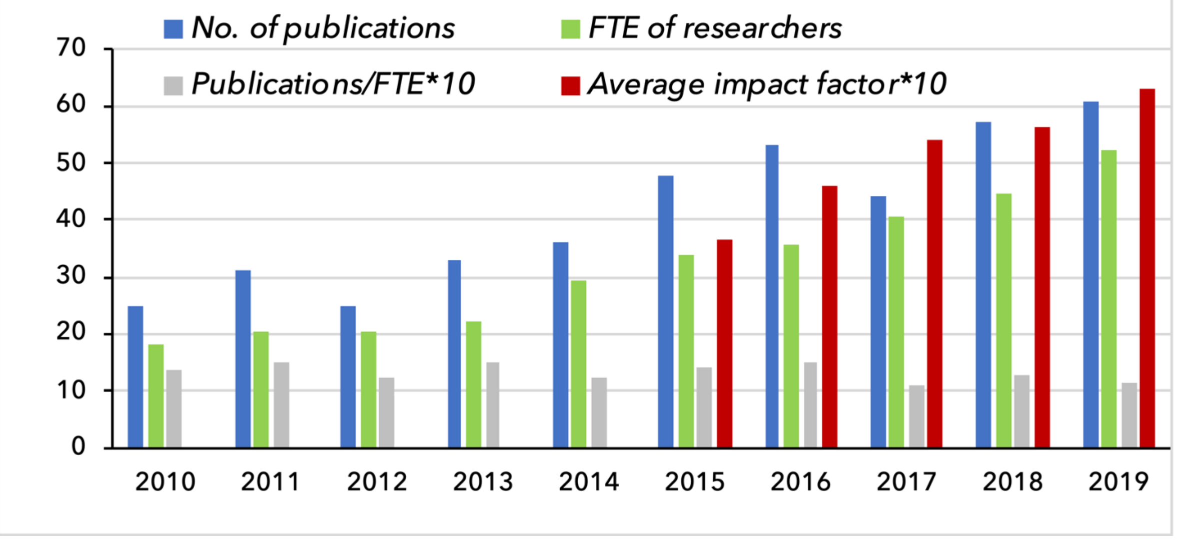
Description of the main research directions investigated by the institute

In this chapter, we first outline the institute´s output in scientific journals and applications and briefly discuss how it is evolving in time and its main components. Then we highlight the main research directions and draft the research interests of both Teams with citing the key references.

Overall situation

IBT was founded in 2008 and has evolved into a well-established member of the Czech Academy of Sciences since then. Since its beginnings, the research strategy followed two branches reflected in this report as activities of Team 1 and Team 2. The current organizational structure of the institute, with nine senior laboratories, two junior laboratories and three infrastructure (service) laboratories, is functional and sustainable. The evaluated period between years 2015 and 2019 can be characterized as stabilization of IBT with two new junior laboratories created. Together with the successful relocation from the old and limiting premises in the bio-medical campus Krc to the new BIOCEV center in Fall 2015 led to a gradual and measurable growth of the scientific output.

The scientific output of IBT is summarized by the histogram below for years 2010 - 2019. We do not include data for years 2008 and 2009 because they report on the initial, basically preparatory, phase of IBT's development. The graph shows the absolute number of papers published in impacted journals (blue), the average number of full time equivalents of researchers in a given year (green), the fraction of the above two numbers multiplied by 10 (grey), and finally the average impact factor of the journals where we published in a given year, again multiplied by 10 (red). The last two numbers were multiplied by 10 to keep data on approximately the same scale.



What does the graph tell us? First of all, the number of publications steadily growths during the whole ten-year period. Even when the average number of published papers divided by the numbers of FTEs of scientists (grey bars in the graph above) is basically constant, does not grow, the impact of our research has increased. We estimate it as the average impact factor of journals where we published (red bars), which grew from less than 4 in year 2015 to more than 6 in year 2019. This is quite a significant jump.

In addition, the histogram above does not include data on our outputs classified as transfer of knowledge, which contribute to the productivity of our research teams. IBT's striving for scientific excellence has been therefore accompanied by an increasing output of applications. Most of these results have had commercial benefits and they have grown during the last five years in both quantity and quality. It is detailed in Chapter 10 Research for Practice. In the evaluated period, we filed 8 patents and obtained several tens of other applied results. The most significant result in this context is the discovery of a new class of potential anticancer drugs targeting mitochondria with the most promising compound, MitoTam. The project has attracted the venture capital and the compound MitoTam is currently being under scrutiny in clinical trials.

To get a more detailed view of IBT's research output in general during years 2015-2019, we point to the list of publications, books, and applied results the IBT Teams 1 and 2 submitted in Phase I of the evaluation.

The most important component of all institute's attributes is its staff. Our HR policy is discussed in Chapter 4 and 5 below. Let us just state here that the age structure of IBT employees is very promising, and we have a very good proportion of older experienced scientists with a PhD title (64 and students, mostly doctoral (the total number of PhD students employed in the institute is 42).

IBT benefits from its participation in the BIOCEV center. The newly established research center has despite its short duration acquired a great national and international reputation. The center as a joint project of six institutes of the Czech Academy of Sciences and two faculties of the Charles university is a great place for broad, interdisciplinary collaborations, enables easy access to the state of the art equipment in several areas of life sciences. The number of scientists, students, and technicians working in BIOCEV is sufficient to master various methodologies with top level expertise and the spectrum of the methodologies available *in situ* allows for synergies between different approaches and certain flexibility in the research plans. The staff of the institute has excellent equipment at its disposal, whether it is owned by the IBT or partner institutes of the Czech Academy of Sciences, or by both faculties of the Charles University. In addition, our affiliation with the BIOCEV project and partnership with the Faculty of Science and First Faculty of Medicine of the Charles University provides a great opportunity to attract students and the laboratories of both Teams have been successful in finding good graduate and undergraduate students as well as students of European Erasmus projects. This is essential for the fulfillment of IBT research tasks but it also fulfills an important function of IBT to train the educated and confident generation of future scientists and teachers. Especially Team 1 appreciates the BIOCEV infrastructure Czech Center for Phenogenomics, CCP, which allows close collaboration on development of unique animal (mouse) models.

As already mentioned, the quality and sustainability of our research depends to a significant extent on the availability of service laboratories with top level equipment and a sufficient number of expert staff and correspondence between scientific ideas and their technical execution is essential. IBT itself operates three top level service laboratories: the Centre of Molecular Structure (CMS), Gene Core - Quantitative and Digital PCR and the Service Technology Laboratory. CMS is a state-of-the-art structural biological workplace and, thanks to close cooperation with structural biologists from CEITEC at Masaryk University (Brno), it has succeeded in obtaining the status of an international center of the Pan-European infrastructure Instruct-ERIC. Due to this status, CMS offers international courses and thus increases the visibility of IBT and attracts new projects. Gene Core - Quantitative and Digital PCR is a service laboratory with expertise in PCR techniques and single-cell techniques. Gene Core co-organizes international courses with the company TATAA Biocenter, which, in addition to the visibility, bring the institute new opportunities for cooperation. The Service Technology Laboratory is an organic chemistry laboratory with the staff possessing irreplaceable know-how for commercial development of potential drugs and for the preparation of their preclinical and clinical testing.

Highlights of the research directions of the institute

The IBT research can be categorized into several main directions as outlined below.

- Research of the mitochondrial energetics. This research direction emphasizes targeting cancer cells via their metabolism mainly by impeding the Complex I and by interfering with their iron metabolism. These efforts led to a fundamental discovery of the horizontal gene transfer of mitochondria between cells and to clinical trials of the compound MitoTam.
- Research projects in the field of developmental biology, transcriptional regulation during embryonic development, and development of the neuronal system in vertebrates. The research aims at identification of the disease predispositions evolving during early development of the organisms extending to cancer research and diagnosis with aiming at potentially applicable results.
- Research of male reproductive molecular and subcellular parameters as markers of fertility disorders involved in sperm-egg fusion including detailed characterization of the dynamic rearrangement of cytoskeletal proteins and their partner proteins.
- New method development of RT-qPCR and implementation of high-throughput gene expression profiling and single-cell analysis using RNA-Seq.
- Protein engineering combining techniques of in vitro protein evolution and of computer modeling for targeted modulation of protein properties such as binding affinity to medically important proteins.
- Structure-function studies of proteins including structure-assisted drug design employing modern methods of biophysical and structural molecular analysis including X-ray crystallography.
- Dynamics of biomolecules and imaging of single molecules and force measurement techniques to uncover how the individual structural elements of the cytoskeleton mechanically cooperate to drive diverse cellular processes.
- Studies of the time dimension of biomolecular dynamics from ultra-short (fs) to medium (ms).
- Structural bioinformatics with unique know-how of nucleic acid structures.

Scientific profiles of the research laboratories of the Teams 1 and 2

The detailed scientific profiles of the Teams 1 and 2 and the constituting research laboratories are presented in part 3.4 of the evaluation report, here we just summarize research directions of both Teams and select the representative results that in our opinion document their excellent scientific quality and ability to remain leaders in their research areas. Importantly, our research is based on advanced methodological approaches and brings innovative ideas published in high quality journals as well as translational research with patents that are being transferred into the medical practice.

Team 1

The five research labs of Team 1 are engaged in cutting-edge basic research aimed at providing better understanding of the molecular mechanism of highly socio-economically challenging human pathologies with considerable impact on population health. The focus is to study genesis and progress of the selected pathologies at the subcellular and molecular levels on cell cultures and animal models and identify their relevant diagnostic biomarkers, to uncover novel functions of genes, detect the changes in the localization and modification of relevant proteins, and identify other molecules involved in the pathological states. An important output is identification of novel and efficient therapeutic approaches. This approach is comparable with that of the contemporary top bio-medical research institutions and is likely to deliver breakthrough results published in leading journals and resulting in commercially applicable intellectual property.

Laboratory of Reproductive Biology, Head: Kateřina Komrsková

The main focus of the laboratory is on the molecular mechanisms of fertilization and specific sperm proteins that play a role in sperm maturation, sperm-egg interaction, and early embryo development. We proved the key role of trans-generational epigenetic de-regulation of microRNA expression induced by pollutants in germ cell differentiation. We detect sperm quality in patients with testicular cancer and diabetes mellitus and characterize sperm antibodies in infertile couples. The knowledge is utilized in development of new diagnostic tools for quality assessment and gamete selection for Centers of Assisted Reproduction.

(Vieweg et al., 2015)

(Frolikova et al., 2016)

(Frolikova et al., 2018)

Laboratory of Molecular Therapy, Head: Jiří Neužil

The laboratory has been focusing on several major projects plus additional side projects. The major projects, which have also provided high-level publications, include i) horizontal transfer of mitochondria; ii) design and testing of novel anti-cancer agents; and iii) the role of mitochondrial respiratory complex II in cancer. The group published 56 papers in the last five years.

(Tan et al., 2015)

(Bezawork-Geleta et al., 2018)

(Bajzikova et al., 2019)

Patent: Neužil, Jiří, Werner, L., Štursa, J. Tamoxifen derivatives for treatment of neoplastic diseases, especially with high HER2 protein level. J. Neužil, Institute of Biotechnology of the Czech Academy of Sciences, Smart Brain s.r.o., KKCG AG. 2018. Number of the patent folder: US9896466. Date of the patent award: 2020-02-20.

Laboratory of Gene Expression, Head: Michael Kubista

The laboratory is Europe's leading academic laboratory specialized in high-throughput gene expression profiling and single-cell analysis using real-time quantitative PCR (qPCR) and RNA-sequencing. We have several basic research projects in the field of developmental biology and stem cells, and applied projects in cancer and neurological research. We also develop methods and applications for nucleic acid analyses and we are active in the area of standardization. The group published more than 40 publications in peer-reviewed journals.

(Androvic et al., 2017)

(Stahlberg & Kubista, 2018)

(Valihrach et al., 2020)

Laboratory of Molecular Pathogenetics, Head: Gabriela Pavlínková

The research program of the laboratory is focused on transcriptional regulation during embryonic development, developmental programming, and identification of the molecular causes of disease predispositions, particularly diabetes and heart disease. We are particularly interested in HIF-1, ISL1, SOX2, and NEUROD1 transcription factor networks and how their dysfunction affects embryonic development and can increase pre-dispositions of an individual to diseases such as diabetes, heart disease, or hearing loss. We also analyze the combinatorial effects of the environment and genetic mutations.

(Bohuslavova et al., 2017)

(Cerychova et al., 2018)

(Bohuslavova et al., 2019)

Laboratory of Tumor Resistance, Head: Jaroslav Truksa, junior laboratory

The main scientific topics of the laboratory are i) Understanding the molecular mechanisms that lead to the proliferation, resistance, and recurrent appearance of cancer cells. ii) Describing the biology of cancer stem cells with the interest targeted to their iron metabolism. iii) Developing new ways to affect cancer cells based on targeting their mitochondria and deregulating their iron metabolism. iv) Deciphering the molecular mechanisms that are responsible for the proper functioning of the iron metabolism throughout the body.

(Rychtarcikova et al., 2017)

(Lettlova et al., 2018)

(Tomkova et al., 2019)

Team 2

The main objective of the team is to mechanistically explain fundamental biological processes with potential applications of the results in the diagnosis and treatment of diseases. Our aim is to understand the mechanisms underlying intermolecular interactions in biomolecular systems and, in a broader sense, the relationship between the biomolecular structure and function. The studied molecular systems are produced by advanced techniques of molecular biology and protein engineering; their dynamics, structure, stability, and activity are determined by the state-of-the-art structural and biophysical experimental and computational methods.

Laboratory of structural biology, Head: Cyril Bařinka

We aspire to elucidate molecular details of the structure and function of several pharmaceutically important zinc-dependent hydrolases, including histone deacetylases and glutamate carboxypeptidases, from the single molecule level to their physiological roles in cellular environments. We also use protein engineering and structure-assisted drug discovery to develop macromolecules and small molecule ligands, respectively, that can be used as research tools and/or be advanced into clinical practice. A platform for heterologous expression and purification of recombinant proteins implemented in our laboratory is extensively used not only by us but also by a wide network of our collaborators and in our commercially oriented on-demand protein production.

(Novakova et al., 2016)

(Kutil et al., 2018)

(Kutil et al., 2019)

Laboratory of structure and function of biomolecules, Head: Jan Dohnálek

The laboratory applies integrative structural biology approaches to investigate new enzymes, receptors, and large molecular complexes with biotechnological or biomedical potential. The uncovered structure-function relationships explain basic biological functions and are applicable in nanotechnologies or in the fight against opportunistic human pathogens and diseases. Our structure-function studies of enzymes with biomedical and biotechnological potential have yielded results in three main directions: i) non-specific nucleases; ii) bilirubin oxidase; and iii) novel glycosidases. New enzymes for biotechnology are studied with a world leader in the field - Novozymes A/S.

(Trundová et al., 2018)

(Koval' & Dohnálek, 2018)

(Koval et al., 2019)

Laboratory of ligand engineering, Head: Petr Malý

The laboratory uses methods of directed evolution of proteins for the generation of unique collections of binding proteins targeted to human cytokines and their receptors, serum oncomarkers, bacterial toxins, and insoluble components of fibrin network. A special attention is paid to the development of “non-cognate ligands” of broadly neutralizing human antibodies identified as “protein prints” mimicking epitopes of virus-neutralizing antibodies to be used as promising immunogens for development of novel protective vaccines. By screening of highly complex combinatorial libraries we identified “protein prints” that can be used as strong immunogens for the stimulation of protective antibodies similar to those originally used as molecular targets.

(Mareckova et al., 2015)

(Hlavnickova et al., 2018)

(Petrokova et al., 2019)

Laboratory of biomolecular recognition, Head: Bohdan Schneider

We focus on understanding interactions driving the specific recognition between biomolecules with a potential diagnostic, medical, or biotechnological use. We apply experimental and computational methods of protein engineering, structural biology, bioinformatics, and molecular modeling to study: i) cytokines and their receptors; ii) time-resolved biomolecular dynamic of light-inducible proteins; and iii) a bacterial transposase RAYT and the related REP DNA. We have a unique know-how in structural bioinformatics of nucleic acids. For more, please see the website biorecognition.structbio.org.

(Mikulecky et al., 2016)

(Schneider et al., 2017)

(Zahradnik et al., 2019)

Laboratory of structural bioinformatics of proteins, Head: Jiří Černý, junior laboratory

The laboratory concentrates on the development and application of structural alphabets for annotation, modeling, model building, validation, and refinement of experimental structures and computer models of proteins and nucleic acids. It also contributes to a deeper understanding of structural and functional features of biologically relevant molecules and their interactions by developing novel computational methods and procedures for modeling of biomolecules and their complexes. It is involved in several collaborations on projects covering structural bioinformatics and molecular modeling of biomolecules or small drug-like molecules with both Teams of IBT as well as outside.

(Cerny et al., 2016)

(Peter & Cerny, 2018)

(Cerny et al., 2019)

Laboratory of structural proteins, Head: Zdeněk Lánský, junior laboratory

Cytoskeletal networks form the internal dynamic scaffold of living cells essential for key cellular processes, such as cell division or morphogenesis. Cytoskeletal proteins self-assemble to drive these processes. Our aim is to understand the principles that underpin their collective action resulting in the coherent behavior of the cytoskeletal networks. We reconstitute cytoskeletal networks from individual components *in vitro*. Using genetic manipulations, biophysical methods with single molecule resolution, and mathematical modelling, we quantitatively explain the studied systems.

(Ludecke et al., 2018)

(Siahaan et al., 2019)

(Schmidt-Cernohorska et al., 2019)

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Research activity and characterisation of the main scientific results

In the period of 2015-2019, the team published 131 papers in peer-reviewed journals, three chapters in scientific books, filed two patents, and obtained 13 applied results. The efforts of the team are primarily focused on high-quality basic research with potential application of the results in the diagnosis and treatment of diseases. The scientific profile and the main results of individual groups are presented below.

Laboratory of Reproductive Biology

Our laboratory has extensive knowledge of assessing male reproductive parameters as markers of fertility disorders. We have been studying the molecular mechanisms of reproduction and the nature of specific sperm proteins that play a role in sperm maturation, sperm-egg interaction and early embryo development. Our area of interest covers monitoring of sperm quality in patients with testicular cancer and diabetes mellitus and characterization of sperm antibodies in infertile couples. Many of these antibodies are used in Centres of Assisted Reproduction and have been commercialized. The key role of trans-generational epigenetic de-regulation of microRNA expression induced by pollutants in germ cell differentiation has been proven.

Recently, we have been interested in detailed characterization of the dynamic rearrangement of cytoskeletal proteins and their partner proteins (IZUMO1, CD46, CD9, CD81, CD151 and integrins) (Fig. 1a) involved in sperm-egg fusion (Fig. 1b) (Frolikova et al., 2016) (Frolikova et al., 2018) (Frolikova et al., 2019) (Jankovicova et al., 2016) (Jankovicova et al., 2019) (Jankovicova et al., 2020). In addition, the role of acrosin inhibitor expression and crucial ubiquitin proteasomal complex during sperm maturation has been addressed (Manaskova-Postlerova et al., 2016) (Zigo et al., 2019). Epigenetic aberrations (selected histone modifications and DNA methylation) in spermatozoa, testicular tissues (Fig. 1c) and early embryos after exposure to environmental pollutants have been studied (Brieno-Enriquez et al., 2015) (Vieweg et al., 2015) and new related projects are planned. All the ongoing research is supported by four grants of the Czech Science Foundation (GACR) and by a prestigious international bilateral grant with the German Grant Foundation (GACR-DGF). The outcome of this research is being translated into new diagnostic tools for identification of sperm parameters and selection of high- and low-quality sperm to be used in Centres of Assisted Reproduction (Fig. 1d). This translational research is supported by grants of the Technological Agency of the Czech Republic and the Agency of the Ministry of Health. Two European and two Czech patents have been submitted and are currently under evaluation.

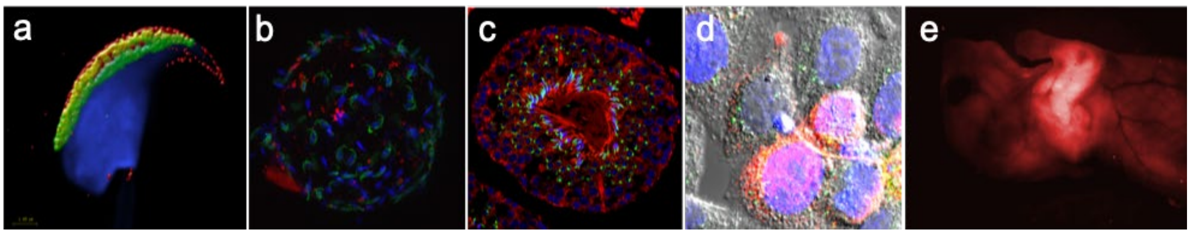

***Fig. 1**. (a) Super-resolution microscopy (SIM) shows the localization of CD46 (green) on the inner and outer acrosomal membrane and β1-integrin (red) on the plasma and outer acrosomal membrane. (b) Super-resolution microscopy (STED) of a mouse oocyte shows the localization of proteins Izumo1 (green), Fcrl (red) and the nucleus (blue). (c) Testis: cross-section using transgenic mouse C57BL/6NAcr3-EGFP/Su9DsRed2, acrosome in developing spermatids and sperm (green), mitochondria (red), nuclei (blue). (d) CHO cells mimic the human egg model co-transfected with both JUNO (red) and fusion protein FcRL (green) with attached human sperm; nuclei (blue). (e) C57BL/6NSu9DsRed2 embryo: Red fluorescent protein expressed in mitochondria is visible under fluorescent light and confirmed by genotyping.*

We participate in productive collaboration based on the knowledge of detailed assessment of reproductive para-meters, monitoring of trans-generational epigenetic inheritance, characterization of epigenetic aberrations and microRNA deregulations, all tailored for specific areas of interest (medical conditions, lifestyle, environmental factors, etc.). In addition, we utilize, in collaboration with commercial subjects, our knowledge of hybridoma technology for production of specific high-affinity monoclonal antibodies (Merck, EXBIO), and 12 prototypes for the detection of relevant molecules have been developed. Moreover, we participate in valuable collaboration based on transgenic mouse lines, property of our laboratory, serving as an indispensable tool for in vivo experiments, specifically transgenic C57BL/6Acr3-EGFP mice expressing green protein (EGFP) in the sperm acrosome (Baert et al., 2019), and transgenic C57BL/6Su9DsRed2 mice expressing red fluorescent protein (RFP) in somatic cell mitochondria (Fig 1c,e) (Bajzikova et al., 2019) (Dong et al., 2017).

Laboratory of Molecular Therapy

Our laboratory has been focusing on several major projects plus additional side projects. The major projects, which have also provided high-level publications, include i) horizontal transfer of mitochondria; ii) design and testing of novel anti-cancer agents; and iii) the role of mitochondrial respiratory complex II in cancer. Our group published 56 papers in the last five years.

The discovery of horizontal transfer of mitochondria in vivo has been our most important discovery to date, since it is a finding of paradigm-shifting nature. In the initial paper, we describe the process, which is based on our result showing delayed tumour formation from cancer cells depleted of their mitochondrial DNA (mtDNA). These so called rho0 cells were found to form tumours only after acquisition of mtDNA from the host, as evidenced by the presence of mtDNA in these tumours with the host homoplasmic polymorphism (Tan et al., 2015). Further, we documented that mtDNA moves from stromal cells to cancer rho0 cells in vivo within whole mitochondria (Fig. 2) (Dong et al., 2017) and that the functional reasons for 'import' of mitochondria by cancer rho0 cells is due to recovery of their respiration; more specifically, we found that the reason is not related to ATP generation (which is provided by aerobic glycolysis) but is critically linked to de novo pyrimidine synthesis, since dihydroorotate dehydrogenase (DHODH), an enzyme critical for this pathway that is a component of the mitochondrial respiratory machinery, needs respiration for its catalytic activity (Bajzikova et al., 2019). This discovery also indicates that DHODH may be a novel, broad-spectrum target for cancer therapy.

After mitochondrially targeted vitamin E succinate (Mito-VES) prepared some 10 year ago, we have synthetized several new anti-cancer agents targeted to mitochondria: mitochondria-targeted metformin (MitoMet) (Boukalova et al., 2016) and mitochondrially targeted tamoxifen (MitoTam) (Rohlenova et al., 2017). Both agents target mitochondrial respiratory complex I. We documented that MitoMet is efficient in killing pancreatic cancer cells expressing wild-type Smad-4, while cells with mutant Smad4 are resistant, and showed that MitoTam is efficient against a number of cancers, epitomized by the hard-to-treat Her2high and triple negative breast cancer. We filed a patent protecting MitoTam as a novel anti-cancer drug, which resulted in launching a Phase 1 clinical trial at the General University Hospital in Prague (EudraCT 2017-004441-25). At present, a Phase 1/1b clinical trial is in the final stages, with its end planned for June 30, 2020; until now, MitoTam has provided benefit (disease stabilization/partial remission) to 12 out of 26 patients tested in Phase 1b (long-term toxicity) (Fig. 3). We are currently planning Phase 2 with expected launch in 2021. Interestingly, we have also found that MitoTam selectively kills not only cancer cells, but also non-cancerous senescent cells via a mechanism involving the mitochondrial transporter ANT2 (Hubackova et al., 2018), and this is likely linked to replication stress (published in 2020: Hubackova S, Davidova E, Boukalova S, Kovarova J, Bajzikova M, Coelho A, Terp MG, Ditzel H, Rohlena J, Neuzil J (2020): Targeting of dihydroorotate dehydrogenase and checkpoint kinase 1 results in suppression of tumor growth via cell cycle arrest induced by replication stress. Cell Death Disease 11, 110). This discovery is a basis for our current studies of MitoTam re-purposing.

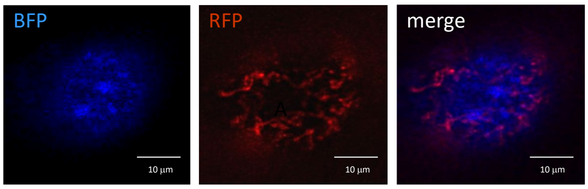

Fig. 2*. B16 rho0 cells with blue fluorescence protein nuclei were grafted in C17BL^{Su9DsRed2} mice with mitochondrial red fluorescent protein, and the tumour cells were recovered and sorted for red and blue fluorescence. The image shows a cancer cell with mitochondria of host origin.*

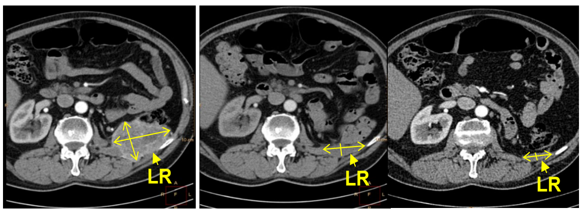

Fig. 3*. A clear cell renal carcinoma patient after left nephrectomy with relapse in the surgical bed was subjected to 3 rounds of 8-week regimen of Mito-Tam treatment, and the CT taken before the trial, after cycles 2 and 3. The red arrows point to the metastatic tumour that decreased over 40-fold.*

Based on our discovery of mitochondrial complex II (CII) as a new target for cancer therapy about 10 years ago, we have studied the biology and function of this complex in cancer (Bezawork-Geleta et al., 2017). We have shown that the catalytic SDHA subunit of CII, in spite of the general notion, does not need the assembly factor SDHAF2 for its flavinylation (Bezawork-Geleta et al., 2016), and we have found that under conditions of stress, CII is present in its partially assembled version, CII-low, comprising only SDHA and SDHAF2 and/or SDHAF4 (Bezawork-Geleta et al., 2018). We have recently characterized pheochromocytoma cells lacking the SDHB subunit of CII and proposed a possible therapeutic approach using ascorbate (published in 2020: Pang Y, Liu Y, Caisova V, Huynh TT, Taieb D, Vanova K, Ghayee HK, Neuzil J, Levine M, Yang C, Pacak K (2020): Targeting SDHB-mutated pheochromocytoma/paraganglioma (PCPG) with pharmacologic ascorbic acid. Clin Cancer Res (in press).

Laboratory of Gene Expression

Our Laboratory of Gene Expression is the leading Czech academic laboratory specialized in high-throughput gene expression profiling and single-cell analysis using RT-qPCR and RNA-Seq. In the period of 2015-2020, the team published more than 40 publications in peer-reviewed journals and one chapter in a scientific book. We have several research projects in the field of developmental biology and neurobiology, and applied projects in cancer research and diagnosis. We are also involved in the development of methods and applications for nucleic acid analyses and standardization protocols for effective workflows.

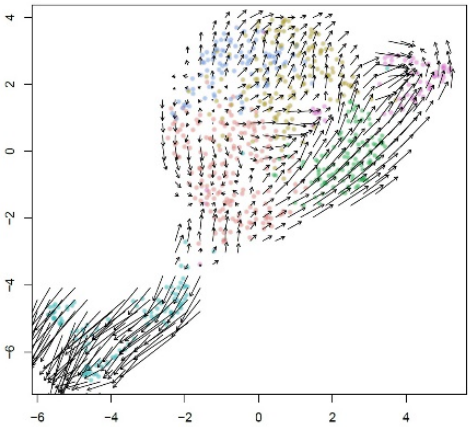

***Fig. 4**. RNA velocity field describes differentiation of astrocytes after ischemic brain injury.*

Our group is very active in the areas of new method development and implementation. In the last five years, our laboratory developed a highly specific, sensitive and cost-effective system to quantify miRNA expression based on two-step RT-qPCR with SYBR-green detection chemistry called two-tailed RT-qPCR (Androvic et al., 2017). Using this approach, we have designed a two-tailed RT-qPCR panel for quality control, monitoring of technical performance, and optimization of microRNA profiling experiments from biofluid samples (Androvic et al., 2019). The technology has already been employed in several collaborative projects and currently is also offered as a service by the Gene-Core facility at the IBT. We are also involved in the process of standardization, having formulated aspects and recommendations for single-cell qPCR (Kubista et al., 2018), and we reviewed methods for single-cell collection and analysis (Valihrach et al., 2018) or for analysis of circulating miRNAs in cancer diagnostics and therapy (Valihrach et al., 2020). We have further compared performance of reverse transcriptases for single-cell studies (Zucha et al., 2019) or participated in multicentre evaluation of circulating plasma miRNA extraction technologies. In the field of single cell analysis, we have analysed factors that regulate the quality of single-cell suspension during dissociation.

In the field of neurobiology, our group focused on characterization of glial cells after brain and spinal cord injuries, during aging and in the progression of neurodegenerative diseases, especially of Alzheimer's disease and amyotrophic lateral sclerosis. We have studied the role of Wnt- and Shh-signalling pathways in prolifera-tion and differentiation of NG2 glial cells, the function of astrocyte in ischemia, the role of Trpv4 and Aqp4 proteins in cell volume regulation, or mechanisms of miRNA regulation in the nervous tissue after spinal cord injury. We have applied the most current approaches for gene expression analysis in the field, such as single-cell gene expression profiling and RNA-Seq (Fig. 4).

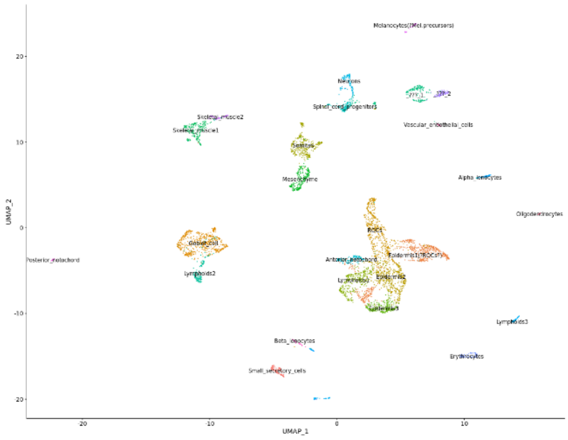

***Fig. 5**. UAMP analysis representing single cell RNA seq of Xenopus wound healing.*

Xenopus laevis eggs and embryos are our prime models in our developmental biology projects. We study localization of maternal coding and non-coding RNAs and proteins using our own methods such as qPCR tomography and Tomo-Seq (Sindelka et al., 2018). Recently, we have utilized other animal models, such as sturgeon and zebra-fish for asymmetrical RNA localization studies. We have also studied regulation of embryonic wound healing and regeneration (Fig. 5), and we have determined a novel role for nitric oxide during healing (Abaffy et al., 2019) and revealed that nitric oxide is important for epidermis formation and function (Tomankova et al., 2017).

Laboratory of Molecular Pathogenetics

Our research programme is focused on transcriptional regulation during embryonic development, molecular mechanisms of developmental programming, and identification of the molecular causes of abnormal embryonic development and disease predispositions. We are particularly interested in HIF-1, ISL1, SOX2, and NEUROD1 transcription factor networks and how their dysfunction affects embryonic development and can increase predispositions of an individual to diseases such as diabetes, heart disease or hearing loss. We also analyse the combinatorial effects of the environment (e.g., diabetes) and genetic mutations. Using mouse models, cellular and single-cell and bulk transcriptome analyses, we analyse molecular mechanisms to identify targets for the development of preventive and diagnostic strategies.

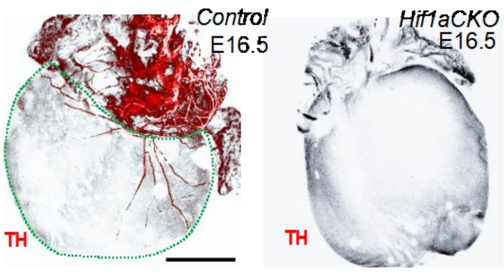

***Fig. 6**. Abnormal development of sympathetic innervation in the Hif1aCKO embryonic heart, as shown by immunolabelling of tyrosine hydroxylase (TH).*

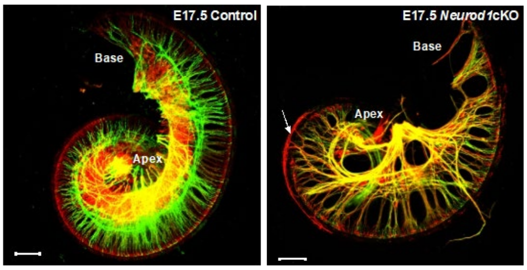

***Fig. 7**. Deletion of Neurod1 results in aberrant formation of the spiral ganglion and innervation in the cochlea of Neurod1CKO compared to controls.*

We have continued our collaboration with Prof Semenza (Nobel Laureate in Physiology and Medicine, 2019; Johns Hopkins University School of Medicine, USA) on the role of HIF-1 pathways in cardiovascular pathologies and in combination with diabetic exposure. We showed that genetic deletion of HIF-1α results in increased cell death and decreased proliferation of neuronal progenitors of the sympathetic system and formation of cardiac sympathetic innervation (Fig. 6) (Bohuslavova et al., 2019). These findings suggest that dysregulated HIF-1α expression may contribute to cardiac dysfunction and disease associated with defects in the cardiac sympathetic system, including sudden cardiac death and heart failure. Together with Prof Kolar's group (Institute of Physiology, CAS) and Prof Kubista's group (IBT, CAS), we showed that a global reduction in the Hif1a gene dosage increases predisposition of offspring exposed to maternal diabetes to cardiac dysfunction, and also underscore HIF-1 as a critical factor in the foetal programming of adult cardiovascular disease (Cerychova et al., 2018).

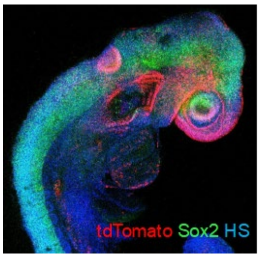

***Fig. 8**. Expression pattern of Sox2 in E9.0 embryos.*

In the last five years, in collaboration with Prof Fritzsch (University of Iowa, USA) and Prof Syka's group (Institute of Experimental Medicine, CAS), we continued our research on the role of Sox2, Isl1 and Neurod1 transcription factors in the neuro-sensory development and function of the auditory system. Our data provide the first insights into the limits of physiology-mediated brainstem plasticity during the development of the auditory system (Fig. 7) (Macova et al., 2019); featured article in J Neurosci). We demonstrated that the absence of primary afferent topology in the inner ear leads to dysfunctional tonotopy of the auditory system. We showed that Isl1 mutation induces alteration in the efferent system and results in an early onset of age-related hearing loss (Chumak et al., 2016) and negatively affects GABA signalling in the brain with correlation to attention deficit hyperactivity disorder (Bohuslavova et al., 2017). We analysed the requirements for SOX2 during neurosensory development (Fig. 8) (Dvorakova et al., 2016). The inner ear provides a simpler model to study SOX2 involvement in neuronal specification, proliferation, and differentiation compared to the brain. We showed differential requirements of SOX2 for neuronal development in the Sox2 conditional deletion mutant, as all early forming vestibular neurons seem to develop normally; however, late-forming spiral ganglion neurons are not formed. Thus, these results address some fundamental questions of cell replacement therapy.

Laboratory of Tumour Resistance

The major research topics investigated in the lab are: i) elucidating the molecular mechanisms underlying cancer resistance, proliferation and recurrence; ii) developing novel approaches that target cancer cells based on mitochondrial targeting and interference with iron metabolism; and iii) deciphering the molecular mechanisms that govern appropriate systemic iron metabolism and the relation-ship between cancer and iron metabolism.

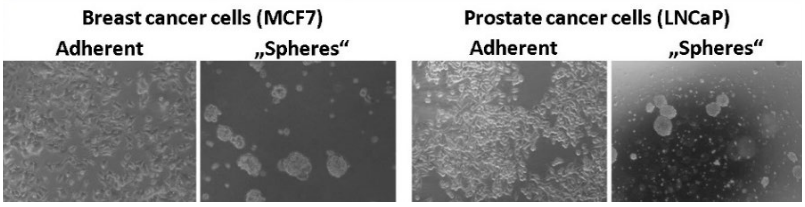

***Fig. 9**. Appearance of cells growing in 3D as so called "spheres" that exhibit properties of tumour-initiating cells.*

Since there are many possible ways how cancer cells can develop resistance, we study several models of resistance. We cultivate and investigate the molecular profiles of tumour-initiating cells (TICs) that grow as three-dimensional (3D) "spheres" and more closely resemble real tumours (Fig. 9).

TICs show upregulation of the ABC transporters that confer resistance to commonly used anti-cancer drugs. We have published a pioneering work about iron metabolism in TICs (Rychtarcikova et al., 2017), where we describe the iron metabolism-related gene signature, document higher iron uptake, higher labile iron pool and accumulation of iron within mitochondria, a markedly higher ROS level and enhanced sensitivity to iron chelation in these cells.

We have also elucidated the molecular mechanisms that underlie tamoxifen resistance in breast cancer cells and we have found an increased level of mitochondrial superoxide and altered mitochondrial structure and function in these cells. In a recent report (Tomkova et al., 2019), we show that these cells exhibit extensive fragmentation of mitochondria and display markedly reduced levels of respiratory super-complexes assembled into supra-molecular structures called "respirasomes", which is associated with their lower enzymatic activity and decreased mitochondrial respiration. These findings thus suggest a link between tamoxifen resistance and decreased mitochondrial function. Importantly, we have shown that high expression of miR-301a-3P in oestrogen-dependent breast cancer cells results in inhibition of oestrogen signalling, thus participating in the transition to tamoxifen resistance (Lettlova et al., 2018).

One of the main topics of our group in collaboration with the Molecular Therapy Laboratory is mitochondrial targeting as effective anti-cancer therapy. In this regard, we have together identified a very strong anti-proliferative effect of MitoVES at doses that are non-apoptotic. The underlying mechanism is a considerable decrease in mtDNA transcription, particularly a decrease in the D-LOOP transcript both in vitro and in vivo (Truksa et al., 2015). The intracellular localization of MitoVES in mitochondria is shown in Fig. 10.

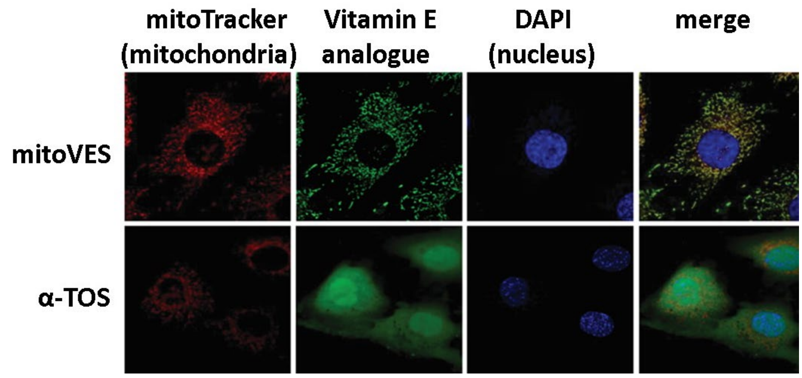

Fig. 9*. Intracellular localization of MitoVES and parental com-pound α-TOS, showing the presence of MitoVES in mitochondria.*

The last topic of the lab is systemic iron metabolism. We use the mouse model of iron deficiency iron refractory anemia (IRIDA) to study the iron metabolism, erythropoiesis and the regulation of erythroferrone (ERFE), transferrin receptor 2 and haemojuvelin in this model. We have been successful in developing the assay to test the ERFE protein level in vivo and reported that its protein level is markedly upregulated in the mouse model of IRIDA despite its inability to respond to erythropoietin and mutated Tmprss6 gene (Frydlova et al., 2017).

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Research activity and characterization of the main scientific results

The primary focus of the team is high-quality basic research in the field of structural biology and protein engineering with potential applications of the results in the diagnosis and treatment of diseases. Over the evaluated period, the team published in total 93 publications in peer-reviewed journals, 2 chapters in scientific books and obtained 17 applied results. The team comprises 6 laboratories, whose research activity over the evaluation period is detailed below. The overview of the publications is in the Appendix 3.6.

Laboratory of structural biology

Our research revolves around recombinant proteins, their production, engineering, structural and functional characterization from the single molecule level to their physiological functions in cellular and organism environments (Fig. 1). A platform for heterologous expression and purification of recombinant proteins implemented in our laboratory is extensively used not only by us but also by a wide network of our collaborators and in our commercially oriented on-demand protein production. We are primarily interested in two major protein families as elaborated below:

(i) A family of peptidases homologous to **glutamate carboxypeptidase II (GCPII), a.k.a. prostate specific membrane antigen (PSMA).**

The human enzyme is implicated in several (patho)physiological processes. In the nervous system, GCPII exerts its peptidase activity by hydrolyzing a peptidic neurotransmitter. Accordingly, GCPII-specific inhibitors have been reported to be neuroprotective in multiple preclinical models of neurodegeneration. Furthermore, over-expression of GCPII in prostate carcinoma makes the enzyme a prime marker for prostate cancer imaging in clinics and a promising target of future therapeutic interventions.

In a series of eight manuscripts we contributed significantly to our understanding of structural features of human GCPII that could be exploited for the rational design of small-molecule GCPII-specific inhibitors. We described an unprecedented binding mode of hydroxamate-based compounds in the internal cavity of GCPII (Novakova et al., 2016) and these inhibitors were later converted into prodrugs improving thus their bioavailability (Rais et al., 2017). In collaboration with our partners, we designed and characterized GCPII-specific compounds of different chemistries (phosphoramidates, carboranes, ureas) and physicochemical characteristics. Additionally, our long-term contribution to the field was recognized by an invitation to co-author a review summarizing 20 years of the development of urea-based radioligands targeting GCPII (Kopka et al., 2017).

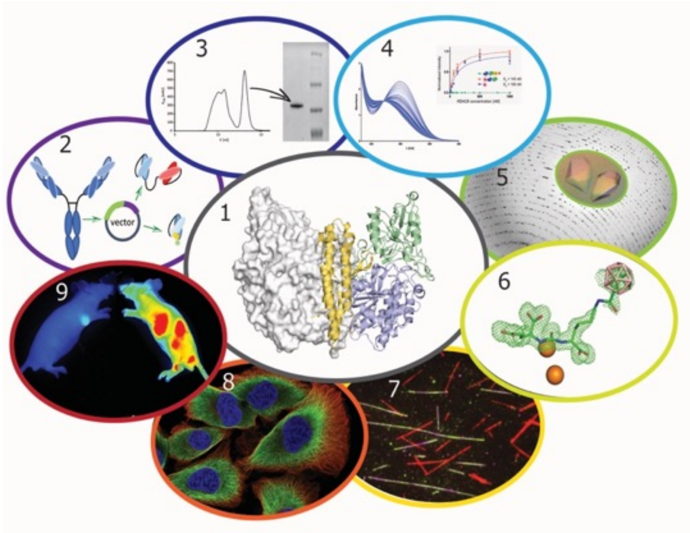

***Fig. 1**. Our research revolves around proteins (1) and includes protein engineering (2), expression/purification (3), biochemical and biophysical characterization (4), X-ray crystallography (5), structure-assisted drug design (6), in vitro (7), cells-based (8) and in vivo (9) assays.*

Protein engineering efforts resulted in three manuscripts describing the development of (i) protein scaffolds that offer a viable alternative to antibodies for biomedical applications (collaboration with A. Skerra, TUM); and (ii) GCPII-specific monoclonal antibodies with exquisite specificity and sub-nanomolar affinity (Banerjee et al., 2019; Novakova et al., 2017). These results have also been patented (US1040624; patent application WO2018129284-A1) and further efforts are aimed at their translation as diagnostics and therapeutics into human medicine.

Several of our projects were/are aimed at the understanding of role(s) of GCPII and its orthologs/paralogs in physiological processes, including uncovering a physiological function of NAALADase L (Tykvart et al., 2015), defining the role of GCPII for angiogenesis (Conway et al., 2016), and elucidating the functional significance of the calcium ion for GCPII stability and hydrolyzing activity (Ptacek et al., 2018).

(ii) A family of zinc dependent **histone deacetylases (HDACs 1 – 11)** that remove acyl/acetyl groups from the lysine side chain, a major post-translational modification found on >10% of proteins of the human proteome. Here we aim at (i) deciphering the structure-function relationship, (ii) defining physiological functions, and (iii) designing specific inhibitors of HDAC6 and HDAC11 isoforms.

Previously, we have established methodology for facile production of all eleven HDAC isoforms that become a cornerstone for our current research. Here developed and implemented *in vitro* assays for profiling isoforms specificity of HDAC inhibitors (Kutil, Mikesova, et al., 2019) (Zessin et al., 2019), established several cell-based assays, an ADMET pipeline, and a structural (X-ray) platform to evaluate HDAC6/inhibitor complexes. This methodological portfolio is exploited in a series of ongoing projects within a network of our academic and corporate collaborators as it complements their expertise in medicinal chemistry and biological (*in vivo*) studies. Within the evaluation period we published total seven manuscripts aimed at the development of HDAC6-selective inhibitors for different applications (neurological disorders, melanomas), with several more manuscripts pending. Furthermore, several of our HDAC-related products (recombinant proteins/antibodies, assays) found their way into a commercial sector revealing thus the commercial outreach of our research.

Our “basic-research oriented” projects are focused on characterization of physiological functions and substrate preferences of HDAC6 and HDAC11. We have discovered that HDAC11 serves as a proficient fatty-acid deacylase and these findings can facilitate the uncovering of additional biological functions of the enzyme ((Kutil et al., 2018); 26 citations in two years). Using human acetylome microarrays and peptide libraries we mapped the substrate specificity of HDAC6, and these data can point out unknown physiological substrates of the enzyme (Kutil, Skultetyova, et al., 2019). We provided a detailed analysis of deacetylation of tubulin, the most prominent physiological substrate of HDAC6, and determined that free tubulin dimers are preferential form deacetylated by HDAC6 (Skultetyova et al., 2017). We discovered the unstructured N-terminus of HDAC6 to be the microtubule-binding domain critical for efficient tubulin deacetylation (Ustinova et al., 2020). We currently follow-up and broaden our research focus by implementing of methods of genetic code expansion to obtain more detailed understanding of substrate recognition by the HDAC family.

Laboratory of structure and function of biomolecules

Our structure-function studies of enzymes with biomedical and biotechnological potential have yielded results in three main directions: (i) non-specific nucleases, (ii) bilirubin oxidase, and (iii) novel glycosidases.

(i) S1-P1 nucleases have a broad range of biotechnological utilizations and there are many questions regarding their mechanism and applicability in treatment of cancer and other

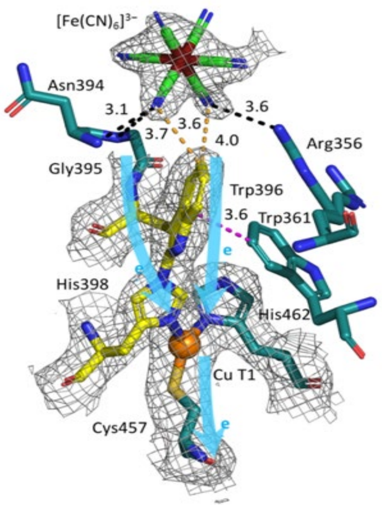

Fig. 2*. Active site of enzyme bilirubin oxidase with unique covalent bond between amino acids tryptophan and histidine (sticks model with carbon in yellow) as observed in our crystal structure.*

diseases. Continued from previous structure-function work we have finalized characterization of tomato nuclease TBN1 by determining 3D structure of its mutant N211D in complex with phosphate in its active center (Stransky et al., 2015) and determining the influence of glycosylation on its function (Podzimek et al., 2018). Eukaryotic nucleases of the S1-P1 type display a strong dependence of their activity and specificity on the level of glycosylation. Artificial increase of glycosylation leads to changes of enzyme specificity. We have determined the first three-dimensional structure of S1 nuclease from *Aspergillus oryzae*, together with functional and ligand binding details (Koval et al., 2016). Here, rules of its interactions with nucleic acids and new enzyme functionality have been discovered for this enzyme, known well for its biotechnological applications. S1-P1 nuclease from the causative agent of the Legionnaires' disease – *Legionella pneumophila* has been expressed and characterized in detail (Trundová et al., 2018). It has been shown to be an RNase with high temperature stability. The first study of this type of nuclease related to a human pathogen has indicated that this nuclease with extreme properties very likely binds to cell membrane. Our further structure-function analysis has yielded new results regarding the role of the surface binding sites of this nuclease (paper in preparation). Our review *Characteristics and application of S1–P1 nucleases in biotechnology and medicine* (Koval' & Dohnálek, 2018) was motivated by our growing experience and interest in this field and by the lack of such comprehensive work. This first review dealing with the properties and application of these enzymes concluded that this biotechnologically essential enzyme type has also an interesting potential in treatment of diseases caused by pathogens and also of some types of cancer.

(ii) Bilirubin oxidase is used in medicine for determination of bilirubin blood level and in industry, biofuel cells, and biosensors for its universal oxidation activity and efficient electron transfer. The enzyme employs a new and unique chemical bond between two amino acids. Using crystallography and mutagenesis we have shown that this Trp–His covalent bond is important for interaction with substrate, depending on the particular substance and environment (Koval et al., 2019). The results have implications for biotechnology and nanomaterials.

(iii) Our studies of new **glycosidases** are focused on proteins with unexplained structural properties and with expected application potential. We have determined the first crystal structure of α-L-fucosidase of GH29 from *Paenibacillus thiaminolyticus* with a unique hexameric arrangement (6 molecules forming one functional unit) and active site complementation (Kovaľová et al., 2019). Its active site is complemented by a tryptophan residue from a neighbour protein chain within the hexamer and its mutation influences its fucosidase and transglycosylation activities. Also, a new type of carbohydrate binding domain has been identified. The results are important for applications of oligosaccharide-modifying enzymes. We have also contributed to determination of X-ray structures of two new, heavily glycosylated, β-D-glucosidases of family GH3 from *Aspergillus* sp. (Agirre et al., 2016), which provided new insights into fungal β-D-glucosidases and a platform for new enzyme variants for industrial application.

In **natural killer cell receptors** we have explained the structural behavior (X-ray crystal structures) of the human cell surface protein LLT1 – a C-type lectin-like ligand of the human receptor NKR-P1 (Skalova et al., 2015). This was the first study with structural insights into

LLT1. Our continued effort in collaboration with the team of O. Vaněk (Charles University, Faculty of Science) has resulted in determination of structures of several other complexes of mammalian cell surface receptors and ligands, awaiting completion of functional analysis.

Intracellular **heme-dependent signal proteins** lack detailed structure-function understanding of their function. In collaboration with the team of M. Martínková (Charles University, Faculty of Science) we have determined the first crystal structures of the globin domain of intracellular oxygen sensor AfGcHK (globin coupled histidine kinase) from soil bacterium *Anaeromyxobacter* sp. Fw109-5 with 5-coordinated and 6-coordinated heme states. By combination of X-ray diffraction and H/D exchange, some characteristics of signalling were explained for the first time (Stranava et al., 2017). Further studies have increased our understanding of the signal transfer and our monomeric crystal structure of the AfGcHK globin domain and mutational analysis of Tyr15 have shown the importance of Tyr15 for globin domain dimerization and its necessity for phosphorylation activity of the enzyme (Skalová et al., 2019).

Our structure-function studies of the **transcription machinery in Gram-positive bacteria** have been focused on new protein partners/factors involved in transcription regulation. Using small angle X-ray scattering and transcription experiments, we have determined new details regarding the domain structure of HelD, an interaction partner of *Bacillus subtilis* RNA polymerase (Koval' et al., 2019). The N-terminal domain is required for ATP-dependent transcription stimulation but not for binding to the RNA polymerase core. The study laid foundations for further work on HelD. We have also contributed to a complex study of conformational behavior of the intrinsically disordered region of the delta protein interacting with RNA polymerase from *Bacillus subtilis*, employing NMR, small-angle X-ray scattering and other biophysical methods for verification of interactions (Kuban et al., 2019). The results have increased our understanding of the features of the charged intrinsically disordered part of the delta protein important in regulation of bacterial transcription.

Laboratory of ligand engineering

One of the most important research activities of the Laboratory of Ligand Engineering during the last five years was dedicated to the development of high-affinity binding proteins targeting paratopes of broadly neutralizing antibodies that mimic epitopes recognized by these antibodies due to shape/charge complementarity. By screening of highly complex combinatorial libraries we identified “protein prints” that can be used as strong immunogens for the stimulation of protective antibodies similar to those ones originally used as molecular targets (Fig. 3). This concept, which has never been used before for the generation of protective vaccines, has recently been demonstrated by us as a novel strategy in the reverse vaccinology. We generated “non-cognate ligands” of HIV-1 broadly neutralizing VRC01 antibody paratope that were identified from our in-lab-designed albumin-binding domain (ABD)-derived combinatorial library. These proteins called VRA ligands mimic gp120/Env epitope located in CD4 binding site and induce gp120-specific and HIV-1-neutralizing antibodies in serum of immunized mice (Kosztyu et al., 2019). As a high-efficacy preventive HIV vaccine is still not available, mainly due to extraordinary mutability of the Env gene, efficient glycan shielding of antigenic epitopes and low immunogenicity of HIV Env glycoprotein, we provided a radical concept for generation of vaccines via small-protein mimetics (see comment by P.J. Klasse *“Non-cognate ligands of Procrustean paratopes as potential vaccine components”*, EBioMedicine, 47, 6-7, 09-2019). As VRA proteins generated in our laboratory possess a promising commercial potential, we submitted Czech patent application (Maly, 2017), which will be extended to a PCT application before September 2020.

Another long-term research activity of the Laboratory is focused on the development of non-immunoglobulin inhibitors of cytokines and their receptors based on small binding proteins. A pivotal role in this effort plays IL-23/IL-17 pro-inflammatory axis which is responsible for several autoimmune disorders including psoriasis and inflammatory bowel disease (IBD). After a successful introduction of our first protein blockers, IL-23 receptor-specific REX protein antagonists (Kuchar et al., 2014) (Krizova et al., 2017) and an international patent by Malý et al. 2017, we streamlined other players in this cascade, IL-23 cytokine and IL-17 receptor A (IL-17RA). We generated a collection of human IL-23-targeted proteins called ILP binders that substantially diminished binding of recombinant p19 protein, alpha subunit of the IL-23, to IL-23 receptor on human THP-1 cells. The most promising candidates inhibited IL-23-driven expansion of IL-17-producing primary human CD4+ T-cells. These novel binders represent unique IL-23-targeted probes useful for IL-23/IL-23R epitope mapping studies and could be used for designing novel p19/IL-23-targeted anti-inflammatory biologics (Krizova et al., 2017; Maly, 2019). We also targeted human IL-17 receptor A (IL-17RA), a downstream member of the IL-23-stimulated/Th-17 cell-mediated pro-inflammatory cascade. We generated a unique collection of human IL-17RA-specific inhibitors called ARS ligands that blocked binding of IL-17 cytokine to recombinant IL-17RA-IgG chimera, inhibited binding of IL-17 cytokine to human keratinocyte HaCaT cells and THP-1 cell line, and suppressed secretion of Gro-alpha (CXCL1) by normal human skin fibroblasts CCD1070Sk upon IL-17A stimulation in vitro (Hlavnickova et al., 2018).

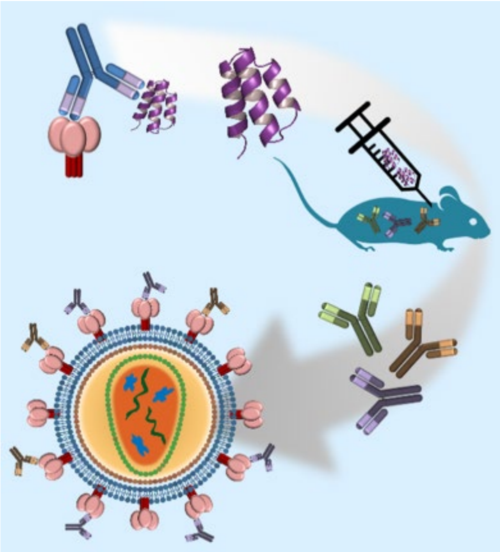

***Fig. 3**. Cartoon summarizing the experimental approach of the Laboratory of ligand engineering.*

As lactic acid bacteria have recently been demonstrated as promising non-toxic and by host tolerable delivery vehicles useful for transport of per-orally administrated therapeutics into the gut, we established collaboration with Dr. Aleš Berlec, PhD. from the Department of Biotechnology at the Jožef Stefan Institute in Ljubljana, Slovenia, for experimental verification of REX, ILP and ARS binders in treatment of intestinal inflammation. In this collaboration, supported by the Czech Academy of Sciences and Slovenian Academy of Sciences and Art (SAZU) by bilateral mobility project (Reg. No. SAZU-16-01, 2016-2018), we developed unique strains of *Lactococcus lactis* that secrete, or display via AcmA peptidoglycan anchor, ILP proteins and retain IL-23-cytokine binding specificity and inhibitory function (Skrlec et al., 2018). In a similar study, we generated a collection of *L. lactis* strains secreting variants of IL-23 receptor-specific REX binding proteins (Plavec et al., 2019). As IL-23/Th17 inflammatory axis plays a substantial role in IBD and Crohn´s disease, all these ILP- and REX-secreting bacteria should be tested in vivo for their anti-inflammatory potential in mouse model of colitis. In a separate project, we developed ABD-derived protein binders targeting Shiga Toxin 1B Subunit and generated recombinant *L. lactis* secreting Shiga Toxin 1B-specific binders (Zadravec et al., 2016). Infections with Shiga toxin (Stx)-producing bacteria may develop into a life-threatening hemolytic uremic syndrome (HUS) with acute renal failure. The developed *L. lactis* strains secreting Stx-binding proteins could serve as tools for removal of Shiga toxins from the intestine.

In another part of our research we developed protein binders useful for in vitro or in vivo imaging. We generated binders targeting human serum prostate cancer oncomarkers including Prostate Specific Protein 94 (PSP94) (Mareckova et al., 2015). In a collaborative project, focused on the development of nanoliposomal systems for rapid diagnosis of thrombi by MRI, we generated a fibrin-specific protein which binds to fibrin fibers of human thrombus and, if immobilized to the liposomal surface, delivers fluorescently labelled protein-liposome complexes under dynamic flow conditions to human thrombus in 3D model of middle cerebral artery (Petrokova et al., 2019). In another project, we collaborated on the development of microfluidic chips based on high-affinity ABD proteins for serum or urine proteins detection (Maly et al., 2016; Semeradtova et al., 2018).

Laboratory of biomolecular recognition

We focus on understanding the interactions driving specific recognition between biomolecules with potential diagnostic, medical or biotechnological use. In our research, we take advantage of experimental and computational methods of protein engineering, structural biology, bioinformatics, and molecular modeling. The central technique of most projects running in the lab is protein engineering: (i) cytokines and their receptors (ii) time-resolved biomolecular dynamic of light-inducible proteins, and (iii) a bacterial transposase RAYT and the related REP DNA. In close collaboration with Laboratory of Dr. Cerny, we work on (iv) structural bioinformatics of nucleic acids. More information about the lab work and results can be found at the website biorecognition.structbio.org.

(i) Proteins of the innate immune system. Cytokines are small signaling proteins essential for proper immune responses during inflammation, infection, trauma or cancer. Any error in their regulation causes serious autoimmune and/or allergic health disorders. We concentrate on the structural aspects of interactions between cytokines from the group of interleukin 10 (family FIL-10) and extracellular parts of their receptors. In the last few years, we have worked on interferon-ɣ and its two receptors. Specifically, we increased the affinity of human IFN-ɣ receptor 1 to its cognate IFN-ɣ (Cerny et al., 2015) and solved a crystal structure of IFN-ɣ receptor 2 (PDB ID 5eh1 (Mikulecky et al., 2016)). Our interest in the system has further spurred the study of evolution of interferons of type II which concluded in a comprehensive overview of evolution of the IFN-ɣ system in fish; the study included the first structure of non-mammalian interferon (PDB ID 6f1e (Zahradnik et al., 2018)).

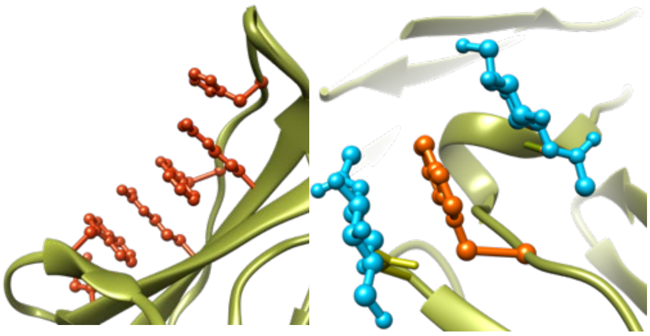

***Fig. 4**. Structure of human IFN-ɣ receptor 2 (PDB 5eh1 (Mikulecky et al., 2016)). Figure on the left shows the stacking motif stabilizing the fold, figure on the right the surface-exposed tryptophan residue sandwiched between two sugar residues and shielded thus from the solvent*

We use techniques of *in vitro* protein evolution, ribosome and yeast display to *de novo* development of specific proteins binding the FIL-10 cytokines and receptors to block or modify their signaling. We have developed a new small protein scaffold that has been trained to bind to interleukin 10 (Pham, Huliciak *et al*. in preparation 2020) and this and other scaffolds have been trained against other FIL-10 cytokines and receptors. By combining modeling approaches, mainly application of the PROSS algorithms, we succeeded in producing stable and non-signaling (PDB ID 6gg1) as well as signaling variants of IL-24 (Zahradnik et al., 2019). The IL-24 related project continues in collaboration with several research groups at the Weizmann Institute of Science and has been extended to protein engineering of other FIL-10 proteins.

(**ii) Time-resolved biomolecular dynamic of light-inducible proteins**. In 2017, two labs of the IBT Team 2 opened a project “Dynamics of biological processes” with the infrastructure ELI-Beamlines in neighboring Dolni Brezany. The goal is to decode the structural dynamics of photo-controlled transcription factors and other proteins. The main workhorse is the light-oxygen-voltage (LOV)-containing protein EL222 but we work also with CarH, coenzyme B12-binding, and CooA, heme-binding transcription factors. We have been approaching the lit-state structural ensemble of EL222 by an integrative structural biology approach combining crystallography, NMR, and small-angle neutron and X-ray scattering (SANS, SAXS). Scattering and fluorometric data indicate light-induced oligomerization of EL222 beyond the dimeric state and possibly large but reversible aggregates. The molecular mechanism of EL222 photoactivation is being approached by time-resolved vibrational spectroscopy methods, particularly femtosecond-stimulated Raman (FSRS) and infrared spectroscopy. As an important step for the understanding of EL222 dynamics, we unveiled the ultrafast (femtosecond-to-nanosecond) dynamics of flavin mononucleotide (FMN), the light-absorbing cofactor of EL222) by advanced quantum chemistry calculations and FSRS experiments (Andrikopoulos *et al.*, PCCP, January 2020, doi: 10.1039/c9cp04918e). The group has collaborated in the commissioning of two high-end optical spectroscopy set-ups at the IBT Centre of Molecular Structure: a time-resolved FTIR instrument coupled to a nanosecond UV/Visible/NIR laser, and a picosecond time-resolved spectrofluorometer. Of a great value for the whole Team 2, IBT and the center BIOCEV has been implementation of protein engineering tools, like site-specific isotopic labeling (SSIL) and genetic code expansion (GCE) technology, in order to introduce non-canonical amino acids carrying spectroscopy-sensitive probes and chemical handles for FTIR, FSRS and fluorescence-based techniques.

(**iii) Transposase RAYT and the related REP DNA oligonucleotides**. We just mention currently a smaller project dealing with the RAYT proteases and REP-related oligonucleotides and (Charnavets et al., 2015). Crystal structures of three REP-related oligoes have been solved and refined (Kolenko et al., to be submitted 2020) with help of our know how of the NA conformational space as described below.

(**iv) Structural bioinformatics** of nucleic acids is a long-standing research interest of the group recently in a close collaboration with Dr. Cerny lab (established 2017) of the IBT Team 2. Nucleic acids are structurally plastic molecules, and their biological functions are enabled by adaptation to their binding partners. We study structural aspects of their recognition by other molecules. To identify structural polymorphisms of nucleic acids we analyzed hundreds of thousands of dinucleotide structures in hundreds of crystal structures. The work is summarized in several publications and the results are available on the website dnatco.org. We have further developed and fundamentally improved our older approaches to the structural analysis of nucleic acids. The main step was a comprehensive description of the structures of DNA dinucleotides by so called Nucleotide Conformers, NtC and the derived Conformational Alphabet of Nucleic Acids, CANA (Schneider et al., 2018). The methodology has been extended to both DNA and RNA (Cerny *et al.*, published in Nucleic Acids Research May 2020, doi: 10.1093/nar/gkaa383). The DNA-limited CANA and NtC classification set was applied to analysis of DNA structures; comparison of structural features of transcription factors and histone-bent DNA revealed significant structural differences between the specific and non-specific interactions between proteins and DNA (Schneider et al., 2017).

A part of our bioinformatic studies focuses on the structure of the solvation shell around biomolecules. The structures of the first hydration shell of amino acids residues in proteins and of DNA dinucleotides are reconstructed by analysis of solvation of large numbers of crystal structures. The results show how hydration depends on the amino acid (Biedermannova & Schneider, 2015) or dinucleotide (Biedermannova et al. to be published) structure and sequence. The results have been summarized in our review (Biedermannova & Schneider, 2016) and are available on the website dnatco.org/wataa (Cerny et al., 2017). We are currently working on the DNA hydration atlas.

Laboratory of structural bioinformatics of proteins

Activities covering structural bioinformatics and molecular modeling of biomolecules or small drug-like molecules resulting from numerous collaborations within the Team II as well as with the Team I are partially discussed in the corresponding parts of the report.

We continued with the development and application of our unique level of description of nucleic acid conformations which resulted in a series of publications (Cerny et al., 2016; Schneider et al., 2018; Schneider et al., 2017). Initially, concentrating still only on DNA, we developed an automated protocol assigning 44 distinct conformational classes called NtC (Nucleotide Conformers) and the DNA structural alphabet CANA (Conformational Alphabet of Nucleic Acids) to describe the DNA structural polymorphism. The NtC classification was used to define a validation score called confal, which quantifies the conformity between an analyzed structure and the geometries of NtC. A novel projection of CANA and confal onto the DNA structure was also developed for simple annotation and validation of nucleic acid structures by non-experts. The structural alphabet was also successfully applied to study the differences and true mechanism of bending of DNA complexed with regulatory proteins and in the nucleosome core particle (NCP). We have identified the specific role of two DNA structural forms, A-DNA, and BII-DNA, discriminating the specific and non- specific binding of DNA to proteins. A-DNA conformations are avoided in non-specific NCP complexes, where the wrapping of the DNA duplex is explained by the periodic occurrence of BII conformation every 10.3 steps. The automated assignment of the NtC classes and CANA codes is freely available for all interested users at dnatco.org and provides a powerful tool for unbiased analysis of nucleic acid structures by structural and molecular biologists. The web service based on our unique automated protocol assigning conformational classes currently allows quantitative assessment and validation of NA conformations and their subsequent analysis by means of pseudo-sequence alignment. The method also allows search for nontrivial structural patterns of nucleic acids. The description of implementation and example use cases for the second version of the DNATCO (dnatco.org/v2) web service were published.

Recently, we have significantly extended and at the same time simplified the structural alphabet which now combines both DNA and RNA and allows analyses of nucleic acid conformations at a unified level. We derived 96+1 NtCs, which describe the geometry of RNA and DNA dinucleotides. NtC classes are grouped into 15 codes of the structural alphabet CANA to simplify symbolic annotation of the prominent structural features of NAs and their intuitive graphical display. Our analysis employing the new extended and universal structural alphabet revealed two surprising results. Notably, over 30% of the nearly six million dinucleotides in the PDB cannot be assigned to any NtC class but we demonstrate that up to a half of them can be re-refined with the help of proper refinement targets. A statistical analysis of the preferences of NtCs and CANA codes for the 16 dinucleotide sequences showed that neither the NtC class AA00, which forms the scaffold of RNA structures, nor BB00, the DNA most populated class, are sequence neutral but their distributions are significantly biased.

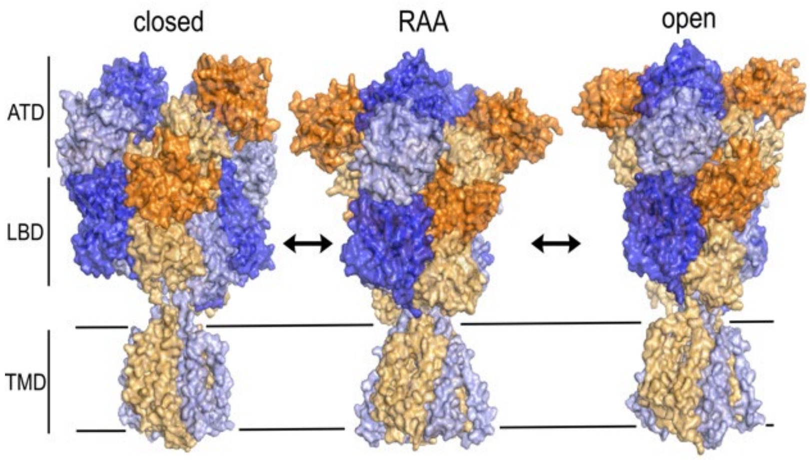

***Fig. 5**. The NMDAR is shown during the structural transition from the closed to the open state of the receptor as captured by molecular dynamics simulations. The snapshots of geometry from the MD simulation were structurally superimposed using the transmembrane domain (TMD) M3 helices as a reference.*

Our development in the field of enhanced sampling simulations of biomolecules resulted in two new theoretical techniques which can significantly accelerate the sampling rate and at the same time improve the accuracy of simulated structural and conformational ensembles. We have developed an enhanced sampling method based on a hybrid Hamiltonian which combines experimental distance restraints with a multiple path-dependent bias (Peter & Cerny, 2019). This simulation method determines the bias-coordinates on the fly and does not require a priori knowledge about reaction coordinates. The method accelerates the sampling of proteins as tested using experimental NMR-restraint data and distance restraints from chemical crosslinking/mass spectrometry experiments. The second enhanced sampling method for molecular dynamics simulation of protein and DNA systems (Peter & Cerny, 2018) is based on a potential of mean force (PMF)-enriched sampling, determined from DNA and protein structures in the PDB. Our results show that the simulation technique enriches the conformational space of biomolecules, provides improved distributions of conformations compared to the underlying forcefield, while we also observe a considerable speed increase in the sampling by factors ranging from 13.1 to 82. Both methods can be combined for further acceleration and higher accuracy of simulations.

As a part of our long-standing collaboration with the Department of Cellular Neurophysiology at the Institute of Physiology studying the N-methyl-D-aspartate (NMDA) receptor we have revealed details of the activation mechanism of the receptor. It is now well established that NMDARs play a crucial role in rapid excitatory synaptic transmission in the mammalian central nervous system and promote persistent changes in synaptic strength. NMDAR states associated with excessive receptor activation, as well as receptor hypo- or hyper-function, are clinically relevant. In our recent publication we report the first complete description of the molecular mechanisms of the NMDA receptor transition from the state where the ion channel is in the open configuration to the unliganded state where the channel is closed (Cerny et al., 2019). Using MD simulations we identified distinct structural states of the NMDA receptor and revealed functionally important residues. The activated (RAA) and open states of the receptor are structurally similar to the liganded crystal structure, while in the unliganded receptor the extracellular domains perform surprisingly large rearrangements leading to a clockwise rotation of up to 45 degrees around the longitudinal axis of the receptor, which closes the ion channel (Fig. 5). The ligand-induced rotation of extracellular - amino-terminal (ATD) and ligand binding (LBD) - domains, transferred by LBD-TMD linkers to the membrane-anchored ion channel is responsible for the opening and closing of the transmembrane ion channel, revealing the properties of NMDA receptor as a finely tuned molecular machine. The study can stimulate the development of new potential drugs modulating NMDA receptor with the necessary functional state specificity.

Laboratory of structural proteins

This junior laboratory was established in the 2015. During the initial five years of the lab existence, the lab research activities comprised three main areas: (i) Regulatory roles of intrinsically disordered microtubule-associated proteins, (ii) Organization principles of higher order microtubule assemblies and (iii) Collective effects in functioning of molecular motors.

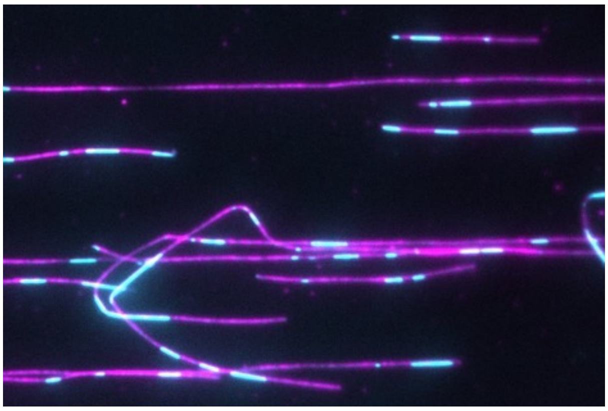

Fig. 6*. Multichannel fluorescence micrograph showing cohesive islands of intrinsically disordered protein tau (cyan) on microtubules (magenta).*

(i) Intrinsically disordered microtubule-associated proteins, such as tau, are essential cytoskeletal regulators important especially in neurons. Their malfunctioning is associated with a number of neurodegenerative diseases, such as the Alzheimer's disease. Recent results suggest that many intrinsically disordered proteins can form compartment by liquid-liquid phase separation. These compartments locally facilitate specific reactions by establishing microenvironments different form the cytoplasm. It is not known whether intrinsically disordered microtubule proteins can phase separate. Consequently, what would be the roles of these compartments is an open question.

We found that microtubule associated protein tau can form liquid-like drops through liquid-liquid phase separation. We found that soluble tubulin partitions into these drops, which locally increases tubulin concentration and enables nucleation of microtubules from the drop and their bundling. Our results thus suggest that condensed compartments of intrinsically disordered microtubule-associated proteins could promote the local formation of microtubule bundles in neurons, acting as non-centrosomal microtubule nucleation centers (Hernandez-Vega et al., 2017).

Knowing that tau can form liquid-like compartments in solution, we next investigated tau condensation on the surface of microtubules. We found that tau molecules on microtubules cooperatively form cohesive islands that are kinetically distinct from tau molecules that bind to microtubules individually (Fig. 6). Importantly, the islands exhibit regulatory qualities distinct from a comparably dense layer of individually bound tau. The islands shield microtubules from particular molecular motors, such as kinesin-1, and microtubule-severing enzymes, such as katanin. Our results thus reveal a microtubule-dependent phase of tau that differentially regulates the access of other microtubule-associated proteins to the microtubule surface (Siahaan et al., 2019).

(ii) The cilium, composed of a **higher order microtubule assembly,** is an organelle crucial for motility, as well as for sensing environmental cues such as signaling molecules, light, and mechanical stimuli. The core structure of the cilium is characterized by nine microtubule doublets, consisting of an incomplete "B-microtubule" coupled to the surface of a complete "A-microtubule". Despite the fundamental role of microtubule doublets, the molecular mechanism governing their formation is unknown. Here we found, using an in vitro assay, a crucial inhibitory role of the C-terminal tail of tubulin in the assembly of the microtubule doublets. Removal of the C-terminal tail of an assembled A-microtubule allowed for the nucleation of a B-microtubule on its surface. We characterized the dynamics of the B-microtubule nucleation and we uncovered a distinctive isotropic elongation of the B-microtubule. We thus found that inherent interaction properties of tubulin provide a structural basis driving flagellar microtubule doublet assembly (Schmidt-Cernohorska et al., 2019).

(iii) Remodeling of microtubule networks underpins essential cellular processes, such as cell division or cell motility. **Microtubule-crosslinking and sliding molecular motors**, such as the members of the kinesin-14 family, are essential regulators of the microtubule network remodeling. In cells, these motors function predominantly in teams. Although these motors have been extensively characterized on the single molecule level, their ensemble dynamics and their collective functioning are much less understood.

Members of the kinesin-14 family typically interact with one microtubule via their non-processive motor domains and with another via their diffusive tail domains. The influence of the tail domains on the performance of the motors is not understood. Here, we showed that diffusive anchorage of the kinesin-14 tail domains considerably impacts velocity and force generated by the motor ensemble. Our data rather suggest a role of kinesin-14 as a flexible element, pliantly sliding and crosslinking microtubules to facilitate remodeling of the mitotic spindle (Ludecke et al., 2018).

Kinesin-14 motors have been shown to collectively slide microtubules at a constant velocity until no overlap remains between them, leading to the breakdown of the initial microtubule geometry. We showed that the sliding velocity of microtubules, driven by ensembles of human kinesin-14 HSET, decreases when microtubules start to slide apart. This results in the maintenance of finite-length microtubule overlaps. We quantitatively explained this feedback using the local interaction kinetics of HSET with overlapping microtubules, which leads to the generation of an entropic force that antagonizes the force exerted by the motors. We thus demonstrated that the spatial arrangement of microtubules can locally regulate the collective action of molecular motors (Braun et al., 2017).

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