transition and pro-fibrotic phenotype of endothelial cells due to the downregulation of Smad2/3.
Further, we focused on atrial fibrosis, one of the most striking features in the pathology of atrial fibrillation, promoted by local and systemic inflammation **(Rudolph 2016)**. We showed that NO_2-OA limits atrial fibrosis and atrial fibrillation during the right atrial electrophysiological stimulation when the left atrial epicardial mapping studies demonstrated preservation of conduction homogeneity by NO_2-OA. These effects were mediated by suppression of Smad2-dependent myofibroblast transdifferentiation and inhibition of Nox2-dependent atrial superoxide formation.

Next, we focused on pathology of pulmonary hypertension that is associated with imbalance in vasoactive mediators and massive remodelling of pulmonary vasculature, representing a serious health complication. We described a novel mechanism of asymmetric dimethyl arginine (ADMA)-induced dysfunction in human pulmonary endothelial and smooth muscle cells related to alternation of STAT3 and hypoxia-inducible factor 1α (HIF-1α) signaling associated with drastic alternations in normal cellular functions (**Pekarová 2015)**. In the following study, we showed that NO_2-OA prevents hypoxia- and ADMA-induced pulmonary endothelial dysfunction by increasing NO production and endothelial NO synthase expression, decreasing pro-inflammatory mediators' production via the STAT3/HIF-1α cascade **(Koudelka 2016)**. Overall, these studies show the pleiotropic effect of NO_2-OA on regulation of EC-macrophage interactions during the immune response and suggest the role of NO_2-OA in the regulation of vascular endothelial immune and fibrotic responses arising during chronic inflammation. Importantly, we brought a new perspective on molecular mechanisms of NO_2-FAs action in pulmonary endothelial dysfunction, which represents a causal link in progression of PH. All together, these findings propose the NO_2-OA may be useful as a novel therapeutic agent for treatment of cardiovascular disorders associated with dysregulation of the endothelial immune response.

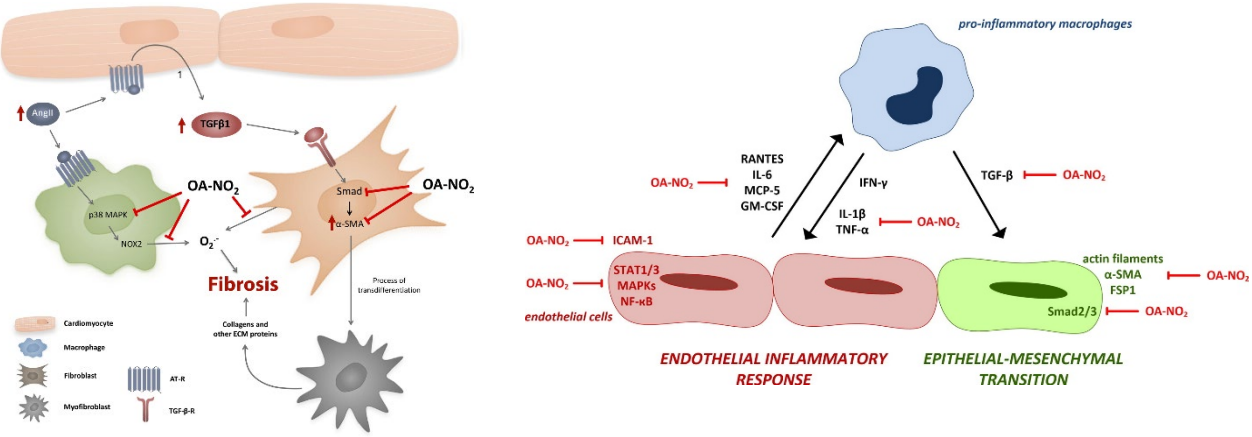

Fig 2: Proposed mechanism of NO_2-FAs mediated modulation of immune response and development of fibrosis and endothelial functions.

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Effect of water-bloom related pollutants on immune system functions and intestinal homeostasis

To build up on our expertise exploring mechanism of immune system activation, we have dedicated part of our research activities to current worldwide risks to human health. Massive expansion of water blooms and toxic cyanobacteria as their major component in sources of drinking and recreational water present one of these health hazards. There is currently only very little information on how they and their metabolites affect human heath under conditions of chronic i.e. toxically subacute exposures that are, however, the most common and dangerous situations **(Kubíčková 2019)**. This research is a current primary focus of Dr. Šindlerová group in which members explore mechanisms of immunomodulatory effects of different types of metabolites and toxins produced by cyanobacteria and cyanobacterial water blooms. We reported significant pro-inflammatory potency of selected metabolites recognized as toxins (microcystin (MC), cylindrospermopsin (CYN)) in very low non-toxic concentrations **(Adamovský 2015, Moosová 2018).** Our results brought a novel insight into the disruptive mechanism of MC showing that it may activate immune cells via Toll-like receptor 2 (Figure 3). Other toxin, CYN, potentiates the effect of LPS indicating potential increase of the overall toxicity of environmental mixtures.

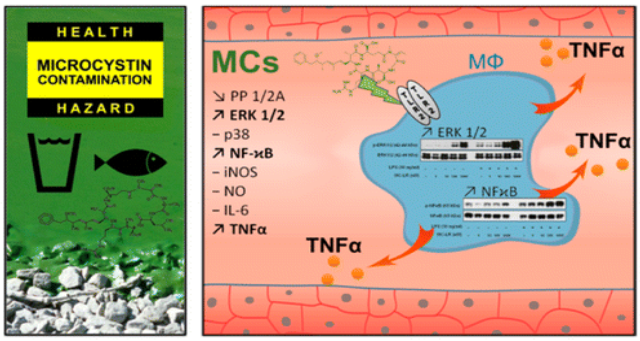

Fig 3: New proposed mechanism of macrophage activation by microcystin.

Besides, there are also other metabolites and parts of the cyanobacteria and water bloom mass that are considered to negatively affect human health. We have broadened our focus on the effects of LPS of cyanobacteria and water bloom in general as suspected significant pathological factors in contaminated water to influence human health. We study LPS isolated either from complex cyanobacterial water blooms obtained from recreational water bodies or cyanobacterial laboratory cultures. Interestingly, in contrast to predictions of some previous works by other authors we have proved pro-inflammatory potential of highly purified LPS from *Microcystis aeruginosa* **(Moosová 2019)**.

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Effects of inflammation mediators and alternated physical conditions on cell differentiation

To evaluate importance of inflammatory mediators and physical factors alternated during the inflammatory process on regeneration, particularly cardiac regeneration, we have employed a unique system of differentiation of embryonic stem (ES) cells into various lineages *in vitro* that represents an important tool for studying the mechanisms underlying cell differentiation as a key process of regeneration. In collaboration with Dr. Pachernik from MU, we have modified the protocol for mouse ES cell differentiation into different lineages using unique ES gene deficient cell types and techniques **(Radaszkiewicz 2016 and 2017).**

We have evaluated effects of altered oxygen levels, hypoxia, since it is a common condition of regenerating tissue under inflammatory conditions. Hypoxia was suggested as an important factor regulating self-renewal and differentiation. At the same time, increased levels of ROS are present at site of inflammation, both from accumulated phagocytes and from mitochondria of other cell types of affected tissue, that affects cell proliferation and differentiation. Nevertheless, information about the exact hypoxia- and ROS-mediated regulatory mechanism of stem cell fate during regenerative process is still missing.

Primarily, we have found that the stabilization of HIF-1α inhibited the proliferation of mouse ES cells and enhanced downregulation of the expressions of pluripotency markers **(Binó 2016).** Next, we have discovered that hypoxia affects differentiation of stem cells into cardiomyocytes through promotion of expression of myosin heavy chain (MHC) β **(Binó 2017).** These effects were shown to be driven by HIF-1α, however, the ChIP analysis did not confirm the direct interaction of HIF-1α with putative HIF response elements predicted in the MHCα and β encoding DNA region. Further analyses showed the significance of the mTOR signaling in induction of Myh7 expression and a hypoxia-triggered reduction of antisense RNA transcripts associated with the Myh7 gene locus.

Next, we focused on the transcription factor HIF-1, that is not only a master regulator of hypoxic response, but also a regulator of the response of cells to stress conditions. Interestingly, we showed HIF-1α deficiency attenuates cardiomyogenesis **(Kudová 2016).** HIF-1α deficiency significantly altered cell differentiation into cardiomyocytes based on parameters mentioned in the previous study and also the quality and quantity of mESC-derived cardiomyocytes. Interestingly, this was accompanied by significantly lower expression of the endoderm markers. Thus, we concluded that HIF-1α deficiency attenuates spontaneous cardiomyogenesis through the negative regulation of endoderm development. Based on this, we tried to encrypt the role of HIF1α in neural fate during differentiation. We identified that neural differentiation is inhibited through HIF1α/β-Catenin signaling in our model **(Večeřa 2017).** HIF-1α deficiency lead to abnormally increased neuronal characteristics during

differentiation that was accompanied by the disruption of β-catenin signaling. The knock-in of HIF-1α, as well as β-catenin ectopic overexpression in HIF1α-/- cells, induced a reduction in neural differentiation. Interestingly, direct interaction between HIF1α and β-catenin was demonstrated for the first time.
In our following research we focused on other important signaling pathway that mediates response to complex inflammatory environment which is MAPK p38alpha kinase activated in cells in response to various extracellular factors released during inflammation. We found that depletion of p38alpha kinase upregulates NADPH oxidase 2/NOX2/gp91 expression and promotes the production of ROS in our model mouse ES cells **(Binó 2019).** At the same time, we showed that hypoxia downregulates MAPK/ERK but not STAT3 signaling during ES cell differentiation **(Kučera 201).** This was driven by increased formation of ROS formation and was surprisingly independent of HIF-1α-signaling.
We also studied effects of opioid peptides such as dynorphins and enkephalins, since levels of these peptides are altered by inflammatory conditions **(Šínová 2019)**. We found that expressions of both κ- and δ-opioid receptors significantly increased during cell differentiation together with change in their localization. However, selected peptides dynorphin A, methionine-enkephalins and leucin-enkephalins exhibited no significant effects on the course of mouse ES cell differentiation. Overall, data do not support the notion that opioid peptides have a significant potential to affect tissue regeneration through modulation of cell differentiation.
Other molecule of interest was melatonin, as a molecule not only involved in the regulation of circadian rhythms but also with strong ROS scavenging capacity, that is suggested to have protective effects against tissue damage and support of regeneration. We showed that melatonin promotes cardiomyogenesis **(Kudová 2016).** We found that melatonin significantly upregulated the expression of cardiac markers (MHC 6 and 7) as well as the percentage of MHC-positive cells. We provided new evidence of a time-specific inhibition of HIF-1α stabilization as an essential feature of this melatonin-promotion of cardiomyogenesis since melatonin decreased HIF-1α stabilization and transcriptional activity and, in contrast, induced HIF-2α stabilization. Interestingly, the deletion of HIF-1α completely inhibited the pro-cardiomyogenic effect of melatonin.
Taken together, results uncover new mechanisms underlying the maturation of cardiac progenitor cells that can help in the development of novel strategies for using melatonin in cardiac regeneration therapy.

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Unique technologies and evaluation of novel potential therapeutic approaches to treat human diseases.

In addition to models and methodologies applied in studies mentioned above, we have several other unique methodologies that are used for evaluation of mechanisms of inflammation-based pathologies. These are also employed in our collaboration with external partners in studies focused on evaluation of new therapeutic approaches.

Fluidic and microfluidic devices to study pathological processes in vessels

Group of Dr. Víteček focuses on development of unique fluidic systems that provide unique opportunity to study blood flow and shear stress in pathophysiology of cardiovascular system.

The first topic is related to perception of mechanical cues related to blood flow and proper reaction to them. In particular, we are interested in the relationship of the flow disturbances and vascular inflammation and the impact of shear stress onto cell fate. To conduct such studies, unique microfluidic chips with circular cross section channels are being constructed and colonized with endothelial cells to study vascular physiology in *in vitro* models (fig. 4A). An adjacent investigation of this topic focuses on the relation between hemodynamics and fate of arterial stenosis and aneurysm. The idea of this effort is to develop a prognostic tool to predict life-threatening pathologies (e.g. atherosclerotic plate or aneurysm rupture). On this subproject which takes advantage of 3D realistic patient-based vascular models (fig. 4B), we cooperate with Dr. Aleš Hejčl.

The second topic focuses on devices to study thrombolysis and development of new thrombolytics. Thromboembolic events are behind the most serious cardiovascular diseases (ischemic stroke, myocardial infarction and embolism). Current treatment involves catheterization or intravenous thrombolysis. Despite obvious advantages of intravenous thrombolytics, the approach suffers from limited efficiency. The aim of the project is to determine factors behind the limited efficiency using an *in vitro* fluidic model. During the evaluation period, an elucidation of basic aspects of a thrombolytic enzyme – alteplase was carried out in static *in vitro* model. Currently, fluidic models mimicking exactly the morphological features of affected human blood vessels are employed. This approach allows to identify biochemical and biophysical factors that are critical for sufficient thrombolysis. The work is carried out in cooperation with Prof. Mikulík (FNUSA-ICRC), the director of the research cluster STROKE (see below - the part Participation in large collaborations). Within activity of this cluster we contribute to improvement of ischemic stroke treatment by means of *in vitro* thrombolysis evaluation. Thus, implementation of our findings in clinical research may contribute to improved clinically applicable thrombolytics.

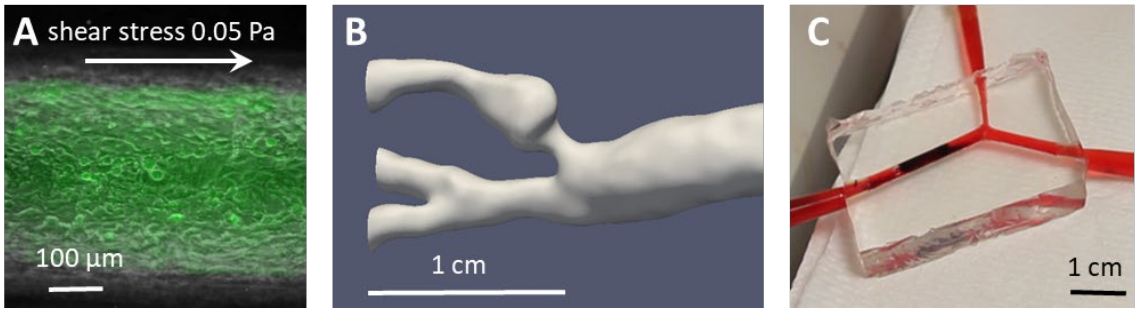

Figure 4.: A Endothelial cell culture in a circular cross-section channel grown under flow conditions. **B** 3D realistic model of stenosis on internal carotid to study hemodynamics. **C** Fluidic model mimicking middle cerebral artery to study thrombolysis.

Hyaluronan based medicinal materials and therapeutics

This topic focuses on evaluation of hyaluronan (HA) regulatory functions in the organism with the goal to improve our knowledge of HA effects on cells, tissue and whole organism to find theoretical background and inspiration for HA based therapeutics. This topic is closely being solved with biotech company Contipro.
We evaluated what is the role of HA in inflammation based pathological processes, with particular focus on HA of different molecular weight **(Šafránková 2018)**. Further, employing our different *in vitro* and *in vivo* models allowing to explore the mechanism of immune system activation and tissue regeneration we study basic mechanisms on cell, tissue and organism functions of modified HA, e. g. HA-fatty acid derivatives, HA based films, nonwoven fabrics and HA- based nanomicellar structures with a goal to suggest new materials for therapeutic and medicinal use **(Huerta-Ángeles 2018, Chmelař 2019)**.
During the last year of the evaluation period, new project focused on potential of HA-based derivatives in prevention of pro-fibrotic processes in peritoneum started. Peritoneal adhesions represent one of the major complications following intra-abdominal and pelvic surgery leading to symptoms such as abdominal pain, bowel obstruction and infertility. Chronic inflammation mediated by myeloid cells, with significant role of PMNLs, participates in development of this process. To study peritoneal adhesions, we developed new *in vivo* models of localized and "diffuse" adhesions and fibrotic processes in mouse peritoneum. To reveal the molecular mechanisms of peritoneal adhesions development, primary cultures of mesothelial cells and peritoneal fibroblasts were isolated and transformed to a pro-fibrotic phenotype, and then protein markers and signaling pathways are studied. The role of PMNLs infiltration in fibrotic lesions was evaluated by flow cytometry; production of pro-inflammatory and fibrinolytic soluble mediators was measured. Changes in cell metabolism of participating cell types are being studied. Understanding the underlying molecular mechanisms is necessary for implementation of effective strategies to prevent this pathological process of peritoneal fibrosis. Newly developed soluble HA-based antiadhesive barriers started to being tested in cooperation with Contipro a.s.

Relevant references:

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Chmelař J, Mrázek J, Hermannová M, **Kubala L, Ambrožová G, Kocurková A,** Drmota T, Nešporová K, Grusová L, Velebný V. Biodegradable free-standing films from lauroyl derivatives of hyaluronan. Carbohydr Polym. 2019 Nov 15;224:115162.

Šafránková B. Hermannová M, Nešporová K, Velebný V, **Kubala L.** Absence of differences among low, middle, and high molecular weight hyaluronan in activating murine immune cells in vitro. Int J Biol Macromol. 2018 Feb;107(Pt A):1-8.

Specific isoforms of adenylate cyclases as a potential therapeutic target

Cyclic AMP (cAMP) is an important intracellular second messenger, which is produced by adenylate cyclases (ACs). Ten mammalian AC isoforms identified so far, differ in their tissue distribution and biochemical regulation. The known activator of membrane AC isoforms 1 to 8 is labdane diterpene forskolin. Importantly, signaling pathways regulated by cAMP are recognized as important regulators of functions of cells involved in immune response and inflammation. Particularly, three dominant AC isoforms (AC3, AC7 and AC9) are suggested. However, the significance of individual isoform on function of specific leukocytes subsets is not well understood. Thus, one of our aims is to clarify the importance of different AC isoforms in regulation of functions of selected leukocyte subpopulation (PMNLs, T- and B-cells) and to elucidate possibilities how to precisely modulate production of cAMP in these cell types.
This research should provide solid background for drug development based on modulation of specific AC isoform activity. This is done with close collaboration with medicinal chemists Assoc. prof. Kamil Paruch and Dr. Jakub Švenda, from Faculty of Science at MU, Brno who developed unique synthesis of forskolin that allows to synthetize unique forskolin derivatives. We screen these newly synthesized derivatives for their potential to specifically modulate activation of particular AC isoform. For this purpose, we developed clones of eukaryotic cells overexpressing selected isoforms of adenylate cyclases used for live cell- and isolated membrane-based screening of modulation of the enzymatic activity of different AC isoforms **(Hylse 2017)**.

Relevant references:

Hylse O, Maier L, Kučera R, **Perečko T, Svobodová A, Kubala L,** Paruch K, Švenda J. A Concise Synthesis of Forskolin. Angew Chem Int Ed Engl. 2017 Oct 2;56(41):12586-12589.

Organic semiconductors for modulation of cell functions and development of sensors

Our other scientific topic is exploration of bio-interfaces based on organic semiconductors. This effort focuses on aspects of bridging electronic circuits with living cells via unique interfaces based on organic semiconductive materials. In cooperation with Brno University of Technology (Prof. Martin Weiter) new material formulations of organic semiconductors were established and thoroughly characterized. Then sensors and actuators based on these materials were constructed and concept proof was carried out. The specific part of work provided by our team involved biological characterization of these sensors or actuator. The overall goal is to develop organic electronics-based devices to determine or modulate (patho)physiological processes in cells **(Salyk 2017, Střítеský 2018a, Střítеský 2018b, Šafaříková 2018)**.

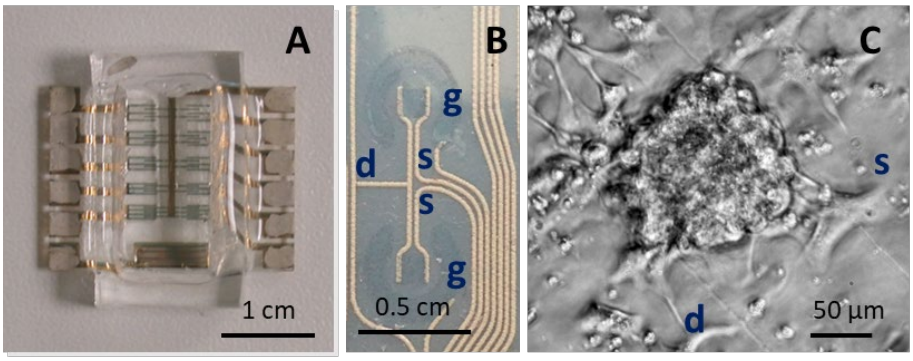

Figure 5.: A Proof of concept sensor/actuator platform to work with animal cells. **B** Layout of a stable platform of organic electrochemical transistors based on PEDOT:PSS (Salyk 2017). **C** Murine cardiomyocytes syncytium integrated with an organic electrochemical transistor (Stříteský 2018a). (s) – source, (d) – drain and (g) - gate electrodes.

Relevant references:

Šafaříková E, Švihálková Šindlerová L, Stříteský S, **Kubala L,** Vala M, Weiter M, **Víteček J.** Evaluation and improvement of organic semiconductors' biocompatibility towards fibroblasts and cardiomyocytes, Sensors and Actuators B: Chemical 2018, 260, 418-425, https://doi.org/10.1016/j.snb.2017.12.108

Stříteský S, Marková A, **Víteček J, Šafaříková E.,** Hrabal M, Kubáč L, **Kubala L,** Weiter M, Vala M. Printing Inks of Electroactive Polymer PEDOT:PSS: The Study of Biocompatibility, Stability and Electrical Properties. Journal of Biomedical Materials Research: Part A, 2018 106A: 1121–1128http://onlinelibrary.wiley.com/doi/10.1002/jbm.a.36314/full, (a)

Stříteský S, Vala M, David J, **Šafaříková E, Víteček J,** Weiter M. The bottom gate, bottom contact DPP(TBFu)2 solution processed transistor for bioelectronics applications. Chemical Papers 2018 72 (7) 1635–164, (b)

Salyk O, **Víteček J,** Omasta L, **Šafaříková E,** Stříteský S, Vala M, Weiter M, Organic Electrochemical Transistor Microplate for Real-Time Cell Culture Monitoring, Applied Sciences, 2017, 7, 998; doi:10.3390/app7100998

Research activity and characterisation of the main scientific results

During the last five years, the team performed research oriented into several directions, mostly according to the plan, described in last evaluation report, namely: 1) secondary structures involved in arrangement and functional properties of telomeric sequences; 2) sequence-dependent properties of cytosine i-motifs; 3) the influence of nucleosides mutations or substitutions on the secondary structures of nucleic acids. The detailed research activities and selected outputs are described accordingly, though there is always an overlap between the directions. Altogether, the team members published 27 publications in international impacted journals and the team contribution ranged from purely in-house performed research, through most frequent equal contribution with one partner, up to brief methodological or intellectual complement to the project of other team(s).

We continued in the studies of conformational properties of telomere sequences. Significant amount of our research was done in collaboration with the team of Dr. Lukáš Trantírek and, to a lesser extent also of Prof. Radek Marek, at the CEITEC, Brno, CR. They focus on NMR studies of nucleic acids, which represents ideal complementary methodology to those adopted by our team. Together we described unique behaviour of C. elegans telomeric sequences indicating that not the primary sequence itself, but the ability to form stable non-B-DNA secondary structure is the key shared property of telomeric sequences that is involved in the function of telomeres. The results were published in Školáková et al., Nucleic Acids Res, 2015. The team's share is about 50% and consists both in experimental work, namely spectroscopic techniques, and in intellectual work including manuscript preparation. We also extended our former works on the structure of guanine quadruplex formed by guanine-rich strand of the human-type telomere sequence, where we followed the influence of various types of naturally occurring base lesions, often formed as a result of ionizing radiation, on the structure and thermodynamic stability of guanine quadruplex formed. Such a lesion, when resulting into conformational change, might influence the interactome and potential druggability of the quadruplex. We showed that the loop adenine conversion into abasic site leads to switch in conformation of the resulting quadruplexes and this effect is much more pronounced when clustering of lesions, described for double stranded DNA as a result of ionizing radiation, occur. The results were published in Kejnovská et al., Nucleic Acids, Res, 2017, with dominant team's share and minor intellectual or experimental (1H NMR) contribution of Dr. Trantírek, Dr. Fiala and Dr. Sagi. We also tried to employ another spectroscopic method, a Raman spectroscopy, for determination of guanine quadruplex conformations. We simultaneously followed environment induced conformational transitions of several variants of human telomere sequence by circular dichroism spectroscopy and by Raman spectroscopy. The involvement of Raman spectroscopy could potentially provide an information about the conformational changes on a level of functional groups. This research was conducted in long-standing collaboration with Dr. Peter Mojzeš at the Charles University in Prague and published in Palacký et al., J Raman Spectroscopy, 2019. The collaborator, Dr. Mojzeš, participated intellectually and provided access to the Raman spectrometers housed at the MFF CUNI. As the telomere DNA is repetitive sequence, there are also other ways, how to deal with guanine lesions. Besides incorporating the lesion into quadruplex, effect of which we described earlier, the lesion-affected repeat might be excluded either into linker region between two quadruplexes, or it can be excluded into loop of the quadruplex. We described how such exclusions of lesion-affected repeats

influence the biophysical properties of the resulting quadruplex and we published the results in Dvořáková et al., BBA General Subjects, 2017. The output was prepared purely by the team. There are reports that the in-cell environment differs from that used for most in vitro quadruplex studies, especially in terms of molecular crowding and dehydration. Others and we have shown before that the conformation of guanine quadruplexes might differ in water solutions and in crowding/ dehydrating solutions. Recently, we showed that various guanine substitutions or lesions prevent this conformational change of guanine quadruplexes, including the telomere ones, into the parallel arrangement preferred in the dehydrating conditions. The results were reported in Bednářová et al., Chem Eur J, 2019. The output was prepared purely by the team.
Though most of our research focused on guanine-rich strand of telomere DNA, mainly due to the existence of single stranded G-rich overhang, dominant part of the telomere is double stranded and thus involves also cytosine-rich strand, prone to form cytosine i-motif, a structure based on hemiprotonated C:C+ pairs under more-less acidic pH. We followed the structure of four-repeat C-rich human telomere sequence with selected nucleosides substituted with various naturally occurring bases, potentially forming because of ionizing radiation. Simultaneously we used this C-rich telomere sequence as a model for simulating strand-slippage and re-arrangements effects within i-motif to uncover the adaptability of i-motif on the base lesions. We observed significant resistance of human telomere i-motif, in terms of thermodynamic stability, towards many of these substitutions. This work, published in Dvořáková et al., Nucleic Acids Res, 2018, served also as a background for consecutive detailed study of i-motif primary sequence requirements. The work was prepared with dominant team's share and minor intellectual contribution of the collaborator, Dr. Sagi. In the first part of the work, we designed extensive set of oligonucleotides to uncover, mostly using CD spectroscopy, elementary rules of i-motif structure, formation kinetics and thermodynamic stability as a function of primary sequence. In the second part, we analysed an unusual multi-state melting behaviour of i-motifs formed by longer, but still naturally relevant, blocks of cytosines. At only slightly acidic or neutral pH values, these sequences undergo several time and temperature-dependent transitions between different i-motif structures. As an explanation, we proposed a kinetic vs thermodynamic equilibrium between i-motifs of various C:C+ pair content. The results were published in Školáková et al., Nucleic Acids Res, 2019 with dominant team's share and a small experimental contribution (1H NMR) of Dr. Krafčík from the team of Dr. Trantírek.
The former results, published by DBNA team on the effect of various base lesion in G-rich strand of the human telomere sequence on the properties of guanine quadruplex, were summarized and put into broader context of recent literature in a review Konvalinová et al., Biochimie, 2017, prepared purely by the team members.
As an initial part of newly derived direction, currently carried out mostly by master students under supervision of Dr. Renčiuk, focusing on the functional roles of non-B structures and established as an inherent complement to structural studies, we followed the role of guanine quadruplex in the promoter of the *oct4* gene. We showed that its presence positively regulates the expression of the gene and the expression might be modulated by variability in the primary sequence on one hand and presence of G4 ligand on the other. The study was performed in equal collaboration, both experimental and intellectual, with the team of Dr. Lukáš Trantírek and published in Renčiuk et al., BBA-Gene Regulatory Mechanisms, 2017.
Members of the team also significantly contributed to a research focusing on the interaction of non-B structures of nucleic acids with proteins, namely with p53 protein under supervision of Dr. Marie Brázdová, IBP AS CR and with IFI16 protein under

supervision of Dr. Václav Brázda, IBP AS CR. The role of team members consisted in experimental (spectroscopic, electrophoretic) characterization of the structures of the protein target nucleic acids, analysis and interpretation of the data and preparation of the respective part of the results for publication. The outputs of the p53 project were published in Marek et al., Biosci Rep, 2016; Adámik et al., Biochimie, 2016; Brázdová et al., Plos ONE, 2016 and Helma et al., Molecules, 2019. The results of IFI16 project were published in Haroniková et al., Plos ONE, 2016.

Similar type of experiments, involving spectroscopic characterisation of non-B conformations of nucleic acids, and supportive type of contributions was applied also for collaborative studies of the electrochemical behaviour of non-B DNA structures of nucleic acids, supervised by Prof. Trnková (Trnková et al., Electroanalysis, 2019; Hudcová et al., Eur. Biophys. J. Biophys. Lett., 2015), Prof. Fojta (Vidláková et al., Anal Bioanal Chem, 2015), Prof. Vacek (Dorčák et al., Analyst, 2016), Dr. Ostatná (Vargová et al., Anal Chim Acta, 2016) and Dr. Doneux (De Rache et al., Electrochimica Acta, 2015). The DBNA team contribution consisted mostly in experimental part with subsequent data analysis and preparation of the respective part for publication.

Team member, dr. Kejnovská also performed a characterisation of the nucleic acid conformation for the project that used long-read SMRT (PacBio) sequencing for identification of non-B DNA conformations and the results were published in Guiblet et al., Genome Res, 2018.

Finally, Michaela Krafčíková and Silvie Foldynová-Trantírková, assigned to DBNA team due to the participation in collaborative OP project funded by ERDF and supervised by Dr. Jean-Louis Mergny and prof. Fojta (project SYMBIT), significantly participated, both experimentally and intellectually, on a results published by team of other project partner, prof. Lukáš Trantírek. The research focused on an in-cell detection of non-B DNA structures and their interactions with small molecule ligands, using a unique methodology of an in-cell nuclear magnetic resonance. The results show that two of three *in vitro*-validated ligands retain their ability to form stable interactions with their model target DNA *in cellulo*, whereas the third one loses this ability due to off-target interactions with genomic DNA and cellular metabolites. These results were published in Krafčíková et al., J Am Chem Soc, 2019. Michaela and Silvie also participated on several other publications of the team of Dr. Trantírek.

Research activity and characterisation of the main scientific results

In the period 2015 – 2019 we have published more than 100 papers (~17 papers with IF 10 or higher and several others in the range 8-10). The 2015-2019 papers have so far received around 1 300 citations **with all forms of auto-citations excluded**. Among the most interesting outcomes we note the following.

Revelation of the basic principles of DNA and RNA guanine quadruplex (G4) folding which differs from common simplistic few-state models popular in experimental literature. We have shown that G4 folding is more complex than often assumed. We have shown that the only possibility how to explain the experimental data is the kinetic partitioning mechanism (KPM). We have described the basic principles of KPM that are specific for G4 systems. We explained that the KPM for G4 molecules means competition among d iverse G4 folds that act as long-living (dominant) free-energy basins on the folding landscape. However, we have also suggested a key role that is played by diverse short-living transient (transitory) ensembles. These ensembles are entirely beyond the detection limits of contemporary experimental techniques but, in analogy to transition states or transition state ensembles, they can decisively contribute to the k_{on} and k_{off} folding rates of all basins on the landscape and thus also to the equilibrium constants. In case of G4 folding landscapes, the transitory ensembles can be exceptionally multidimensional and thus resist any dimensionality reduction descriptions; that is why we avoid calling them “states”.

Significant advancement is our contribution to the formulation of formamide scenario of the origin of life, in a close collaboration with laboratories of E. di Mauro and R. Saladino in Italy (who first noted the possibility of the formamide pathway around 2001), and Martin Ferus in Prague. Formamide scenario is a continuous chemical pathway of the emergence of the first RNA molecules from the simplest inorganic compounds via the formamide precursor rather than through the traditional HCN-based chemistry. The 2015 PNAS (joint with Ferus’s lab) paper demonstrating straightforward synthesis of NA bases from formamide via radical chemistry upon high-energy events (asteroid impact and lightning) has received world-wide press release via Associated Press. Since that we have published number of other papers dealing with key aspects of the formamide pathway, including accumulation of formamide under geochemically relevant conditions, synthesis of the nucleic acids building blocks in formamide-rich environment as well as template-free polymerization of 3’-5’ cyclic nucleoside monophospates to the first RNAs. The formamide scenario is still a new field, but its popularity is steadily growing. However, we also published papers on other possible scenarios, mainly dealing with the possible role of photochemical processes.

Substantial improvement of simulation force fields for nucleic acids. Current status is that our force fields OL3 (from 2010) and OL15 (from 2015) are recommended as first-choice force fields for RNA and DNA, respectively, in the AMBER program suite, perhaps the most widely used biomolecular software package (see the Table on p. 35 in https://ambermd.org/doc12/Amber20.pdf). However, based on major testing of mainly RNA simulation force fields in the period 2015-2019, we made a key strategic conclusion that the present force-field form is at its limits of being further tunable. In other words, we argue that it is not possible to obtain further improvements of mainly the RNA simulations by the traditional reparametrization efforts considering mainly modifications of the dihedral potentials, since the improvements are negated by newly emerging problems. We have also convincingly demonstrated that some force-field versions released by other groups are plagued by serious side-effects which often were not recognized by their authors. Thus, close to the end of the evaluation period, we

suggested a major change in the paradigm of the further RNA force-field development. What we propose is to introduce a new force-field term for tuning of H-bonding that is orthogonal to the presently used basic force-field terms. This should allow to increase flexibility of the parametrization and achieve some improvements without the unintended side-effects. Importantly, we have suggested a first specific version of such a term and implemented it specifically for RNA molecule. It can be routinely used in MD simulations. It shows promising improvements for notoriously problematic short RNA model systems where rigorous primary experimental data is available while avoiding deteriorations for larger folded RNAs. We suggest it is presently the best available force field for RNA simulations with potential for further improvement.

Insights into the role of structural dynamics and partial disorder in several protein-NA systems. Our simulations show how structural dynamics allows a given protein to read multiple RNA targets (studies done mainly for RRM class of proteins) or separate binding affinity from reactivity (RuvC Holliday junction resolvase and generation of PPT primer by HIV-1 reverse transcriptase). In this area we collaborate with several established experimental laboratories, in the evaluated period for example with F.H.T. Allain from ETH Zurich and M. Nowotny from IIMCB Warsaw.

Series of studies experimenting with application of recently-emerging modern QM and QM/MM methods to nucleic acids. Our studies clearly demonstrate the advantages of QM methods over the MM description but also highlight the limitations stemming from the sampling issue. That is why we have also been experimenting with QM/MM dynamical simulations, though, with contemporary computers, these methods cannot be yet applied to nucleic acids. They can be, however, very insightful in studies of simple prebiotic chemical reactions, including those in the interstellar space or under suspected conditions on exoplanets.

161-printed pages invited Chemical Reviews paper (2018, IF 54). It is widely considered as currently the most authoritative paper in the field of RNA MD simulations and modelling. It is not a mere literature overview of the field, but mainly a conceptual paper of the field of atomistic simulations of nucleic acids which also defines its future goals. In contrast to many other reviews in the literature, we very openly discuss limitations of the MD simulation technique.

What follows is the complete list of all papers published in the period 2015-2019 separated in five thematic areas. Note that many of the papers fit to more than one area and thus the division is approximate. The list is used to specify the contribution by Sponer's group, which is done in the following way. All authors who are primarily from the group located at IBP are **in bold.** It is applied for authors primarily employed in Sponer's group at the time of publication as well as for former group members who have done the work while they were primarily employed in the lab and they affiliate the lab on the particular paper (i.e., in case of some delay of the publication). Additional authors who are marked in red (in by about 30 papers) are researchers primarily employed at the Palacky University, Olomouc, where J. Sponer is supervising the research devoted to nucleic acids since 2008; majority of these authors have part-time positions also at Sponer's IBP group. Only papers devoted to the IBP topics are listed (i.e., other papers of these authors are not included). For each paper, all corresponding authors are marked with symbol "*" which best separates the contributions from the IBP group on each paper.

RNA simulations, protein-RNA complexes and related topics

Suddala, K. C.; Price, I. R.; Dandpat, S. S.; **Janeček, M**.; Kührová, P.; **Šponer, J**.; Banáš, P.; Ke, A.;* Walter, N. G.*, Local-to-global Signal Transduction at the Core of a Mn2+ Sensing Riboswitch. **Nature Commun** 2019, 10, 4304. **IF 11.880**

Ripin, N.*; Boudet, J.; Duszczyk, M. M.; Hinniger, A.; Faller, M.; **Krepl, M**.; Gadi, A.; Schneider, R. J.; **Šponer, J**.; Meisner-Kober, N. C.*, Allain F.H.T.*, Molecular Basis for AU-rich Element Recognition and Dimerization by the HuR C-terminal RRM. **Proc Natl Acad Sci USA** 2019, 116, 2935-2944. **IF 9.58**

Pokorná, P.; Krepl, M.; Bártová, E.; **Šponer, J**.*, Role of Fine Structural Dynamics in Recognition of Histone H3 by HP1γ(CSD) Dimer and Ability of Force Fields to Describe Their Interaction Network. **J Cheml Theory Comput** 2019, 15, 5659-5673. **IF 5.313**

Legartová, S.; Lochmanová, G.; Zdráhal, Z.; Kozubek, S.; **Šponer, J**.; **Krepl, M**.; **Pokorná, P**.; Bártová, E.*, DNA Damage Changes Distribution Pattern and Levels of HP1 Protein Isoforms in the Nucleolus and Increases Phosphorylation of HP1β-Ser88. **Cells** 2019, 8, 1097. **IF 5.656**

Campagne, S.; **Krepl, M.; Spone**r, J.; Allain, F.H.T.*, Combining NMR Spectroscopy and Molecular Dynamic Simulations to Solve and Analyze the Structure of Protein–RNA Complexes. **Methods Enzymol**, 2019, 614, 393-422 **IF 2.002**

Bochicchio, A.; **Krepl, M.*;** Yang, F.; Varani, G.; **Sponer, J**.; Carloni, P.,* Molecular Basis for the Increased Affinity of an RNA Recognition Motif with Re-engineered Specificity: A Molecular Dynamics and Enhanced Sampling Simulations Study. **Plos Comput Biol** 2018, 14, e1006642. **IF 3.955**

Krepl, M.*; Vögele, J.; **Kruse, H**.; Duchardt-Ferner, E.; Wöhnert, J.*; **Sponer, J**.* An Intricate Balance of Hydrogen Bonding, Ion Atmosphere and Dynamics Facilitates a Seamless Uracil to Cytosine Substitution in U-turn of the Neomycin-sensing Riboswitch. **Nucleic Acids Res** 2018, 46, 6528–6543. **IF 11.561**

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Research activity and characterization of the main scientific results

During the evaluated period, our work concentrated primarily on elucidation of **molecular and cellular mechanisms** involved in various aspects of **cancer cell biology and therapy** (A), potential **therapeutic and/or preventive approaches** linked with lipid metabolism (B), as well as on potential role of **carcinogenic and/or endocrine-disrupting environmental chemicals** (C) contributing to oncogenic disease development. Although we were working on quite a large number of projects as outlined below, we used common technology and methodology approaches, which allowed us to optimally use the resources of our department/institute. The principal results that we have obtained are commented individually below and the results are grouped here according to a major focus of the respective publications that reported our findings. We primarily report here those **publications, which originated in our department** (mostly with corresponding author from our team); in addition, we also participated in a number of collaborative studies – regarding these, we refer to the list provided for the bibliometric analyses.

A) Cancer progression, tumor cells and their plasticity/heterogeneity; novel therapeutic approaches

1) Plasticity/heterogeneity of cancer cells.

The **cell plasticity**, in tight association with stemness, contributes to the homeostasis, **evolution of early neoplastic lesions**, and **cancer dissemination**. The ability of tumor cells to adapt to dynamic changes in the microenvironment is considered to be a key requirement for their survival and outgrowth. The process which most likely interlinks the cancer cell plasticity with their dissemination capability, and adaptation to microenvironmental factors is the **epithelial-to-mesenchymal transition (EMT)** and other types of plasticity often associated with disease progression and therapy resistance. In our work in this area, we focused on the **cell surface glycoprotein Trop-2**, which is commonly overexpressed in carcinomas and represents an exceptional antigen for targeted therapy. We provided evidence that surface Trop-2 expression is functionally connected with an epithelial phenotype in breast and prostate cell lines and in patient tumor samples. We further showed that Trop-2 expression is suppressed epigenetically or through the action of epithelial-to-mesenchymal transition transcription factors and that deregulation of Trop-2 expression is linked with cancer progression and poor patient prognosis. Moreover, we introduced a high-throughput screening platform to identify surface antigens that associate with epithelial-mesenchymal plasticity. We found specific set of **surface molecules that are lost in breast cancer cells that underwent EMT** *in vivo* and we propose that such surface signature expression reflects the epithelial-mesenchymal plasticity in breast cancer. Heterogenity and plasticity of solid cancer is represented also by its stromal compartment. Therefore, we also focused on the identification of **fibroblasts and cancer-associated fibroblasts** from human cancer tissue using surface markers. Regarding the plasticity of cancer cells, we also established and

characterized two new **iPSC cell lines derived from fetal and prostate cancer-derived fibroblasts** which may be used for differentiation into different prostate-specific cell types in differentiation studies.

Major relevant publications:

* corresponding author from our department

* Šimečková S., Kahounová Z., Fedr R., Remšík J., Slabákova E., Suchánková T., Procházková J., Bouchal J., Kharaishvili G., Král M., Beneš P., Souček K. (2019) *High Skp2 expression is associated with a mesenchymal phenotype and increased tumorigenic potential of prostate cancer cells*. Sci. Rep. 9: 5695.

* Remšík J., Fedr R., Navrátil J., Binó L., Slabáková E., Fabian P., Svoboda M., Souček K. (2018) *Plasticity and intratumoural heterogeneity of cell surface antigen expression in breast cancer*. Br. J. Cancer 118: 813-819.

* Remšík J., Binó L., Kahounová Z., Kharaishvili G., Šimečková S., Fedr R., Kučírková T., Lenárt S., Muresan X.M., Slabáková E., Knopfová L., Bouchal J., Král M., Beneš P., Souček K. (2018) *Trop-2 plasticity is controlled by epithelial-to-mesenchymal transition*. Carcinogenesis 39:, 1411-1418.

Knopfová L., Biglieri E., Volodko, N., Masařík M., Hermanová M., Glaus Garzon J.F., Ducká M., Kučírková T., Souček K., Šmarda J., Beneš P., Borsig L. (2018) *Transcription factor c-Myb inhibits breast cancer lung metastasis by suppression of tumor cell seeding.* Oncogene 37: 1020-1030.

* Kahounová Z., Kurfürstová D., Bouchal J., Kharaishvili G., Navrátil J., Remšík J., Šimečková S., Student V., Kozubík A., Souček, K. (2018) *The fibroblast surface markers FAP, anti-fibroblast, and FSP are expressed by cells of epithelial origin and may be altered during epithelial-to-mesenchymal transition.* Cytometry Pt A 93A: 941-951.

* Kahounová Z., Slabáková E., Binó L., Remšík J., Fedr R., Bouchal J., Kurfürstová D., Vrtel R., Student V., Jurečková L., Porokh V., Hampl A., Souček K. (2018) *Generation of human iPSCs from human prostate cancer-associated fibroblasts IBPi002-A*. Stem Cell Res. 33: 255-259.

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2) Novel therapeutic approaches and synthetic lethality.

Modern anticancer drugs equipped with selective biological activity often effectively treat only the tumors whose viability is critically dependent on the presence of a particular biological process. Due to heterogeneity of tumors and since only a small proportion of tumors has been treatable by modulation of a single biological process, it seems that in most malignancies it will be necessary to modulate (preferably in a synergic manner) several crucial biological pathways. One of the approaches that have come into focus in the last period is the **concept of synthetic lethality** where the required phenotype (selective apoptosis and cell death of tumor cells) is caused by a synergic modulation of two or more biological processes. We studied this and other related novel therapeutic approaches in several project supported by the Czech Science Foundation and/or Czech Health Research Council. **Checkpoint kinase 1 (CHK1)** is a master regulator of cell division which is involved in all defined cell cycle checkpoints: G1/S, intra-S, G2/M, and the mitotic spindle checkpoint. Due to such prominent role, a CHK1 kinase targeting is being considered as an attractive concept for the development of new anticancer therapies. In this **multidisciplinary and multi-institutional project**, we took of advantage of established and fruitful collaborations with our clinical and academic partners (Masaryk Memorial Cancer Institute, FNUSA-ICRC, Masaryk University and others). We also described novel mechanisms of potentiation of CHK1 inhibition effects in combination with platinum-based chemotherapy drugs. The effects of platinum-based and other DNA-damaging drugs were also pursued in combination with other treatments, including activation of peroxisome-proliferator-activated receptor γ, or death ligand TRAIL. Importantly we reported synthesis and **profiling of new CHK1 inhibitor - MU380, a nontrivial analogue of clinically profiled compound SCH900776**, possessing the highly unusual N-trifluoromethylpyrazole motif, which was envisioned not to undergo metabolic oxidative dealkylation and thereby provide greater robustness to the compound. Overall, MU380 represents **a novel state-of-the-art CHK1 inhibitor** with a high potency, selectivity, and an improved metabolic robustness to oxidative N-dealkylation, and we showed his potency in many different cancer models including preclinical *in vivo* studies. Our work was also extended to inhibitors of casein kinase 1δ/ε (CK1δ/ε), which is a key component of non-canonical Wnt signaling pathways. These have been shown to drive pathogenesis of chronic lymphocytic leukemia (CLL) and in one of our studies, using preclinical mouse model, we successfully documented that **CK1 may serve as a novel therapeutic target in CLL**, acting in synergy with B-cell receptor inhibitors. Importantly, we **established workflow for medium throughput characterization of newly prepared compounds with anti-cancer potential, which was used in many collaborative studies** with organic and medicinal chemists (examples of these studies are listed below).

Major relevant publications:

* corresponding author from our department

* Lauková J., Kozubík A., Hofmanová J., Nekvindová J., Sova P, Moyer M.P., Ehrmann J., Hyršlová Vaculová A. (2015) *Loss of PTEN Facilitates Rosiglitazone-Mediated Enhancement of Platinum(IV) Complex LA-12-Induced Apoptosis in Colon Cancer Cells.* PLoS One 10: e0141020.

* Samadder P., Suchánková T., Hylse O., Khirsariya P., Nikulenkov F., Drápela S., Straková N., Vaňhara P., Vašíčková K., Kolářová H., Binó L., Bittová M., Ovesná P., Kollár P., Fedr R., Ešner M., Jaroš J., Hampl A., Krejčí L., Paruch K., Souček K. (2017) *Synthesis and Profiling of a Novel Potent Selective Inhibitor of CHK1 Kinase Possessing Unusual N-trifluoromethylpyrazole Pharmacophore Resistant to Metabolic N-dealkylation*. Mol. Cancer Ther. 16: 1831-1842.

* Herůdková J., Paruch K., Khirsariya P., Souček K., Krkoška M., Vondálová Blanářová O., Sova, P., Kozubík A., Hyršlová Vaculová A. (2017) *Chk1 Inhibitor SCH900776 Effectively Potentiates the Cytotoxic Effects of Platinum-Based Chemotherapeutic Drugs in Human Colon Cancer Cells*. Neoplasia 19: 830-841.

Maier L., Khirsariya P., Hylse, O., Adla, S. K., Černová, L., Poljak, M., Krajčovičová, S., Weis, E., Drápela, S., Souček, K., Paruch, K. (2017) *Diastereoselective Flexible Synthesis of Carbocyclic C-Nucleosides.* J. Org. Chem. 82: 3382-3402.

* Vondálová Blanářová O., Šafaříková B., Herůdková J., Krkoška M., Tománková S., Kahounová Z., Anděra L., Bouchal J., Kharaishvili G., Král M., Sova P., Kozubík A., Hyršlová Vaculová A. (2017) *Cisplatin or LA-12 enhance killing effects of TRAIL in prostate cancer cells through Bid-dependent stimulation of mitochondrial apoptotic pathway but not caspase-10*. PLoS One 12: e0188584.

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Janovská P., Verner J., Kohoutek J., Bryjová L., Gregorová M., Dzimková M., Skabrahova H., Radaszkiewicz T., Ovesná P., Vondálová Blanářová O., Němcová T., Hoferová Z., Vašíčková K., Smyčková L., Egle A., Pavlová S., Poppova L., Plevová K., Pospíšilová Š, Bryja V. (2018) *Casein kinase 1 is a therapeutic target in chronic lymphocytic leukemia.* Blood 131: 1206-1218.

Boudný M., Zemanová J., Khirsariya P., Borský M., Verner, J., Černá J., Oltová A., Šeda V., Mráz M., Jaroš J., Jašková Z., Spunarová M., Brychtová Y., Souček K., Drápela S., Kašpárková M., Mayer J., Paruch K., Trbušek M. (2019) *Novel CHK1 inhibitor MU380 exhibits significant single-agent activity in TP53-mutated chronic lymphocytic leukemia cells*. Haematologica 104: 2443-2455.

Němec V., Hylsová M., Maier L., Flegel J., Sievers S., Ziegler S., Schroder M., Berger B.T., Chaikuad A., Valčíková B., Uldrijan S., Drápela S., Souček K., Waldmann H., Knapp S., Paruch K. (2019) *Furo[3,2-b]pyridine: A Privileged Scaffold for Highly*

Selective Kinase Inhibitors and Effective Modulators of the Hedgehog Pathway. Angew. Chem. Int. Ed. Engl 58: 1062-1066.

B) Lipids, lipid metabolism and its potential implications in colon cancer development, prevention or therapy

1) Lipidome of colon cancer cells and its alterations by fatty acids

In yet another project supported by the Czech Science Foundation and the Czech Health Research Council, we focused primarily on the analyses of mechanisms underlying potential **anti-tumor and chemopreventive action of polyunstaurated fatty acids** (such as docosahexaenoic acid – DHA) and **short-chain fatty acids**, in particular butyrate, which is a major regulator of colon epithelium homeostasis. We identified the ability of colon cancer cells to differentiate (in response to butyrate) as a **key factor determining their apoptotic responses**. This is linked with cell-specific alterations of specific groups of lipids, namely **cardiolipins** and **sphingolipids** (in particular specific ceramide classes). We identified a wide range of cardiolipin species in the colon cells, which composition was significantly modified after DHA and butyrate treatment. Clearly, both dietary agents together, through modulation of **expression/activites of enzymes involved in synthesis of fatty acids or ceramides**, induce **dynamic alterations of lipid metabolism**, thus contributing to distinct cellular differentiation or apoptotic responses in colon cancer cells. The distinct fates of colon adenocarcinoma cells are associated also with their different phospholipid and sphingolipid profiles. We established that *in vitro* cultured colon cancer cells reflect well some of the alterations observed also in primary cancer cells isolated from CRC patients. The modulatory role of **butyrate, this important regulator of colonic epithelium homeostasis** controlling both **PUFA incorporation and metabolism** should thus be taken into account, when designing nutritional intervention strategies or current trends focusing on the role of microbiome in CRC development and progression. These studies have been carried out in our department, in a close collaboration with the team of dr. Machala at the Veterinary Research Institute (who supervised targeted lipid analyses); our clinical collaborators provided patient material. We include here also one paper finished and accepted in 2019, but published later this year.

Major relevant publications:

* corresponding author from our department

* Tylichová Z., Straková N., Vondráček J., Hyršlová Vaculová A., Kozubík A., Hofmanová J. (2017) *Activation of autophagy and PPARγ protect colon cancer cells against apoptosis induced by interactive effects of butyrate and DHA in a cell type-dependent manner: The role of cell differentiation*. J. Nutr. Biochem., 39: 145-155.

Hofmanová J., Slavík J., Ovesná P., Tylichová Z., Vondráček J., Straková N., Hyršlová Vaculová A., Ciganek M., Kozubík A., Knopfová L., Šmarda J., Machala M. (2017) *Dietary fatty acids specifically modulate phospholipid pattern in colon cells with distinct differentiation capacities*. Eur. J. Nutrition, 56(4): 1493-1508.

* Tylichová Z., Slavík J., Ciganek M., Ovesná P., Krčmář P., Straková N., Machala M., Hofmanová J., Kozubík A., Vondráček J. (2018) *Butyrate and docosahexaenoic acid interact in alterations of specific lipid classes in differentiating colon cancer cells*. J. Cell. Biochem., 119(6): 4664-4679.

Machala M., Procházková J., Hofmanová J., Králiková L., Slavík J., Tylichová Z., Ovesná P., Kozubík A., Vondráček J. (2019) *Colon cancer and perturbations of sphingolipid metabolism*. Int. J. Mol. Sci., 20(23): 6051.

* Hofmanová J., Slavík J., Ovesná P., Tylichová Z., Dušek L., Straková N., Hyršlová Vaculová A., Ciganek M., Kala Z., Jíra M., Penka I., Kyclová J., Kolář Z., Kozubík A., Machala M., Vondráček J. (2020) *Phospholipid profiling enables to discriminate tumor- and non-tumor-derived human colon epithelial cells: phospholipidome similarities and differences in colon cancer cell lines and in patient-derived cell samples*. PLoS One, 15(1): e0228010.

2) Interactions of lipids with metabolism of xenobiotics

The project, supported by the Czech Science Foundation, has brought novel findings about the mechanisms underlying the interactions among toxic and carcinogenic dietary contaminants, polycyclic aromatic hydrocarbons (PAHs), and dietary polyunsaturated fatty acids, together with gut microbiome products, such as short-chain fatty acid butyrate. The goal of this project was to clarify the mechanisms potentially contributing to **interactive effects of butyrate with fatty acids, such as DHA, and with genotoxic PAHs, here represented by benzo[a]pyrene (BaP), an important carcinogen**. We were able to identify and describe in detail a number of **cellular and molecular mechanism playing key roles in interactions of these compounds** in epithelial cell models derived from the colon tissue. We succeeded in identification of an unconventional role of Wnt/β-catenin signaling pathway in regulation of bioactivation and genotoxicity of BaP in colon cell models. For the first time, we were able to describe **interactive effects of butyrate and BaP in a battery of colon cell models, and its impact on mutagenic/genotoxic effects of this important carcinogen**. Our work has enabled us to describe in detail **molecular mechanisms** responsible for the **impact of butyrate on expression and activity of enzymes participating in metabolism of carcinogens**, in particular of cytochrome P450 family 1 (CYP1) nzymes. These included in particular the **inhibition of histone deacetylases** by butyrate. A successful description of these mechanisms within this project is important not only for our general understanding of toxic modes of action of environmental pollutants, such as PAHs, but it may in future serve to facilitate also an **improved evaluation of risks associated with exposition of human population to**

these highly dangerous contaminants, which can be found at significant levels e.g. in heat-processed foods. These studies have been designed, and a majority of experiments and preparation of manuscripts have been carried out in our department. Our collaborating partners from the Veterinary Research Institute and Institute of Experimental Medicine (IEM CAS) contributed to specialized analyses of BaP metabolites and DNA adducts. In all papers, both first and corresponding author were from our department.

Major relevant publications:

* corresponding author from our department

* Kabátková M., Zapletal O., Tylichová Z., Neča J., Machala M., Milcová A., Topinka J., Kozubík A., Vondráček J. (2015) *Inhibition of β-catenin signalling promotes DNA damage elicited by benzo[a]pyrene in a model of human colon cancer cells via CYP1 deregulation.* Mutagenesis, 30(4): 565-576.

* Zapletal O., Tylichová Z., Neča J., Kohoutek J., Machala M., Milcová A., Pokorná M., Topinka J., Moyer M. P., Hofmanová J., Kozubík A., Vondráček J. (2017) *Butyrate alters expression of cytochrome P450 1A1 and metabolism of benzo[a]pyrene via its histone deacetylase activity in colon epithelial cell models.* Arch. Toxicol., 91(5): 2135-2150.

* Zapletal O., Procházková J., Dubec V., Hofmanová J., Kozubík A., Vondráček J. (2019) *Butyrate interacts with benzo[a]pyrene to alter expression and activities of xenobiotic metabolizing enzymes involved in metabolism of carcinogens within colon epithelial cell models.* Toxicology, 412C: 1 -11.

* Tylichová Z., Neča J., Topinka J., Milcová A., Hofmanová J., Kozubík A., Machala M., Vondráček J. (2019) *n-3 Polyunsaturated fatty acids alter benzo[a]pyrene metabolism and genotoxicity in human colon epithelial cell models.* Food Chem. Toxicol., 124: 374-384.

C) Carcinogenicity, endocrine disruption and toxic modes of action of organic pollutants

Toxic compounds, found in the environment or in food, which belong among efficient ligands of the aryl hydrocarbon receptor (AhR), may negatively affect human health. The identification of molecular and cellular mechanisms underlying the toxic effects of these organic pollutants is essential information necessary for the definition of adverse outcome pathways, which are relevant for both individual toxicants and their complex environmental mixtures.

1) Impact of toxic AhR ligands on adult liver progenitor cells

We studied the impact of toxic ligands of Ah receptor (AhR) on a specific cell subpopulation within the liver – **adult liver progenitors** that are activated during liver diseases or during the action of toxicants. We focused particularly on studying potential **interactions of the AhR with the effectors of the Hippo signaling pathway, proteins YAP1 and TAZ**, which play a key role in regulation of development and regeneration of the liver. The most important results of the project included definition of the first suitable human model for studies of effects of AhR ligands on liver progenitors. Activation of the AhR in adult liver progenitors leads to **stimulation of cell proliferation**, which is accompanied with **an increased sensitivity of cells to apoptosis**, as well as with **deregulation of a number of constituents and/or transcriptional targets of signaling pathways involved in control of progenitor cell behavior**. These include Wnt/β-catenin, TGF-β, EGFR or insulin signaling pathways, which have all been implicated in developmental and tumorigenic processes in the liver. Together, these results indicate that persistent toxic AhR ligands, such as TCDD, can induce **complex alterations of the physiological role(s) of adult liver progenitors**. Furthermore, the project has brought description of a novel role of YAP1 as a protein that not only controls proliferation/survival of liver progenitors, but which activity also determines the type of impact of the AhR ligands on these cells. This research, supported by another project funded by the Czech Science Foundation, contributed to our understanding of **cellular and molecular mechanisms underlying the effects of AhR ligands on activity of adult liver progenitors**. A detailed knowledge of these mechanisms is of major importance not only for studies of **compounds acting as chemical carcinogens**, but it may become useful also for evaluation of risks associated with exposition of human population to these highly dangerous contaminants. These studies have been designed, and a majority of experiments and preparation of manuscripts have been carried out in our department. Our collaborating partners from the Veterinary Research Institute, Palacký University, and Institute of Experimental Medicine (IEM CAS) contributed to specialized analyses (especially transcriptomics), or to writing of manuscripts. In all papers (except for the multiple author review), both first and corresponding author were from our department.

Major relevant publications:

* corresponding author from our department

* Svobodová J., Kabátková M., Šmerdová L., Brenerová P., Dvořák Z., Machala M., Vondráček J. (2015) *The aryl hydrocarbon receptor-dependent disruption of contact inhibition in rat liver WB-F344 epithelial cells is linked with induction of survivin, but not with inhibition of apoptosis*. Toxicology, 333: 37-44.

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A., Marignani P.A., Memeo L., Modello C., Naus C.C., Ponce-Cusi R., Raju J., Roy D., Roy R., Ryan E., Salem H.K., Scovassi I., Singh N., Vaccari M., Vento R., Vondráček J., Wade M., Woodrick J., Bisson W.H. (2015) *Mechanisms of environmental chemicals that enable the cancer hallmark of evasion of growth suppression*. Carcinogenesis, 36 (Suppl. 1): S2-S18.

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2) Endocrine disrupting effects of organic pollutants and versatile roles of the AhR

In another project supported by the Czech Science Foundation, we focused primarily on the **interplay between the AhR and estrogen receptors (ER)** as a key interaction defining the effects of important environmental pollutants **on estrogen signaling**, which may contribute to their potential endocrine disrupting effects and carcinogenesis. We focused primarily on two important groups of **endocrine disrupting compounds (EDCs)** – environmental **polycyclic aromatic hydrocarbons (PAHs)** with distinct AhR-mediated activities, highly important group of combustion particle-associated environmental pollutants, and an emerging group of airborne pollutants (in particular within indoor air) - **low-chlorinated polychlorinated biphenyl congeners (LC-PCBs)**. Our results highlight the **diversity of interactions of PAHs** with cell signaling mechanisms linked to steroid receptor functions. The effects of PAHs may include: **their potential direct impact on ER**α and ERβ; **activation of the AhR as a factor modulating activities of the ERs**; and, in particular, **the AhR-driven metabolism of PAHs, which may yield both genotoxic PAH metabolites and highly abundant hydroxylated metabolites, directly activating ER**α and/or ERβ. In particular, we have shown, for the very first time using specific AhR knockout models, that the AhR-dependent metabolism plays a key role in ER-mediated effects of PAHs with moderate AhR mediated activities. The balance of these effects seems to determine the fate of cells through activation/inactivation of specific downstream signaling mechanism, controlling cell proliferation, survival or migration behavior. Our work was extended also to the **AhR activity of strong novel AhR ligands among PAHs**, as well as their complex mixtures originating in various combustion processes. Finally, we have also evaluated effects of other classes of pollutants interacting with ERs. Here, our principal finding was that **LC-PCBs** (and their metabolites) were able to **stimulate ER signaling and ER-dependent cell**

proliferation effects; however, here, the effects of PCBs are not dependent on the AhR or AhR-dependent metabolism. Their estrogenic activities, and suppression of the AhR-mediated activity, were found to be among the relevant modes of action of airborne LC-PCBs. Our results, providing information about individual toxic potencies of environmentally relevant LC-PCB congeners, may in future contribute to refine the risk assessment of this yet poorly characterized group of PCBs. These studies have been designed, and preparation of manuscripts have been carried in our department, in a close collaboration with our partners at. the Veterinary Research Institute (all papers) and Institute of Experimental Medicine CAS (last paper). On four papers, the first author was from our department. We further contributed to additional studies on this subject, not listed here. We include here also one important paper finished in 2019, but accepted and published later this year.

Major relevant publications:

* corresponding author from our department

* Vondráček J., Pěnčíková K., Neča J., Ciganek M., Grycová A., Dvořák Z., Machala M. (2017) *Assessment of the aryl hydrocarbon receptor-mediated activities of polycyclic aromatic hydrocarbons in a human cell-based reporter gene assay.* Environ. Pollut., 220(Pt A): 307-316.

* Hýžďalová M., Pivnička J., Zapletal O., Vázquez-Gómez G., Matthews J., Neča J., Pěnčíková K., Machala M., Vondráček J. (2018) *Aryl hydrocarbon receptor-dependent metabolism plays a significant role in estrogen-like effects of polycyclic aromatic hydrocarbons on cell proliferation.* Toxicol. Sci., 165(2): 447-461.

* Pěnčíková K., Svržková L., Strapáčová S., Neča J., Bartoňková I., Dvořák Z., Hýžďalová M., Pivnička J., Pálková L., Lehmler H.-J., Li X., Vondráček J., Machala M. (2018) *In vitro profiling of toxic effects of prominent environmental lower-chlorinated PCB congeners linked with endocrine disruption and tumor promotion.* Env. Pollution, 237: 473-486.

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hydrocarbons are potent inducers of the AhR-dependent signaling in human cells. Environ. Pollution, 266 (Pt.2): 115125.