# Description of the main research directions investigated by the institute

Research pursued at IMG combines continuation of the long-term topics developed by well-established groups and new themes brought by new group leaders. The Institute perceives the diversity of topics as its strength because it creates an inspiring environment that attracts young promising scientists. On the other hand, it is evident that the basis of important discoveries is the synergy between collaborating laboratories. Several major research directions are thus crystallizing at IMG. The main research directions and their achievements in the evaluated period have been as follows:

## Cancer biology and genome maintenance mechanisms

The topic includes identification and characterization of oncogenes or tumour suppressor genes (and alteration of these genes in transforming cells), DNA repair mechanisms including DNA damage response, and the role of other factors that are responsible for cellular transformation and tumour formation.

**Therapeutic resistance of cancer cells; RECQ5 helicase suppresses transcription-associated genomic instability (the team of J. Bartek, Z. Hodny, and P. Janscak)**

The long-term research interests of the IMG team are mechanisms of the cellular DNA damage response in relation to human diseases including cancer and ageing. In recent years, the efforts of the team were dedicated to unravelling circumstances of DNA 'irreparability' and resulting incessant DNA damage response, which guides a cell to a specific phenotype. Among the most important results obtained in the evaluation period was the contribution to the mechanisms of therapeutic resistance of cancer cells ***(Kyjacová L, Hubáčková S, Krejčíková K, Strauss R, Hanzlíková H, Dzijak R, Imrichová T, Šímová J, Bartek J, Reiniš M, Hodný Z: Radiotherapy-induced plasticity of prostate cancer mobilizes stem-like non-adherent, Erk signalling-dependent cells. Cell Death Differ 2015, 22(6): 898-911)***.

Transcription-replication conflicts (TRCs) represent a significant source of genomic instability in cells experiencing DNA replication stress. However, the question how a replication fork restarts DNA synthesis upon a TRC remains elusive. The IMG team found that RECQ5 helicase binds to RNA polymerases I and II and suppresses transcription-associated genomic instability. This occurs by promoting resolution of conflicts between transcription and replication machineries promoting replication fork progression through actively transcribed genes ***(Urban V, Dobrovolna J, Hühn D, Fryzelkova J, Bartek J, Janscak P: RECQ5 helicase promotes resolution of conflicts between replication and transcription in human cells. J Cell Biol 2016, 214(4): 401-15)***.

**Identification of the primary source of genome damage detected by sensor protein PARP1 (the team of K. Caldecott)**

DNA single-strand breaks (SSBs) are amongst the most frequent DNA lesions arising in cells threatening genetic integrity and cell survival. ADP ribosylation catalysed by PARP enzymes that occurs at the site of the breaks is a ubiquitous post-translational modification involved in a number of critical physiological processes such as DNA replication and repair, cellular differentiation, and carcinogenesis. Inherited defects in ADP-ribose metabolism often cause human disease such as cancer, immunodeficiencies, and neurodegeneration. The IMG team showed that the intermediates of DNA replication so-called Okazaki fragments are the primary source of DNA SSBs in cells. The work has important implications for cancer research, because it identifies these obligatory DNA replication intermediates as the likely source of synthetic lethality that is triggered in certain cancer cells by PARP1 inhibition ***(Hanzlikova H, Kalasova I, Demin AA, Pennicott LE, Cihlarova Z, Caldecott KW. The importance of poly(ADP-Ribose) polymerase as a sensor of unligated Okazaki fragments during DNA replication. Mol Cell 2018, 71(2):319-331)***.

**Deciphering the role of the WIP1 (proto)oncogene in cancer development (the team of L. Macurek)**

The IMG team previously identified clinically relevant frameshift or non-sense mutations in the *PPM1D* gene (the gene encodes WIP1 phosphatase) resulting in production of a C-terminally truncated stabilized form of WIP1. Importantly, the phosphatase regulates modification/stability of the principal tumour suppressor p53. The team developed a new transgenic mouse model that mimics the mutations observed in humans. Additionally, a cohort of human patients suffering from colon carcinoma was analysed. Moreover, the effect of WIP1 inhibition was tested using organoids derived from the colon tumours. The results showed that truncated WIP1 increased the number of intestinal polyps induced in the APCmin mouse model of induced intestinal cancer; *PPM1D/WIP1* mutations were predominantly identified in a subgroup of tumours with microsatellite instability driven by oncogenic BRAF-V600E mutation. Interestingly, tumours carrying truncated PPM1D/WIP1 were more sensitive to WIP1 inhibition, and the inhibition improved sensitivity to the commonly used chemotherapeutic 5-fluorouracil ***(Burocziova M, Burdova K, Martinikova AS, Kasparek P, Kleiblova P, Danielsen SA, Borecka M, Jenikova G, Janečková L, Pavel J, Zemankova P, Schneiderova M, Schwarzova L, Ticha I, Sun XF, Jiraskova K, Liska V, Vodickova L, Vodicka P, Sedlacek R, Kleibl Z, Lothe RA, Korinek V, Macurek L: Truncated PPM1D impairs stem cell response to genotoxic stress and promotes growth of APC-deficient tumours in the mouse colon. Cell Death Dis 2019, 10(11): 818)***.

**The function of the C/EBPa transcription factor in granulopoiesis and haemato-oncological disorders (the team of M. Alberich Jorda)**

Haematopoiesis is a continuous and tightly regulated process in which haematopoietic stem cells (HSCs) develop into mature blood cells. The HSC pool is limited and its integrity needs to be preserved throughout life. The IMG research group investigated the regulation of HSC maintenance/fate by C/EBPα transcription factors (C/EBPα plays a crucial role in HSC fate and the commitment of HSCs into the myeloid lineage). Using gene expression profiling and chromatin-immunoprecipitation followed by sequencing, the team identified *EVI2B* (ecotropic virus integration site 2) as a novel target gene of C/EBPα. During granulocytic differentiation, EVI2B levels are upregulated, and prevention of this upregulation results in a block of neutrophilic production in mouse and human cells. Importantly, in the acute myeloid leukaemia (AML) patient samples with mutated C/EBPa, EVI2B levels are low, thus possibly contributing to the block of granulocytic development characteristic of this disorder ***(Zjablovskaja P, Kardosova M, Danek P, Angelisova P, Benoukraf T, Wurm AA, Kalina T, Sian S, Balastik M, Delwel R, Brdicka T, Tenen DG, Behre G, Fiore F, Malissen B, Horejsi V, Alberich-Jorda M: EVI2B is a C/EBPα target gene required for granulocytic differentiation and functionality of hematopoietic progenitors. Cell Death Differ 2017, 24(4): 705-716)***.

**Molecular mechanisms controlling self-renewal and transformation of intestinal and hematopoietic stem cells (the team of V. Korinek, L. Lanikova, and T. Valenta)**

In the small intestine, epithelial Paneth cells secrete Wnt ligands and thus potentially form the stem cell niche that normally could maintain the epithelial homeostasis. On the other hand, in the colon, the identity of cells comprising the stem cell niche is unknown. The IMG team in collaboration with the laboratory of K. Basler (Institute of Molecular Life Sciences, Zurich, Switzerland) showed that sub-epithelial mesenchymal cells expressing transcription factor Gli1 form the “enigmatic” niche for intestinal epithelial stem cells in the colon. In addition, single-cell RNA sequencing and immunohistocytochemical analyses revealed that Gli1-expressing cells represent a heterogeneous cell population consisting of myofibroblasts and lipofibroblasts, some of them secreting Wnt ligands. Importantly, $Gli1^{+}$ cells are enriched during tissue regeneration upon intestinal damage or in tumour tissue ***(Degirmenci B, Valenta T, Dimitrieva S, Hausmann G, Basler K: GLI1-expressing mesenchymal cells form the essential Wnt-secreting niche for colon stem cells. Nature 2018, 558(7710): 449-453).***

Myeloproliferative neoplasms (MPN) represent a group of disorders arising due to the genetic defect(s) in haematopoietic stem cells. While the concept of somatic driver mutations in MPNs is well established, contribution of other factors, such as germ-line variants that modulate the risk of MPN development by promoting acquisition of additional somatic mutations, is less understood. The IMG team demonstrated that germ-line (or acquired) mutations in the gene encoding Janus kinase 2 (JAK2) enhances the oncogenic JAK2/STAT signalling and causes a specific clinical course of the disease in MPN patients. In more detail, it was shown that besides well-characterized mutation JAK2 V617F frequently detected in polycythaemia vera (PV) patients, the course of the diseases may be influenced by additional JAK2 mutations that may contribute to leukaemic transformation of PV cell clones ***(Lanikova, L., Babosova, O., Swierczek, S., Wang, L., Wheeler, D.A., Divoky, V., Korinek, V. and Prchal, J.T.: Coexistence of gain-of-function JAK2 germ line mutations with JAK2V617F in polycythemia vera. Blood 2016, 128, 2266-2270)***.

## Developmental biology

Several IMG research groups are pursuing developmental biology topics. Besides experimental mice, the groups use other animal models such as zebrafish, medaka, chicken, amphioxus, and several invertebrate models (e.g., jellyfish, marine ragworm).

**Developmental mechanisms for the eye lens formation and neural crest induction (the team of Z. Kozmik)**

Eye development is a “classical” model suitable for investigating how a spatiotemporal control of gene expression regulates cell specification. The IMG team discovered that during eye lens formation, expression of transcription factor Pax6, a "master regulator" of the eye, is regulated by Meis1 and Meis2 transcription factors ***(Antosova B, Smolikova J, Klimova L, Lachova J, Bendova M, Kozmikova I, Machon O, Kozmik Z: The Gene Regulatory Network of Lens Induction Is Wired through Meis-Dependent Shadow Enhancers of Pax6. PLoS Genet 2016, 12(12): e1006441)***.

The neural crest (NC) is crucial for the evolutionary diversification of vertebrates. NC cells are induced at the neural plate border by a coordinated action of several signalling pathways, including Wnt/β-catenin. NC cells are normally generated in the posterior neural plate border, whereas the anterior neural fold is devoid of NC cells. Using the mouse model, the team showed that active repression of Wnt/β-catenin signalling is required for maintenance of neuroepithelial identity in the anterior neural fold and for inhibition of NC induction ***(Mašek J, Machoň O, Kořínek V, Taketo MM, Kozmik Z: Tcf7l1 protects the anterior neural fold from adopting the neural crest fate. Development 2016, 143(12): 2206-16)***.

**Ex vivo growth and manipulation of haematopoietic stem cells obtained from the zebrafish model (the team of P. Bartunek)**

The IMG team established *in vitro* and *in vivo* systems to study the self-renewal and differentiation of embryonic, haematopoietic, and neural stem cells. Cytokines, genetic engineering tools and small molecules were employed to manipulate these cells from various vertebrate model organisms. Zebrafish became a popular model organism to study the genetic underpinnings of haematopoiesis. However, the main disadvantage of this model was unavailability of culture conditions to study haematopoietic cells *ex vivo*. The team optimized culture conditions and generated recombinant zebrafish cytokines that enable growth and differentiation of zebrafish haematopoietic cells in cultures for the first time ***(Svoboda O, Stachura DL, Machonova O, Zon LI, Traver D, Bartunek P: Ex vivo tools for the clonal analysis of zebrafish hematopoiesis. Nat Protoc 2016, 11(5): 1007-20)***. The cytokines and additional tools are either available through *Addgene* or directly distributed by the IMG laboratory.

**Contribution of retrotransposons to gene expression remodelling and gene evolution (the team of P. Svoboda)**

The IMG team studies evolution of genes and their regulation mainly in the context of the female germline in mice. During the 2015-2019 period, among interests of the team were long non-coding RNAs (lncRNAs) and comparative analysis of mammalian maternal transcriptomes with a particular focus on the contribution of retrotransposons to gene expression in the germline and its evolution. This work yielded the most significant article of the group, which identified the largest contribution of a single retrotransposon family to gene expression remodelling and gene evolution discovered so far ***(Franke V, Ganesh S, Karlic R, Malik R, Pasulka J, Horvat F, Kuzman M, Fulka H, Cernohorska M, Urbanova J, Svobodova E, Ma J, Suzuki Y, Aoki F, Schultz RM, Vlahovicek K, Svoboda P: Long terminal repeats power evolution of genes and gene expression programs in mammalian oocytes and zygotes. Genome Res 2017, 27(8): 1384-1394)***.

## Molecular retrovirology

Retrovirology has been the most traditional research area at IMG. The research topic remains strong and productive, as documented by the following results published by **the team of J. Hejnar**.

The main model of the IMG team is represented by avian leucosis viruses. Moreover, the team studies the genetics and genomics of chicken, the natural host of the avian leucosis viruses. The team showed that transcriptionally active proviruses were found close to the active promoters and enhancers, and proviruses in other locations were mostly silenced. This is an important finding indicating that retroviral vectors aimed for gene therapy in humans have to be designed with altered integration preference so that they integrate into intergenic regions. At these positions, the proviruses should be protected from DNA methylation and transcriptional silencing ***(Senigl F, Maman Y, Dinesh RK, Alinikula J, Seth RB, Pecnova L, Omer AD, Rao SSP, Weisz D, Buerstedde JM, Aiden EL, Casellas R, Hejnar J, Schatz DG: Topologically Associated Domains Delineate Susceptibility to Somatic Hypermutation. Cell Rep 2019, 29(12): 3902-3915.e8)***.

The expertise related to the epigenetic control of retroviral infection was employed in the studies of retroviral integration in mammals. The IMG team and its collaborators examined the dynamics of DNA methylation of HIV-1 proviruses. The study clearly showed that the epigenetic control is an important mechanism of transcriptional silencing of HIV-1, which creates a latent reservoir of proviruses in infected patients ***(Trejbalová K, Kovářová D, Blažková J, Machala L, Jilich D, Weber J, Kučerová D, Vencálek O, Hirsch I, Hejnar J: Development of 5' LTR DNA methylation of latent HIV-1 provirus in cell line models and in long-term-infected individuals. Clin Epigenetics 2016, 8: 19)***.

Mining the newly released mammalian genomes provided an excellent opportunity to identify molecular fossils – rare copies of old endogenous retroviruses preserved from ancient infections of the evolutionary lineages. The team developed original bioinformatic tools and methods for discovering such endogenous retroviral copies and described the evolution and diversification of lentiviral copies in recent dermopteran species ***(Hron T, Farkašová H, Padhi A, Pačes J, Elleder D: Life history of the oldest lentivirus: characterization of ELVgv integrations in the dermopteran genome. Mol Biol Evol 2016, 33(10): 2659-69)***. The team also described the first endogenous deltaretrovirus (HTLV-related retrovirus) discovered in the genome of Miniopterus bats and constructed the tree of this retrovirus family. This contribution was of “heuristic” importance and shed light on genome evolution, “endogenization” of retroviruses, and virus-host co-evolution ***(Farkašová H, Hron T, Pačes J, Hulva P, Benda P, Gifford RJ, Elleder D: Discovery of an endogenous Deltaretrovirus in the genome of long-fingered bats (Chiroptera: Miniopteridae). Proc Natl Acad Sci U S A 2017, 114(12): 3145-3150)***.

Finally, the IMG team contributed significantly to the field of transgenesis in chicken. The team developed an original strategy of orthotopic male germ line transplantation into the testes of recipients ***(Trefil P, Aumann D, Koslová A, Mucksová J, Benešová B, Kalina J,***

***Wurmser C, Fries R, Elleder D, Schusser B, Hejnar J: Male fertility restored by transplanting primordial germ cells into testes: a new way towards efficient transgenesis in chicken. Sci Rep 2017, 7(1): 14246)***.

## Molecular and cellular immunology

Immunology represents one of the most important IMG research areas. In recent years, it has shifted from the "classical" (transplantation) immunology mainly to molecular immunology, with emphasis on the mechanisms of immunoreceptor signalling, and detailed analysis of the specific cell types involved in the immune responses.

**Virtual memory T-cell production mechanisms (the team of O. Stepanek)**

The main team focus is on the T-cell-mediated adaptive immune responses and T-cell diversity. Virtual memory T-cells are foreign antigen-inexperienced T-cells that have acquired memory-like phenotype and constitute 10-20 % of all peripheral $CD8^+$ T-cells in mice. Their origin, biological roles, and relationship to naïve and foreign antigen-experienced memory T-cells are incompletely understood. By analysing T-cell receptor repertoires and using retrogenic monoclonal T-cell populations, the team described the molecular mechanisms driving formation of virtual memory T-cells ***(Drobek A, Moudra A, Mueller D, Huranova M, Horkova V, Pribikova M, Ivanek R, Oberle S, Zehn D, McCoy KD, Draber P, Stepanek O: Strong homeostatic TCR signals induce formation of self-tolerant virtual memory CD8 T cells. EMBO J 2018, 37(14))***.

**PSTPIP2 has a central position in the signalling network of neutrophil activation (the team of T. Brdicka)**

The main focus of the IMG team is on the biology of membrane adaptor proteins in leucocytes. Membrane adaptors are transmembrane (or membrane-associated) proteins that have no enzymatic activity. However, their intracellular parts contain various interaction motifs, which recruit signalling enzymes and other proteins to the proximity of cellular membranes. The team is interested in different aspects of adaptor protein function, starting with molecular and biochemical properties and ending with physiological functions in living organisms and roles in diseases. One of the membrane adaptors that was identified by the team was PSTPIP2. Interestingly, PSTPIP2 deficiency in the mouse model resulted in autoinflammatory disease chronic multifocal osteomyelitis, characterized by sterile inflammation of hind paw and tail bones and surrounding soft tissues. Detailed analysis of this phenotype demonstrated that neutrophils from PSTPIP2-deficient mice showed generalized hyper-responsiveness to multiple stimuli acting through different receptors, indicating the role of PSTPIP2 in neutrophils ***(Drobek A, Kralova J, Skopcova T, Kucova M, Novák P, Angelisová P, Otahal P, Alberich-Jorda M, Brdicka T: PSTPIP2, a protein associated with autoinflammatory disease, interacts with inhibitory enzymes SHIP1 and Csk. J Immunol 2015, 195(7): 3416-26)***.

**Molecular mechanisms of the autoimmune polyendocrine syndrome; early embryo haematopoiesis (the team of D. Filipp)**

The IMG team was focused on central and peripheral T-cell tolerance and autoimmunity, and embryonic haematopoiesis. Autoreactive T-cells aided by autoantibodies cause an organ-specific autoimmune disease in multiple organs, but the disease pathogenesis is poorly understood. One example is the autoimmune polyendocrine syndrome type (APS-1), a rare autosomal recessive disorder caused by loss of central immunological tolerance due to mutations in the autoimmune regulator (*AIRE*) gene. Capitalizing on joint expertise and collaborations with leading European investigators, the team described the molecular and cellular mechanisms behind gut-related immunopathology of APS-1. The results provided important clues for development of new diagnostic biomarkers and therapeutic options for gut autoimmunity diseases ***(Dobeš J, Neuwirth A, Dobešová M, Vobořil M, Balounová J, Ballek O, Lebl J, Meloni A, Krohn K, Kluger N,***

***Ranki A, Filipp D: Gastrointestinal autoimmunity associated with loss of central tolerance to enteric α-defensins. Gastroenterology 2015, 149(1): 139-50)***.

The team identified Toll-like receptors (TLRs) and their adaptor proteins as functionally important molecules during early stages of embryonic development. Using genetic labelling in live animals, the team demonstrated that the *Tlr2* locus is indeed activated at early stages of embryonic development in emerging haematopoietic progenitors. Notably, when Tlr2-positive progenitors were genetically labelled at embryonic day 8.5, they were able to give rise to erythroid, myeloid, as well as lymphoid haematopoietic lineages that persisted in the peripheral blood of adult animals. The results indicate that Tlr2 can be utilized as a reliable marker of early haematopoietic precursors ***(Balounová J, Šplíchalová I, Dobešová M, Kolář M, Fišer K, Procházka J, Sedlacek R, Jurisicova A, Sung HK, Kořínek V, Alberich-Jorda M, Godin I, Filipp D: Toll-like receptor 2 expression on c-kit cells tracks the emergence of embryonic definitive hematopoietic progenitors. Nat Commun 2019, 10(1): 5176)***.

**Mechanisms of mast cell chemotaxis (the team of Petr Draber)**

The long-term research interest of the IMG team lies in the molecular mechanisms governing signal transduction from plasma membrane immunoreceptors, specifically the high-affinity IgE receptor (FcεRI), to the cytoplasm. In relation to new functions of non-T cell activation linker (NTAL) adaptor protein, discovered at IMG, in chemotaxis of mast cells, the team discovered the role of RHOA/ERM/β1-integrin and PI3/AKT signalling pathways in this process ***(Halova I, Bambouskova M, Draberova L, Bugajev V, Draber P: The transmembrane adaptor protein NTAL limits mast cell chemotaxis toward prostaglandin $E_2$. Sci Signal 2018, 11(556): eaao4354)***.

## Molecular genetics, genomics, and disease modelling

The theme represents a research direction with a long tradition at IMG; the main topics include elucidation of the phenomenon of mouse subspecies male hybrid sterility, cancer transcriptomics, and pathological mechanisms of rare diseases.

**Involvement of PRDM9 in hybrid sterility and in meiotic chromosome synapsis (the team of J. Forejt)**

The main topic of the IMG team is the role of meiotic recombination and homologous chromosome synapsis in infertility of interspecific hybrids and in the origin of species. Hybrid male sterility is the most studied but still poorly understood form of reproductive isolation, which restricts gene flow between incipient species. The phenomenon is an essential prerequisite for successful speciation. In 2009, the group identified *Prdm9* as the first hybrid sterility gene in vertebrates (Mihola et al. Science 2009). In the evaluated period, the team mainly focused on detailed analysis of the role of Prdm9 in meiotic recombination. To investigate the relation between Prdm9-controlled meiotic arrest and asynapsis, random stretches of consubspecific (belonging to the same subspecies) homology were inserted into several autosomal pairs in sterile hybrids. Interestingly, 27 or more megabases of consubspecific homology fully restored synapsis in a given autosomal pair, indicating that two or more double-strand breaks within symmetric hotspots per chromosome are necessary for successful meiosis ***(Gregorova, S, Gergelits, V, Chvatalova, I, Bhattacharyya, T, Valiskova B, Fotopulosova V, Jansa, P, Wiatrowska, D, Forejt, J: Modulation of Prdm9-controlled meiotic chromosome asynapsis overrides hybrid sterility in mice. Elife 2018, 14(7): e34282)***.

**Recovery of male fertility by a loss of meiotic epigenetic factor PRMD9 (the team of Z. Trachtulec)**

Infertility is a major health issue, as it affects about 186 million people in reproductive age worldwide. In mouse, the deficiency in Prdm9 results in complete meiotic arrest of both sexes of all tested “classical” mouse strains. The IMG team investigates the effects of

chromatin marks, especially histone methylations, accompanying the sites of programmed meiotic double-stranded DNA breaks (and other meiotic epigenetic modifications) on the quantity and quality of germ cells as well as on offspring production in several animal species. Strikingly, using genetic manipulation and cross breeding, the team showed that in some (specific) genetic backgrounds, the Prdm9-deficient mice are fertile. One of the possible mechanisms of infertility suppression could be more efficient DNA repair of relocated recombination sites to crossovers. The results imply that drugs (or conditions) inducing increase in DNA breaks could improve fertility in humans ***(Mihola O, Pratto F, Brick K, Linhartova E, Kobets T, Flachs P, Baker CL, Sedlacek R, Paigen K, Petkov PM, Camerini-Otero RD, Trachtulec Z: Histone methyltransferase PRDM9 is not essential for meiosis in male mice. Genome Res 2019, 29(7): 1078-1086)***.

**Cancer transcriptomics, Scrimer, Ancient mitochondrial genomes database (the team of M. Kolar)**

The focus of the service-research group is genomics, transcriptomics, bioinformatics and database development. The team was involved in a long-term collaborative project focused on identification of specific genetic markers in cancer tissue isolated from patients with head and neck squamous cell carcinomas and skin cancer ***(Kodet O, Lacina L, Krejčí E, Dvořánková B, Grim M, Štork J, Kodetová D, Vlček Č, Šáchová J, Kolář M, Strnad H, Smetana K Jr: Melanoma cells influence the differentiation pattern of human epidermal keratinocytes. Mol Cancer 2015, 14(1): 1)***.

With the next-generation sequencing methods, it has become possible to obtain genome-wide sequence data even for non-model species. Currently, there is no available software for automated design of PCR and genotyping primers from next-generation sequence data. The group developed the Scrimer “pipeline” that automates multiple steps: adaptor removal, read mapping, selection of single nucleotide polymorphisms (SNPs) and multiple primer design from transcriptome data ***(Mořkovský L, Pačes J, Rídl J, Reifová R: Scrimer: designing primers from transcriptome data. Mol Ecol Resour 2015, 15(6): 1415-20)***.

The group also generated an open database of ancient mitochondrial genomes. It is the most comprehensive published database focused entirely on human populations from prehistoric and early historic times. The database brings the full “mitogenome” sequences together with extensive metadata and advanced search and mapping tools. The database is used by geneticists, anthropologists, archaeologists, and other researchers from all over the world, having over 10,000 unique users in 2019 ***(Ehler E, Novotný J, Juras A, Chylenski M, Moravcík O, Paces J: AmtDB: a database of ancient human mitochondrial genomes. Nucleic Acids Res 2019, 47(D1): D29-D32)***.

The group also published many articles in collaboration with other groups from the Institute (see the group summary).

**Molecular mechanisms of the Netherton syndrome (the team of R. Sedlacek)**

Although thematically distinct, all research of the IMG team is based on the usage of mouse models as a tool to reveal gene functions in the complexity of the whole organism. The topics include ubiquitin ligases, skeleton and tooth development, transcription factors, proteases, and rare disease models. One of the major focuses of the team were kallikrein-related peptidases (shortly kallikreins; Klks). The team generated a set of single or compound Klk knockout mice allowing deciphering the functional proteolytic network in the skin (the disrupted genes were *Klk5*, *Klk7*, *Klk8*, *Klk11*, *Klk13*, and *Klk14*). The Netherton syndrome (NS) is a rare hereditary disease characterized by hair and skin anomalies and increased susceptibility to atopic eczema. To generate a mouse model of NS, a mutation identified in the NS individuals in the coding sequence of the *Spink5* gene (the gene encoded protease inhibitor LEKT1) was introduced into the mouse genome. Subsequently, Spink5 mutant mice in combination with Klks-deficient animals were used to identify the NS pathological mechanisms. Interestingly, simultaneous disruption of the *Klk5* and *Klk7* genes suppressed the pathological effect of mutant LEKT1, indicating possibilities of therapeutic

intervention in humans suffering from NS ***(Kasparek P, Ileninova Z, Zbodakova O, Kanchev I, Benada O, Chalupsky K, Brattsand M, Beck IM, Sedlacek R: KLK5 and KLK7 ablation fully rescues lethality of Netherton syndrome-like phenotype. PLoS Genet 2017, 13(1): e1006566)***.

**Analysis of neurotransmitter-mediated signalling (the team of J. Blahos)**

The IMG team was focused on the role of signalling initiated by the interaction between neurotransmitters, namely endocannabinoids, and the glutamate G-protein coupled receptors (GPCR). They used molecular biology, pharmacology, and genetic tools to uncover the signalling mechanisms and function of metabotropic glutamate receptors (mGluRs). The team identified SGIP [Src homology 3-domain growth factor receptor-bound 2-like (endophilin) interacting protein 1] as a novel partner of CB1R (type 1 cannabinoid receptor). SGIP1 is functionally linked to clathrin-mediated endocytosis and its overexpression in the hypothalamus leads to energy regulation imbalance resulting in obesity. The team reported that SGIP1 prevents the endocytosis of activated CB1R and, consequently, alters signalling via the receptor ***(Hájková A, Techlovská Š, Dvořáková M, Chambers JN, Kumpošt J, Hubálková P, Prezeau L, Blahos J: SGIP1 alters internalization and modulates signalling of activated cannabinoid receptor 1 in a biased manner. Neuropharmacology 2016, 107: 201-14)***.

**Genotype-phenotype association study of the Bardet-Biedl syndrome (the team of O. Stepanek and M. Huranova)**

Besides its major topic related to T-cell biology, the group studies the molecular mechanisms of ciliogenesis, particularly the role of an octameric ciliary transport complex called the 'BBBsome'. Alterations in the BBSome composition (or structure) are associated with the Bardet-Biedl syndrome (BBS), a recessive hereditary disease causing multiple organ anomalies. Since there is a link between the function of the BBSome and the immune/haematopoietic system, research topics in the groups are interlinked. The team applied a meta-analysis approach to study the genotype-phenotype association in humans using a proprietary database of all reported BBS patients. The analysis revealed that the identity of the causative gene (and the character of the mutation) partially predicts the clinical outcome of the disease. Besides its potential use for clinical prognosis, the analysis revealed functional differences of particular BBS genes in humans ***(Niederlova V, Modrak M, Tsyklauri O, Huranova M, Stepanek O: Meta-analysis of genotype-phenotype associations in Bardet-Biedl syndrome uncovers differences among causative genes. Hum Mutat 2019, 40(11): 2068-2087)***.

## Biology of the cell nucleus, cytoskeleton, and cellular flagellum

Biochemistry and cell biology of the cytoskeleton and cytoskeleton-associated proteins represent one of the "traditional" topics of the Institute. However, two newly formed groups (led by M. Gregor and V. Varga) brought new impetus to this theme. The topic of the nucleus organization and splicing mechanisms are studied in groups of P. Hozak and D. Stanek, respectively.

**The structure and functions of the nuclear lipid islets (the team of P. Hozak)**

Different processes are spatially and temporarily organized in cell nuclei and many nuclear functions are critically dependent on this order. The IMG team pioneered a novel and original concept of involvement of phosphatidylinositol 4,5-bisphosphate (PIP2) in gene transcription and nucleolar organization. The team aimed to map structural and regulatory molecules of the nucleus with relation to the intranuclear phosphoinositides. The team described a novel type of nuclear structure – nuclear lipid islets (NLIs). They are of 40-100 nm in size with a lipidic interior, and PIP2 molecules comprise a significant part of their surface. Most of NLIs have RNA at the periphery. Consistent with that, RNA is required for their integrity. The NLI periphery is associated with Pol II transcription machinery, including

the largest Pol II subunit, transcription factors and nuclear myosin 1 (NM1/NMI). Importantly, the PIP2-NM1 interaction is important for Pol II transcription ***(Sobol M, Krausová A, Yildirim S, Kalasová I, Fáberová V, Vrkoslav V, Philimonenko V, Marášek P, Pastorek L, Čapek M, Lubovská Z, Uličná L, Tsuji T, Lísa M, Cvačka J, Fujimoto T, Hozak P: Nuclear phosphatidylinositol 4,5-bisphosphate islets contribute to efficient RNA polymerase II-dependent transcription. J Cell Sci 2018, 131(8): 211094)***.

**Regulatory sequences requirement for splicing of long non-coding RNAs (the team of D. Stanek)**

The IMG team was primarily focused on RNA splicing, particularly on the formation of spliceosomal small nuclear ribonucleoproteins (snRNPs) and RNA splicing regulation. The team also studied splicing of non-coding RNAs and elucidated a “puzzling” fact why long non-coding RNAs (lncRNAs) are less efficiently spliced than protein-coding pre-mRNAs. The team discovered that the splicing efficiency of lncRNAs is more dependent on the 3’ splice site strength than pre-mRNAs, which suggested that lncRNAs are more dependent on basic splicing signals. Indeed, further analysis confirmed that lncRNAs contain less binding sites for splicing enhancers SR proteins. The results indicated that lncRNAs lack the cooperative interaction network that enhances splicing, which rendered their splicing outcome more dependent on the optimality of splice sites ***(Krchnáková Z, Thakur PK, Krausová M, Bieberstein N, Haberman N, Müller-McNicoll M, Stanek D: Splicing of long non-coding RNAs primarily depends on polypyrimidine tract and 5' splice-site sequences due to weak interactions with SR proteins. Nucleic Acids Res 2019, 47(2): 911-928)***.

**The expression and function of □-tubulin isoforms in neuroblastoma cells (the team of Pavel Draber)**

The main research interest of the IMG team were the molecular mechanisms governing the regulation of microtubule nucleation and organization in normal and pathological conditions. The organization of dynamic microtubules, formed by αβ-tubulin dimers, is controlled by microtubule organizing centres (MTOCs). One of the critical components of MTOCs is γ-tubulin, which along with γ-tubulin complex proteins (GCPs) forms nucleation complexes necessary for microtubule nucleation. The team mainly studied the regulation of γ-tubulin nucleation complexes by signal transduction molecules (kinases and phosphatases) and newly discovered centrosomal adaptor proteins (GIT/βPIX). The team showed that enhanced expression of γ-tubulin isoform 2 in neuroblastoma cells was triggered by oxidative stress induced by mitochondrial inhibitors and that γ-tubulins associate with mitochondria ***(Dráberová E, Sulimenko V, Vinopal S, Sulimenko T, Sládková V, D'Agostino L, Sobol M, Hozák P, Křen L, Katsetos CD, Dráber P: Differential expression of human γ-tubulin isotypes during neuronal development and oxidative stress points to a γ-tubulin-2 prosurvival function. FASEB JOURNAL 2017, 31(5): 1828-1846)***.

**Analysis of the eukaryotic flagellum composition (the team of V. Varga)**

The IMG team was focused on the protein composition and formation of eukaryotic flagella and cilia. These closely related and evolutionarily conserved organelles are – due to their motility, sensory and signalling functions – essential for many organisms. In humans, cilia are found on the surface of a vast majority of cell types and their malfunctions are manifested by a wide range of symptoms, including developmental abnormalities, sterility, chronic respiratory problems, blindness, and polycystic kidney disease. The team aimed to elucidate poorly understood aspects of basic biology of the cilia and flagella, in particular those related to their cytoskeleton. The model systems were parasitic protozoan Trypanosoma brucei and ciliated mammalian cells. The group identified a number of novel constituents of the flagellum tip, sub-localizes them and characterizes their contribution to the flagellum functions ***(Varga V, Moreira-Leite F, Portman N, Gull K: Protein diversity in discrete structures at the distal tip of the trypanosome flagellum. Proc Natl Acad Sci U S A 2017, 114(32))***.

**The role of plectin in biliary tree architecture and stability in cholestasis (the team of M. Gregor)**

The main focus of the IMG team in the past five years has been cytoskeleton-dependent regulation of cell-cell contacts in simple epithelia, regulation of cell-matrix adhesions, and cytoskeleton (and adhesion)-mediated signalling in epithelial-mesenchymal transition, cell migration, and invasiveness. Using a liver-specific knockout mouse model in combination with an "array" of cell biology techniques (e.g., CRISPR/Cas9 targeted cell lines, traction force and atomic force microscopy, magnetic tweezer rheology, cell stretching, and FRET-based tension sensors), the team analysed the liver-specific role(s) of cytoskeletal linker protein plectin. The team demonstrated that plectin plays an essential role in the adaptation of liver tissues to cholestasis (cholestasis is a condition where the bile flow from the liver is blocked or reduced}. In cholestasis experimental models, plectin deficiency led to biliary epithelial instability, and mutated mice failed to activate a cholestasis-induced adaptive response ***(Jirouskova M, Nepomucka K, Oyman-Eyrilmez G, Kalendova A, Havelkova H, Sarnova L, Chalupsky K, Schuster B, Benada O, Miksatkova P, Kuchar M, Fabian O, Sedlacek R, Wiche G, Gregor M: Plectin controls biliary tree architecture and stability in cholestasis. J Hepatol 2018, 68(5): 1006-1017)***.

## Structural biology

The topic is covered by **the team of P. Maloy Rezacova**. The main interest of the team lies in structural studies of various proteins of biological or medicinal interest using protein crystallography. The structural knowledge is used to understand the protein function and (in some projects) also in modulating the protein function by design of specific inhibitors. In a structure-based drug discovery project, the team targets human enzymes, and the knowledge of protein structures provides a platform for rational design of specific inhibitors. Since the laboratory expertise is unique within the Czech Republic, the team also participated by its structural biology expertise in many other projects.

The team contributed to structural analysis of important interactions of lens epithelium-derived growth factor LEDGF/p75. LEDGF/p75 is a transcriptional co-activator that contributes to the regulation of gene expression by tethering other factors to actively transcribed genes on chromatin. Its chromatin-tethering activity is "hijacked" in two important disease settings: HIV and mixed lineage leukaemia. The basis for the biological regulation of LEDGF/p75's interaction to binding partners has remained unknown. During the evaluated period, the team uncovered molecular features crucial for interaction with LEDGF/p75 with several binding partners ***(Tesina P, Čermáková K, Hořejší M, Procházková K, Fábry M, Sharma S, Christ F, Demeulemeester J, Debyser Z, De Rijck J, Veverka V, Řezáčová P: Multiple cellular proteins interact with LEDGF/p75 through a conserved unstructured consensus motif. Nat Commun 2015, 6(6): 7968)***.

In another project, the team focused on design of novel and original inhibitors targeting a therapeutically relevant isoenzyme of human carbonic anhydrase (CA). A series of more than 70 sulfamides incorporating carborane clusters were developed. The lead compounds from this series inhibited CA with Ki values in the low nanomolar or subnanomolar range, with some inhibitors being more than 1000-fold more selective for tumour-specific CA isoform (CAIX) than CAII present in normal tissue. Importantly, the compounds demonstrated favourable *in vitro* toxicological and pharmacokinetic profiles and reduced the tumour size in mice ***(Grüner B, Brynda J, Das V, Šícha V, Štěpánková J, Nekvinda J, Holub J, Pospíšilová K, Fábry M, Pachl P, Král V, Kugler M, Mašek V, Medvedíková M, Matějková S, Nová A, Lišková B, Gurská S, Džubák P, Hajdúch M, Řezáčová P: Metallacarborane Sulfamides: Unconventional, Specific, and Highly Selective Inhibitors of Carbonic Anhydrase IX. J Med Chem 2019, 62(21): 9560-9575)***.

## Research activity and characterization of the main scientific results

### A. Retroviral receptors and restriction factors

We showed that certain alterations of viral envelope glycoproteins prime the virus for receptor-independent cell infection. Viruses with primed envelopes are less stable (in terms of temperature and pH resistance) than wild types, but can evolve towards new receptor specificity and host range extension. This was demonstrated and analyzed in avian leukosis retroviruses, but can be valid for all enveloped viruses. This research was done entirely in our laboratory.

Retroviral host range extension is coupled with Env-activating mutations resulting in receptor-independent entry.

Lounková A, Kosla J, Přikryl D, Štafl K, Kučerová D, Svoboda J.Proc Natl Acad Sci U S A. 2017 114(26):E5148-E5157

Based on our previous finding that single amino-acid, W38 of the chicken Na+/H+ exchanger, is critical for the avaian leukosis virus type J (ALV-J) entry. Therefore, we used the presence of W38 as a marker of species susceptible to ALV-J. We identified New World quails as a species that can be infected and serve as a natural reservoir of the virus. This is important in assessing the risk of cross-species transmission of the virus. This study was done exclusively in our group.

We also described the lack of genetic polymorphism within the chicken Na+/H+ exchanger (NHE1), the receptor for avian leukosis virus type J (ALV-J). All chicken breeds examined in this study contain the critical W38 of chNHE1 and we assume that there are no sources of polymorphism to be used for the husbandry towards the resistance to ALV-J. Although purely descriptive, this study points to the biotechnological solution of anti-ALV-J resistance and precedes the next effort towards the resistance to ALV-J in chicken. This study was done almost exclusively in our laboratory. M Vinkler from the Faculty of Science, Charles University of Prague, contributed with DNA samples from his collection of chicken breeds.

Knowing that Tva, Tvc, and chNHE1 (Na+/H+ exchanger isoform 1) are receptors for avian leukosis virus (ALV) subgroups A, C, and J, respectively, we examined the possibility that CRISPR/Cas9-mediated knock down of aforementioned genes results in genetic resistance to ALVs. We demonstrated the anti-ALV resistance in cultured chicken cells and elaborated the CRISPR/Cas9 technology necessary for the *in vivo* gene KO. This paper directly precedes the PNAS paper Koslová et al., 2020. This study was done exclusively in our group.

Next, we have shown the resistance to avian leukosis virus type J (ALV-J) created by single amino-acid deletion in the receptor, Na+/H+ exchanger type 1 (NHE1). The receptor has been manipulated by CRISPR/Cas9-mediated homologous recombination and the mutation introduced into the chicken by orthotopic transplantation of primordial germ cells. This is the first gene editing in chicken worldwide and the second example of anti-virus resistance based on receptor mutagenesis.This research is based on the long-term collaboration between our laboratory and Biopharm, a private company with a strong research division. The focus on anti-ALV-J comes from the traditional model explored in our lab, Biopharm contributed with the orthotopic PGC transplantation. Both first and correspondence authorships are from our laboratory.

Identification of New World Quails Susceptible to Infection with Avian Leukosis Virus Subgroup J.

Plachý J, Reinišová M, Kučerová D, Šenigl F, Stepanets V, Hron T, Trejbalová K, Elleder D, Hejnar J.
J Virol. 2017, 91(3). pii: e02002-16

Genetic Diversity of NHE1, Receptor for Subgroup J Avian Leukosis Virus, in Domestic Chicken and Wild Anseriform Species.

Reinišová M, Plachý J, Kučerová D, Šenigl F, Vinkler M, Hejnar J.

PLoS One. 2016, 11(3):e0150589.
Genetic Resistance to Avian Leukosis Viruses Induced by CRISPR/Cas9 Editing of Specific Receptor Genes in Chicken Cells.
Koslová A, Kučerová D, Reinišová M, Geryk J, Trefil P, Hejnar J. Viruses. 2018, 10(11). pii: E605.
Precise CRISPR/Cas9 editing of the NHE1 gene renders chickens resistant to the J subgroup of avian leukosis virus.
Koslová A, Trefil P, Mucksová J, Reinišová M, Plachý J, Kalina J, Kučerová D, Geryk J, Krchlíková V, Lejčková B, Hejnar J. Proc Natl Acad Sci U S A. 2020 pii: 201913827

There is a new avian leukosis virus emerging in Chinese chicken breeds, which does not cluster to the classical ALV subgroups. We identified that all these new isolates use the chicken Tva as a cell receptor and share it with ALV subgroup A. Based on the receptor usage, superinfection interference experiments, and the different host range we postulate the new ALV-K subgroup. Identification of ALV-K receptor is important for the future antiviral strategies and husbandry of resistant chicken lines.
This study was done exclusively in our group.
The Novel Avian Leukosis Virus Subgroup K Shares Its Cellular Receptor with Subgroup A.
Přikryl D, Plachý J, Kučerová D, Koslová A, Reinišová M, Šenigl F, Hejnar J.
J Virol. 2019, 93(17). pii: e00580-19.

Only a few antiviral genes (called viral restriction factors) have been described in birds, mostly because birds lack counterparts of highly studied mammalian restriction factors. Tetherin/BST-2 is a restriction factor, originally described in humans, that blocks the release of newly formed virus particles from infected cells. We discovered the BST-2 sequence in domestic chicken and described its antiviral activity against a prototypical avian retrovirus, avian sarcoma and leukosis virus (ASLV). Analysis of avian BST-2 genes will shed light on defense mechanisms against avian viral pathogens. This work has been done together with the team of klaus Strebel at NIH, United States.
Antiviral Activity and Adaptive Evolution of Avian Tetherins. Krchlíková V, Fábryová H, Hron T, Young JM, koslová A, Hejnar J, Strebel K, Elleder D. J Virol 2020, 94(12), pii e00416-20.

**B. Epigenetic control of retroviruses**

Epigenetic control, particularly DNA methylation, is an important mechanism of transcriptional silencing, which creates the latent reservoir of HIV-1 proviruses in infected patients. Dynamics of the DNA methylation has been examined here and our research provides data important for the current concepts of functional HIV-1 cure such as kick and kill or block and lock strategies. The central idea and the cell-line models came from our laboratory. We teamed-up with additional co-authors in order to obtain samples of patient DNAs (ML, JD), cultivate the ex vivo samples under BSL3 conditions (WJ) and analyse our results statistically (VO). Both first and correspondence authorships are from our laboratory.
Development of 5' LTR DNA methylation of latent HIV-1 provirus in cell line models and in long-term-infected individuals.
Trejbalová K, Kovářová D, Blažková J, Machala L, Jilich D, Weber J, Kučerová D, Vencálek O, Hirsch I, Hejnar J. Clin Epigenetics. 2016, 8:19.

Expression od the ERVWE1 provirus and display of syncytin-1 are typical for placental trophoblast. We have previously described epigenetic silencing of ERVWE1 in non-placental tissues. Here, we describe hypomethylation of ERVWE1 and aberrant expression of its spliced mRNA in germ line tumors, particularly seminomas. This is the best description of aberrant expression of an endogenous retrovirus, which could help in differential diagnostics of

embryonal carcinoma with the seminoma component. The aforementioned findings can be explained by increased expression of Tet dioxygenases (mostly Tet1), the DNA demethylation enzymes in germ line tumors. Decreased levels of methylcytosine and hydroxymethylcytosine were detected immunohistochemically and by qRT-PCR. We coin the term anti-methylation phenotype in opposite to the methylator phenotype of Tet- or IDH1/2-defficient tumors, which have worse prognosis than normal tumors. The seminal ideas and the molecular analyses of Tet expression arose in our laboratory. Z. Vernerová, A. Szabó, and P. Kézl from the Faculty Hospital Kralovske Vinohrady helped us to get the tumor samples and performed immunohistochemistry analyses. R. Amouroux and P. Hájková from MRC London provided their unique expertise in LC/MS analysis of methylcytosine and hydroxymethylcytosine.
DNA hypomethylation and aberrant expression of the human endogenous retrovirus ERVWE1/syncytin-1 in seminomas.
Benešová M, Trejbalová K, Kovářová D, Vernerová Z, Hron T, Kučerová D, Hejnar J. Retrovirology. 2017, 14(1):20
Overexpression of TET dioxygenases in seminomas associates with low levels of DNA methylation and hydroxymethylation.
Benešová M, Trejbalová K, Kučerová D, Vernerová Z, Hron T, Szabó A, Amouroux R, Klézl P, Hajkova P, Hejnar J. Mol Carcinog. 2017, 56(8):1837-1850

We showed that transcriptionally active proviruses were found close to the active promoters and enhancers, proviruses in other locations were mostly silenced. This is an important finding indicating that retroviral vectors aimed for gene therapy in human have to be designed with altered integration preference so that they integrate into intergenic regions. At these positions, the proviruses should be protected from DNA methylation and transcriptional silencing. This research was done entirely in our laboratory.
Accumulation of long-term transcriptionally active integrated retroviral vectors in active promoters and enhancers.
Šenigl F, Miklík D, Auxt M, Hejnar J. Nucleic Acids Res. 2017 Dec 15;45(22):12752-12765.

We compared the integration profiles of three different retroviral species representing alpha-, lenti-, and gamma-retroviruses and selected the proviral copies with long-term stable expression. Combining the epigenetic features at the sites of integration, we concluded that transcriptionally active proviruses accumulate close to gene promoters and enhancers. This finding is important for retrovirus-based gene therapy, because such an accumulation indicates genotoxicity of retroviral vectors. This study was done exclusively in our group.
Proviruses with Long-Term Stable Expression Accumulate in Transcriptionally Active Chromatin Close to the Gene Regulatory Elements: Comparison of ASLV-, HIV- and MLV-Derived Vectors. Miklík D, Šenigl F, Hejnar J.Viruses. 2018, 10(3). pii: E116..

Epigenetics is an important silencing mechanism in endogenous retroviruses and it is involved also in the early steps of endogenization of retroviruses. Young endogenous retroviruses are exemplified with CrERV, which is insertionally polymorphic in the mule deer. Rapid DNA methylation inactivates most CrERV copies with exception of tiny fraction of proviruses integrated close to gene promoters. This is the first study employing the NGS strategy for DNA methylation analysis. This study was done exclusively in our group.
Insight into the epigenetic landscape of a currently endogenizing gammaretrovirus in mule deer (*Odocoileus hemionus*).
Hron T, Fabryova H, Elleder D. Genomics. 2020, 112(1):886-896.

Based on the aforementioned findings, we employed the retroviral vectors for studies on somatic hypermutation and AID targeting. Retrovirus vectors were used because of their unbiased integration, which enabled us to introduce AID-sensitive reporter cassette into representative position in the human genome and seek for the AID hypermutation hot- and

coldspots. We demonstrate that the ectopic (non-Ig locus) AID hypermutation activity targets certain chromatin regions delineated by CTCF-binding sites (so called topologically associated domains). This finding not only points to the sites endangered by the potentially oncogenic mutagenesis, but also elucidates the mechanisms of somatic hypermutation. The seminal ideas and the retrovirus-based approach to somatic hypermutation arose in our laboratory and 60% of the benchwork has been done by F. Šenigl, the first and corresponding author. In course of the research, it become necessary to team up with laboratories of D.G. Schatz, R. Casellas, and E.L. Aiden in order to perform some specialized experiments and computative analyses.
Topologically Associated Domains Delineate Susceptibility to Somatic Hypermutation.
Senigl F, Maman Y, Dinesh RK, Alinikula J, Seth RB, Pecnova L, Omer AD, Rao SSP, Weisz D, Buerstedde JM, Aiden EL, Casellas R, Hejnar J, Schatz DG. Cell Rep. 2019, 29(12):3902-3915.

**C. Paleoretrovirology**

Mining the newly released mammalian genomes provides an excellent opportunity to identify molecular fossils – rare copies of old endogenous retroviruses preserved from ancient infections of the evolutionary lineages. We developed our original bioinformatic tools and methods for discovering such endogenous retroviral copies and we discovered the oldiest endogenous lentivirus in the genome of dermopteran mammals in 2014. Now, we described the evolution and diversification of these lentiviral copies in recent dermopteran species.
Life History of the Oldest Lentivirus: Characterization of ELVgv Integrations in the Dermopteran Genome.
Hron T, Farkašová H, Padhi A, Pačes J, Elleder D.Hron T, et al. Among authors: Elleder D. Mol Biol Evol. 2016, 33(10):2659-69.

Next, we described the first endogenous deltaretrovirus (HTLV-related retrovirus) discovered in the genome of *Miniopterus* bats. We not only discovered this virus fossil, but constructed also the evolution and diversification of the retrovirus family. This contribution is of heuristic importance and sheds light on genome evolution, endogenization of retroviruses, and virus-host co-evolution. The central finding and algorithm of iterative querying the databases with newly released animal genomes came from our laboratory. We had to team-up with additional co-authors in order to obtain samples of bat DNAs (HP, BP) and improve our bioinformatic analysis (PJ, GRJ). Both first and correspondence authorships are from our laboratory.
Discovery of an endogenous Deltaretrovirus in the genome of long-fingered bats (Chiroptera: Miniopteridae). Farkašová H, Hron T, Pačes J, Hulva P, Benda P, Gifford RJ, Elleder D. Proc Natl Acad Sci U S A. 2017, 114(12):3145-3150.

As a continuation of the 2017 discovery of the first endogenous retrovirus in the genome of *Miniopterus* bats, we described a coupple of new endogenous deltaretroviruses in the newly released genomes of different bats, dolphins, civets, and solenodons. Comparison of these deltaretroviral copies enabled to analyse the age and kinship of deltaretroviruses and shed the light on their evolution. We made the central findings and bioinformatic analyses. For writing the paper and interpreting the results we teamed-up with R. Gifford from the University of Glasgow
Deltaretroviruses have circulated since at least the Paleogene and infected a broad range of mammalian species.
Hron T, Elleder D, Gifford RJ. Retrovirology. 2019, 16(1):33.
Remnants of an Ancient Deltaretrovirus in the Genomes of Horseshoe Bats (Rhinolophidae).
Hron T, Farkašová H, Gifford RJ, Benda P, Hulva P, Görföl T, Pačes J, Elleder D.Hron T, et al. Among authors: Elleder D. Viruses. 2018, 10;10(4):185.

We elaborated the cervid endogenous retrovirus (CrERV) as a model for studies on the retroviral endogenization. This study describes that replication-competent virus can be induced from young and polymorphic endogenous retroviruses by co-cultivation of deer and human cells. Evolution of endogenous retroviruses, their tropism, host restriction, and complementation can be studied using this model.
This study was done in our lab with some help with electron microscopy from H. Kabickova (Military Health Institute Prague). Prof. M. Poss contributed with some seminal ideas and hypotheses.
Induction and characterization of a replication competent cervid endogenous gammaretrovirus (CrERV) from mule deer cells.
Fábryová H, Hron T, Kabíčková H, Poss M, Elleder D. Virology. 2015, 485:96-103.

**D. Chicken genomics and transgenesis**

Because we use avaian leukosis viruses as a model in most of our studies, we are also interested in the genetics and genomics of chicken, the natural host of avian leukosis virus. The genomic expertise turned out to be useful in analysing the integration sites and epigenomic features at the site of retrovirus integration. In addition to this, we published several studies on chicken genome, newly identified chicken genes, and the techniques of transgenesis or genome editing in chicken.

We corrected the traditional view that chicken and birds in general lack many genes important in mammals. Thanks to our skills in chicken genomics, we discovered many of the „hidden“ genes in microchromosomal regions extremely enriched in GC. This paper revealed the existence of chicken EpoR, leptin, TNF alpha, Glut4 and other genes and refuted hypotheses how birds replace their activities. Hence, multiple functional studies follow this paper. This study has been performed in collaboration with other colleagues interested in using the chicken model (P. Pajer from the Military Health Institute, J. Pačes and P. Bartůněk from Institute of Molecular Genetics).
Hidden genes in birds. Hron T, Pajer P, Pačes J, Bartůněk P, Elleder D. Genome Biol. 2015, 16:164.
Characterization of Chicken Tumor Necrosis Factor-α, a Long Missed Cytokine in Birds.
Rohde F, Schusser B, Hron T, Farkašová H, Plachý J, Härtle S, Hejnar J, Elleder D, Kaspers B.Rohde F, et al. Among authors: Elleder D. Front Immunol. 2018, 9:605
After TNF-α, still playing hide-and-seek with chicken genes. Elleder D, Kaspers. Poult Sci. 2019, 98(10):4373-4374.

We importantly contributed to the field of transgenesis in chicken. We have elaborated our original strategy of orthotopic male germ line transplantation into testes of recipients sterilized by irradiation. Transplantation of primordial germ cells lead to restoration of spermatogenesis without any background of the recipients´ offsprings. We demonstrated the efficiency of our procedure by introducing the mCherry reporter into the chicken genome. The seminal idea primordial germ cells transplantation arose in our laboratory and we developed the technique in equivalent three-sided collaboration with the group of P. Trefil (BIOPHARM Jílové) and B. Schusser (Technische Universität München, Germany). P. Trefil contributed with the skills of orthotopic male germ line transplantation, B. Schusser provided the expertise of PGC culture.
Male fertility restored by transplanting primordial germ cells into testes: a new way towards efficient transgenesis in chicken.
Trefil P, Aumann D, Koslová A, Mucksová J, Benešová B, Kalina J, Wurmser C, Fries R, Elleder D, Schusser B, Hejnar J. Sci Rep. 2017, 7(1):14246.

## Research activity and characterisation of the main scientific results.

In years 2015-2019 we have significantly increased the quality of our publication in all three area of interest. As documented in the **List of publications** shown below, we have published 15 papers in international journals with IF up to 19.809 (*Dobes et al, Gastroenterology, 2015*). Three additional manuscripts which were an indispensible part of our research activities between 2015-2019 were sent to various impacted international journal for a review process and have been already either accepted for publication or are currently under revision (see below, underlined are members of the lab#13).

1. Ballek, O., Valecka, J., Manning, J., Filipp, D. The pool of preactivated Lck in the initiation of T-cell signaling: a critical re-evaluation of the Lck standby model. ***2015. Immunol Cell Biol., 93: 384-395.***

2. Dobeš, J., Neuwirth, A., Dobešová, M., Vobořil, M., Balounová, J., Ballek, O., Lebl, J., Meloni, A., Krohn, K., Kluger, N., Ranki, A. and Filipp, D. Gastrointestinal Autoimmunity Associated with Loss of Central Tolerance to Enteric □-Defensins. ***2015. Gastroenterology, 149(1):139–150.***

3. Janečková L., Pospíchalová V., Fafílek B., Vojtěchová M., Turečková J., Dobeš J., Dubuissez M, Leprince D, Baloghová N., Horázná M., Hlavatá A., Stančíková J., Šloncová E., Galušková K., Strnad H, Kořínek V., Hic1 tumor suppressor loss potentiates TLR2/NF-kappaB signaling and promotes tissue damage-associated tumorogenesis. ***Molecular Cancer Research, 2015; 13(7):1139-1148.***

4. Stančíková J., Krausová M., Kolář M., Fafílek B., Švec J., Sedláček R., Neroldová M., Dobeš J., Horázná M., Janečková L., Vojtěchová M., Oliverius M., Jirsa M., Kořínek V. NKD1 marks intestinal and liver tumors linked to abberant Wnt signaling. ***Cellular Signaling, 2015;27(2):245-56.***

5. Chlubnová I., Králová B., Dvořáková H., Spiwok V., Filipp, D., Nugier-Chauvin C., Daniellou R., Ferrières V. Biocatalyzed synthesis of difuranosides and their ability to trigger production of TNF-α. ***2016. Bioorg Med Chem Lett. Mar 15;26(6):1550-3.***

6. Ballek, O., Valecka, J., Dobešová, M., Broučková, A., Manning, J., Řehulka, P., Stulík, J. and Filipp, D. TCR triggering induces the formation of Lck-RACK1-actinin-1 multiprotein network affecting Lck redistribution. ***2016. Front. Immunol. 7: 449***.

7. Vavrova, K., Vrabcova, P., Filipp, D., Bartunkova, J., Horvath, R. Generation of T cell effectors using tumor cell-loaded dendritic cells for adoptive T cell therapy. ***2016. Medical Oncology, 33(12):136-43.***

8. Stechova, K., Sklenarova-Labikova, J., Kratzerova, T., Pithova, P., and Filipp, D. Not Only Glycaemic but Also Other Metabolic Factors Affect T Regulatory Cell Counts and Proinflammatory Cytokine Levels in Women with Type 1. ***2017. J. Diabetes Res., 2017:5463273.***

9. Dobeš J, Edenhofer F, Vobořil M, Brabec T, Dobešová M, Čepková A, Klein L, Rajewsky K, Filipp, D. A novel conditional Aire allele enables cell-specific ablation of the immune tolerance regulator Aire. ***2017. Eur. J. Immunol. 48(3):546-548.***

10. Filipp D, Brabec T, Vobořil M, Dobeš J. Enteric α-defensins on the verge of intestinal immune tolerance and inflammation. ***2019. Semin Cell Dev Biol. 88:138-146.***

11. Yamano T*, Dobes J*, Voboril M., Steinert M, Brabec T, Zietara N, Dobesova M, Ohnmacht C, Laan M, Peterson P, Benes V, Sedlacek R, Hanayama R, Kolar M, Klein L*. and Filipp D*. Aire-expressing ILC3-like cells in the lymph node display potent APC features. ***2019. J Exp. Med., 216(5):1027-1037***
**** equal contribution***

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In years 2015**–**2019 we continued in curiosity driven research in all major areas of our interest highlighted in the above section. Compared to the previous evaluated period, i.e. 2010-2014, we dedicated our effort to improve the impact of our research activity by implementing the following four strategies: (i) in general, we strictly set up our priorities and selected those areas of research which were highly novel, promising, competitive, and yet close to our expertise and interest; (ii) particularly, we focused on a deeper and more comprehensive understanding of cellular, molecular and signaling aspects of the mechanisms of central and peripheral tolerance and embryonic homeostasis. Towards this end, we utilized the strategy of generation of several knock-out and knock-in transgenic mouse strains which allowed us to observed biological correlates and consequences of these mechanisms under perturbed and unperturbed

conditions; (iii) we heavily invested in the acquisition of the battery of commercially or academically available transgenic mouse models and created an experimental panel of mutated strains which allowed us to accelerate the rate of discovery; and (iv) we further deepen our ties with collaborating laboratories abroad and locally, organized regular meetings and exchange of ideas, materials and reagents.

**Specifically, in 2015–2019 we focused on the following subjects:**

## 1. Mechanisms of central and peripheral tolerance, autoimmunity

**A.** We have generated a novel Aire$^{fl/fl}$ mouse strain. Aire$^{fl/fl}$ mice was instrumental for the elucidation of several processes associated with establishment of central and peripheral tolerance. It is of note that this strain of mice has been deposited in Jackson Laboratory Animal facility where it is commercially available to a wide research community (JAX Stock No. 031409: B6.Cg-Aire<tm1Dfil>/J) ***(paper #9).***

**B.** Autoimmune polyendocrine syndrome (APS-1) is a rare autosomal recessive disorder caused by the loss of central immunological tolerance due to mutations in the autoimmune regulator (AIRE) gene. Autoreactive T cells aided by autoantibodies cause an organ-specific autoimmune disease in multiple organs, but the pathogenesis is poorly understood. Capitalizing on our joint expertise and collaborations with leading European investigators (Husebye, Ranki, Krohn), we (i) elucidated the molecular and cellular mechanisms behind gut-related immunopathology using the Aire$^{-/-}$ and other relevant transgenic and knock-out mouse models of APS-1; (ii) identified the novel gut-related autoantigens in APS-1 patients; (iii) developed an assays to measure these autoantibodies and correlated the findings to clinical outcomes; and (**iv**) we correlated a dramatic enrichment of Segmented Filamentous bacteria (SFB) and Lactobacilly in the microbiome of intestine and the induction of Th-17 responses in the gut of APS-1 patients as well as mouse model of this disease. These research activities provided important clues for further work to develop new diagnostic biomarkers and therapeutic options for APECED and gut autoimmunity as a whole (***paper #2***).

**C.** Aire serves an essential function for T cell tolerance by promoting expression of tissue-restricted antigens in medullary thymic epithelial cells (mTECs). However, the identity of these Aire expressing cells remains controversial. Some studies reported Aire expression in lymph node DC-like population referred to as eTACs (extrathymic Aire expressing cells). In contrast, we found that expression of Aire protein is exclusively confined to a BM-derived cell population that is clearly distinct from eTACs. Aire protein+ cells have a lymphoid morphology, are lineage negative, independent of Rag and depend on RORγt, thus belong to a ‘novel’ subset of innate lymphoid cells group 3 (ILC3s) subset. Most importantly, Aire+ ILC3s display potent APC features. Our data provide exciting insights into the nature and function of Aire expressing cells in the periphery (***paper #11***). In addition, with a tight collabration with prof. J. Abramson lab from The Weizmann Institute of Sciences in rehovot, Israel, we have shown that Aire-ILC3 cells are critical for effective IL-17 adaptive immune responses to blood-borne pathogens (***paper #17)***.

**D.** We have also engaged in collaborative research with the team of Prof. Štechová from the Department of Internal Medicine, 2nd Medical Faculty of Charles University and University Hospital Motol, Prague, in the investigation of immune homeostasis and its disturbance in the type 1 diabetes (T1D). Notably, we studied various glycaemic and lipid metabolic factors that affect T regulatory cell counts and proinflammatory cytokine levels in women with T1D. Our results showed an intricate relationship between glycaemic, lipid and immune parameters in T1D female patients. We showed that one important factor that negatively affects immune parameters such as Treg counts, TNF-alpha production and vitamin D levels is the total time spent in the hyperglycaemic state reflected in HbA1c values. Our data also suggests that complementing

glycaemic data with lipid clinical parameters can provide clinicians with a more comprehensive view on the immunometabolism of T1D patients and their long-term prognosis **(*paper #8)***.

## 2. Toll-like receptors and embryonic hematopoiesis

**E.** The premise of this work is the finding that Toll-like receptors (TLRs) and their adaptor proteins are functionally expressed during the early stages of embryonic development. It turned out that all TLRs and their adaptor proteins are expressed during early mouse embryogenesis (E7.5-12.5). Embryonic TLR2+ ckit+ progenitors undergo myelopoiesis in vivo and in vitro and respond to TLR2 stimulation by an enhanced proliferation and myeloid differentiation rate in a MyD88-dependent manner. TLR2+ ckit+ hematopoietic progenitors that exhibit the attributes of erythro-myeloid progenitors (EMPs) are readily detectable in the developing yolk at E7.5 and coexpress CD41, the marker indicative of commitment to hematopoietic development and Runx1, the transcription factor essential for the emergence of hematopoiesis. Using in vivo genetic labeling we demonstrate that the Tlr2 locus is indeed activated at early stages of embryonic development (E7.75-8.5) in emerging hematopoietic progenitors which contribute to not only embryonic but also adult hematopoiesis. Notably, when TLR2+ progenitors were genetically labelled at E8.5, they were able to give rise to erythroid, myeloid as well as lymphoid hematopoietic lineages that persisted in the peripheral blood of adult animals for >16 weeks. Moreover, the progeny of these cells persisted in the peripheral blood of primary and secondary recipients for >16 weeks, suggesting their long-term repopulating activity. Together, our results demonstrate that Tlr2 locus is active in yolk sac (YS)-derived EMPs and thus TLR2 can be utilized as reliable marker of early hematopoietic precursors and that Tlr2 locus is already active in E8.5 hematopoietic precursors that give rise to adult long-term HSCs **(*paper #12)***.

**F.** We generated a novel transgenic “knock in” mouse model suitable for inducible targeted depletion of TLR2$^{+}$ EMPs. In this model, the red fluorescent protein and diphtheria toxin receptor sequences are linked via a P2A sequence and inserted into the *Tlr2* locus before its stop codon. Using this model, we show that a timely-controlled deletion of TLR2$^{+}$ EMPs results in a marked decrease in both erythroid as well as myeloid lineages and, consequently, in embryonic lethality at E12.5. These findings validate the importance of EMPs in embryonic development **(*paper #18).***

## 3. Lck, lipid rafts and TCR proximal signalling

**G.** We completed our investigation into a novel mechanism regulating the proximal TCR signaling whereby the coengagement of TCR and CD4 leads to the recruitment of scaffold protein RACK1 which links the signaling to cytoskeletal network for permissive signaling. We performed several experiments necessary for the completion of manuscript describing the phenomenon concerning the formation Lck-RACK1-actnin-1 multiprotein network affecting Lck subcellular redistribution. Results presented in this study are the very first to characterize the role of RACK1 in early T-cell activation. RACK1-Lck complex formation in primary CD4+ lymph node T-cells is activation-inducible, transient and wanes shortly after activation. We showed that Lck and RACK1 co-redistribute with the same kinetics to the forming immunological synapses. In this context, our biochemical data showed that the formation of RACK1 Lck complexes, as well as the subsequent translocation of Lck to light DRM depends on an intact microtubular network. Interestingly, our data showed that Lck formed complexes with RACK1, irrespective of its kinase activity status, suggesting that conformational changes of TCR and/or CD4 may play a role in their induction. While the underlying mechanism awaits some resolution, we also demonstrated that it is only when these complexes contain kinase active Lck, they recruit alpha-actinin-1. The binding of alpha-actinin-1 to RACK1-Lck complexes adds another layer of complexity to the schematic of

TCR signaling, and is worth of further intense studies. Importantly, knock-down of RACK1 in primary CD4+ T-cells profoundly hampered the translocation of Lck to light DRMs. Thus, our data demonstrated that RACK1 fulfills the role of an adaptor protein that is involved in the regulation of Lck redistribution tolight DRMs through the linking of TCR/CD4-Lck to the cytoskeletal network **(*paper #6)*.**

**H.** In a parallel line of research, in collaboration with the research team of Prof. Bartůňková from Department of Immunology, 2nd Medical Faculty and Faculty Hospital Motol, Charles University, we contributed to studies with aim to generate oncoantigen targeting T cell effector cells via tumor cell-loaded dendritic cells for adoptive T cell therapy, more specifically, for the treatment of prostate cancer. In this study, tumor specific T cells are effectively activated and expanded when their cognate antigens are presented in the context of tumor cell-loaded matured DCs. In addition, a significant fraction of these CD4 and CD8 T cells, enriched in the final product, exhibits an early memory phenotype. This population proved to be functional and capable of both IFN-gamma production and lysis of target tumor cells. Moreover, after re-stimulation, the final T cell culture expresses a high level of Ki-67 which is indicative of recent antigen-specific activation and proliferation. This report highlighted the optimization of the Adoptive Cell Transfer (ACT) protocol using blood from healthy donors and described the generation of a mixture of tumor targeting T cells that exist in a continuum of differentiation stages which might be optimal for ACT purposes and may be widely applicable for the treatment of different malignancies. This study was published in the journal of Medical Oncology (***paper #7)***.

**I.** This investigtion describes the use styrene-maleic acid (SMA) amphipathic copolymers for studying membrane proteins within their native lipid environment. The results support the use of SMA as a potentially better (less artifact prone) alternative to detergents for studies on membrane proteins and their complexes, including membrane rafts (***paper #13)***.

The papers which are not commented in this section represent those research activities where the members of our lab were involved in the reserach with collaborating laboratories and where we provided our expertise, model, reagents and where we actively contributed to writing and/or editing the manuscripts.

## Research activity and characterisation of the main scientific results

**GABAb receptor**

In contrast to many other G protein-coupled receptors, metabotropic gamma-aminobutyric acid receptor (GABAB) cell surface stability is not subject to agonist-promoted internalization, but is constitutively internalized. We report that the ubiquitin-specific protease 14 (USP14) interacts with the GABAB(1b)subunit's second intracellular loop. Cell surface ubiquitination precedes endocytosis, after which USP14 acts as an ubiquitin-binding protein that targets the ubiquitinated receptor to lysosomal degradation and promotes its deubiquitination.

**mGluR receptor**

In our studies, we mapped two sites on the G-alpha subunit that govern specificity toward these receptors. One of these regions is the extreme C-terminus, also recognized by other GPCRs. Therefore, our study showed that this region allows distinguishing the G-protein signaling in all known classes of GPCRs. Also, another region, named the L9 loop on G-alpha subunit was shown to be important for signaling specificity of mGluRs. We next identified a portion of the receptor that is in contact with this extreme C-terminus of G-alpha subunit. This is the second intracellular loop. Some of these studies were concluded upon my return to Prague. A fruitful collaboration with Jean-Philippe Pin and Laurent Prezeau at Montpellier continues today.

**SGIP1**

The endocannabinoid system (ECS) is involved in synaptic plasticity regulation with a plethora of physiological consequences. Cannabinoid receptor 1 (CB1R), a G-protein coupled receptor (GPCR), is a central molecule of ECS. GPCRs are regulated by universal mechanisms, including phosphorylation mediated by G protein-coupled receptor kinases (GRKs) that trigger interactions with beta-arrestin, and initiate endocytosis, via clathrin mediated endocytosis (CME) in the case of CB1R. Besides signaling molecules common for all GPCRs, several proteins have been reported to interact with CB1R and to influence specific functions. CB1 receptor interacting protein (CRIP1a) regulates CB1R signaling and endocytosis, Adaptor protein 3 (AP-3), affects processing and signaling of the internalized pool of CB1Rs, and G-protein-associated sorting protein 1 (GASP1) controls lysosomal trafficking of down-regulated CB1R.

SGIP1 constitutes the muniscin family, together with the ubiquitous FCH/F-BAR domain only protein 1 and 2 (FCHO1/2). Muniscins interact with other molecules involved in CME; endophilin, AP-2, intersectin and Eps15. FCHO1/2 initiates plasma membrane invagination during the initiation of CME, while SGIP1 opposes this process. Hypothetically, the apparent difference between SGIP1 and FCFO1/2 domain organization within their N-termini may explain their contrasting effects. FCHO1/2 have the N-terminal portion folded to form F-Bar domains that are involved in the initiation of plasma membrane invagination during early stages of CME pit formation, while the N terminus of SGIP1 contains membrane phospholipid-binding (MP) domain that has no sequence similarity to the F-Bar motives and probably interacts with the plasma membrane differently than the F-Bar domains of FCHO1/2. Most likely, the interaction of MP domain with plasma membrane does not impose invagination of the membrane within the nascent pit formation.

SGIP1 is highly conserved across species, abundantly expressed in the nervous system, and enriched in compartments adjoining presynaptic boutons, in which it constitutes over 0.4% of protein content. We did not detect SGIP1 when using our antibodies on immunoblots from peripheral tissues. In mice, SGIP1 and CB1R have discernible overlapping expression patterns in most brain regions, including those involved in mood control for example, in prefrontal cortex, striatum, and hippocampus, and nociception, namely in the hypothalamus and other pain processing circuits. SGIP1 co-immunoprecipitates with CB1R from brain homogenates, and in neurons, the two molecules co-localize in presynaptic compartments. One recognized physiological role of SGIP1 relates to regulation of energy homeostasis. Elevated levels of SGIP1 mRNA in the hypothalamus of the Israeli sand rat (*Psammomys obesus*) correlate with obesity of the animals held in captivity, and genetic variations within the SGIP1 gene are associated with energy balance disturbances in humans. Also, a possible association of mutations within SGIP1 gene with neurological disorders occurrences have been reported in humans. Interestingly, the ECS is involved in regulation of energy balance, and in addiction. Thus, the CB1R-SGIP1 relationship may be relevant here. On the other hand, SGIP1 deletion does not change the mice weight, thus only overexpression of SGIP1 in hypothalamus is associated with obesity.

CB1R accumulates on axonal plasma membranes in cultured neurons, where it is substantially more stable compared to the receptors found on the neuronal soma. In Mazoni's lab they used single-quantum dot microscopy to study CB1R properties on the surface of cultured cortical neurons. They recognized an immobile CB1R fraction that remained on the plasma membrane in the vicinity of synapses for at least 30 minutes following agonist stimulation. Therefore, in presynaptic compartments, a portion of CB1R is resistant to agonist-induced internalization, and has low mobility. Our hypothesis is that this observation may be mechanistically explained by the SGIP1-CB1R interacion.

**Publications**

N*icolas Lahaie, Michaela Kralikova, Laurent Prézeau, Jaroslav Blahos*, Michel Bouvier**
Post-endocytotic Deubiquitination and Degradation of the Metabotropic γ-Aminobutyric Acid Receptor by the Ubiquitin-specific Protease 14.*
**Journal of Biological chemistry 2016,** Mar 25;291(13):7156-70
* corresponding and senior co-authors

We detected the interaction between GABB receptor and USP14, and initiated the studies in our laboratory. The study was concluded in collaborative effort, and both groups contribution was about equal, which is also expressed by shared senior co-authorship.
Mechanisms controlling the metabotropic γ-aminobutyric acid receptor (GABAB) cell surface stability are still poorly understood. In contrast with many other G protein-coupled receptors (GPCR), it is not subject to agonist-promoted internalization, but is constitutively internalized and rapidly down-regulated. In search of novel interacting proteins regulating receptor fate, we report that the ubiquitin-specific protease 14 (USP14) interacts with the GABAB(1b)subunit's second intracellular loop. Probing the receptor for ubiquitination using bioluminescence resonance energy transfer (BRET), we detected a constitutive and phorbol 12-myristate 13-acetate (PMA)-induced ubiquitination of the receptor at the cell surface. PMA also increased internalization and accelerated receptor degradation. Overexpression of USP14 decreased

ubiquitination while treatment with a small molecule inhibitor of the deubiquitinase (IU1) increased receptor ubiquitination. Treatment with the internalization inhibitor Dynasore blunted both USP14 and IU1 effects on the receptor ubiquitination state, suggesting a post-endocytic site of action. Overexpression of USP14 also led to an accelerated degradation of GABABin a catalytically independent fashion. We thus propose a model whereby cell surface ubiquitination precedes endocytosis, after which USP14 acts as an ubiquitin-binding protein that targets the ubiquitinated receptor to lysosomal degradation and promotes its deubiquitination.

We detected the interaction between GABB receptor and USP14, and initiated the studies in our laboratory. The study was concluded in collaborative effort, and both groups contribution was about equal, which is also expressed by shared senior co-authorship.

*Alena Hajkova, Sarka Techlovska, Michaela Dvorakova M, Janice Noda Chambers, Jiri Kumpošt, Pavla Hubalkova, Laurent Prezeau, Jaroslav Blahos**
SGIP1 alters internalization and modulates signaling of activated cannabinoid receptor 1 in a biased manner.
**Neuropharmacology 2016** Aug;107:201-214
(*Corresponding author )
This was our first report about CB1R-SGIP1 relationship with mostly *in vitro* approach. Robust internalization follows CB1R stimulation in transfected cells that lack SGIP1. Co-expression of SGIP1 with CB1R interferes with agonist-stimulated CB1R internalization in this system (Hajkova et al., 2016). Functional consequences of SGIP1 association with CB1R result from obstruction of CB1R down-regulation.
G-protein activation and termination of G-protein mediated signaling are not affected by SGIP1. Subsequent events that are facilitated by CB1R C-tail phosphorylation, and would result in CME, are profoundly decreased by SGIP1. SGIP1 stalls CME, and the desensitized receptor remains on the cell surface.
Our current understanding suggests two broad pathways of GPCR signaling, one from the cell surface, and a second signaling wave mediated by the internalized GPCR from intracellular compartments (Daaka et al., 1998). We proposed the following hypothesis that aims at depicting how the relationship between SGIP1 and CB1R affects events that follow the receptor desensitization:
During CB1R desensitization, arrestins interact with the phosphorylated CB1R. The temporary association between phosphorylated CB1R and arrestins terminates as the receptor is internalized. SGIP1 stalls CB1R internalization. Therefore, the arrestin interaction with desensitized CB1R persists longer in the presence of SGIP1. The consequence of stabilizing CB1R at the cell surface by SGIP1 is that dissociation of arrestin from CB1R that follow internalization occurs more slowly.
We also observed that ERK1/2 signaling is decreased in the presence of SGIP1. This decrease is likely the consequence of a lack of the arrestin-mediated ERK1/2 pathway activation from internalized CB1R.
Therefore, SGIP1 adjusts CB1R signaling in a biased manner; it does not influence CB1R mediated G-protein signaling at the cell surface, but it reduces ERK1/2 signaling from internalized CB1R. For a schematic representation of these events, please see our earlier study.
We thus asked, what would be consequences of the SGIP1-CB1R relationship in vivo. To gain insight into the physiological roles of SGIP1, we used a reverse genetic approach in an ongoing research.

*Michaela Dvorakova, Agnieszka Kubik-Zahorodna, Alex Straiker, Radislav Sedlacek, Alena Hajkova, Ken Mackie, Jaroslav Blahos**

**SGIP1 is involved in regulation of emotionality, mood, and nociception and tunes in vivo signaling of Cannabinoid Receptor 1**

**British Journal of Pharmacology,** under review

(*Corresponding author )

First, we developed the animal model, which is time consuming. Then we performed all the behavioral studies that are the merit of this paper. This manuscript was submitted in October 2019, and is in the review process since. Hopefully, our last revision is going to be successful soon, as we addressed most of the reviewers' comments. For the current study, we developed mice with constitutively deleted SGIP1 for behavioral studies, assessing anxiety-related behavior, coping with unescapable situations, and we tested their acute nociception. We also studied the efficiency of CB1R agonists and an opioid in mice lacking SGIP1 in nociception. Observations resulting from this study have the potential to be implicated in pain management.

SGIP1 null mice were used in a reverse genetic approach to investigate the role of SGIP1 in vivo in the behavioral tests. SGIP1$^{-/-}$ mice have phenotypes restricted to particular tasks, namely in tests that examined aspects of mood-related behaviors and nociception, while other modalities, including their mobility, exploratory drive, and working memory, remained intact. These results indicate that the development of the nervous system in SGIP1$^{-/-}$ mice is not profoundly affected. Both male and female SGIP1$^{-/-}$ mice have similar exploratory drives and mobility in the Y-maze test. We also verified the integrity of sensorimotor gating in the SGIP1$^{-/-}$ mice. Sensorimotor processing and working memory are not altered in SGIP1$^{-/-}$ mice, thus dwe exclude altered exploratory drives or impaired sensorimotor gating as causative for the observed differences in further behavioral examinations.

The ECS is involved in controlling mood, processing of fear, and adaptive handling of stressful situations. Our results from the Elevated plus maze and Open field experiments imply an anxiolytic-like phenotype for SGIP1$^{-/-}$ mice, with variability between sexes. This resonates with previous reports.

The anxiolytic-like phenotype of SGIP1$^{-/-}$, we report in this paper, and higher resistance to unescapable situation, are in accordance with pharmacological studies demonstrating anxiolytic-like and antidepressive-like effects of enhanced endocannabinoid transmission by blockage of their metabolic degradation in rodents. Anxiety and depression have a high rate of comorbidity. Previous investigations remarked overlapping molecular pathophysiological mechanisms of both modalities. Perhaps, further studies will elucidate if SGIP1 engages the same pathways leading to two distinctive phenotypes.

Extinction of fear memories is modulated by the ECS. Our current study revealed a strong impact of sex differences in FE. In the FC tests, both in a context and as a response to a cue, results were comparable between SGIP1$^{-/-}$ and WT mice for both sexes. Extinction of the aversive memories was comparable for SGIP1$^{-/-}$ and WT males, but there was a significantly more efficient FE in SGIP1$^{-/-}$ female mice compared to WT females. Differences between sexes were described in this process

Based on our results, we conclude that SGIP1 regulate anxiety levels under specific contexts, possibly via modulation of CB1R signaling.

Next we examined the acute responses and tolerance progression to THC in the cannabinoid tetrad tests. After initial THC treatments, SGIP1$^{-/-}$ and WT mice displayed similar levels of

catalepsy. SGIP1$^{-/-}$ mice developed a tolerance to THC-induced catalepsy at a significantly slower rate. SGIP1 also exhibited enhanced THC antinociception with a significant effect on the first day of dosing that decayed over the next seven days of repeated drug delivery at a rate similar to WT mice. Similarly, THC-induced hypothermia was augmented in SGIP1$^{-/-}$ mice, and they progressively developed tolerance to this effect. In the rotarod tests, the THC treatments on day 1 shortened endurance of SGIP1$^{-/-}$ animals on the rod in comparison with WT mice and animals treated with vehicle, but in day 4 and 7 they performed comparably.
Following the chronic treatment with THC, rimonabant was applied. This resulted in exhibiting abnormal behaviors in SGIP1$^{-/-}$ mice. We observed intense and persistent jumping in this cohort. Interestingly, similar jumping was also reported upon morphine withdrawal and decreased in CB1R$^{-/-}$ mice. Crosstalk between the endocannabinoid system and other signaling pathways, namely the opioid system, may explain this observation on a molecular level.

CB1R activation blunts reactions to painful stimuli. SGIP1$^{-/-}$ mice have prolonged reaction latencies in the In the tail immersion test (TIT), compared to the WT animals. This was observed upon both single and repeated tests. As a part of the tetrad tests, we also studied the effect of THC on nociception. SGIP1$^{-/-}$ mice had elevated latency in the response before THC treatment and enhanced sensitivity to the drug. Also, upon the daily THC administration for 8 days, tolerance to developed more slowly SGIP1$^{-/-}$ males.
In the SGIP1$^{-/-}$ male cohort, lack of SGIP1 synergistically enhanced antinociception to THC and WIN in the TIT. This effect was particularly noticeable in the delayed responses upon increasing CB1R agonist dosage. Re-analyses of the dose responses for WIN suggests, there is synergistic-like effects of WIN with that of SGIP1 deletion.
Morphine in the TIT interacted differently with SGIP1$^{-/-}$ mice than CB1R agonists. While deletion of SGIP1 still enhanced morphine-induced antinociception, the interaction was weaker than for CB1R-meidated antinociception, consistent with an additive type of interaction.
The CB1R antagonist rimonabant pre-treatment resulted in transiently increased nociception in SGIP1$^{-/-}$ males 30 minutes after the injection, but this effect did not persist after one hour and was absent in the tests in consecutive days.
In accord with previous reports, we also detected sex-dependent variances of the ECS effects on nociception (Fattore & Fratta, 2010).
We proposed that SGIP1 may well be a novel regulator of CB1R mediated antinociception.
Results of the behavioral tests described in previous studies, in which the ECS was manipulated chemically or genetically may be related to the present study. Global deletion of CB1R also resulted in a modified exploratory phenotype, hypoactivity, and anxiety-like behavior, namely if the CB1R$^{-/-}$ mice were subjected to highly aversive conditions, and also moderate doses of CB1R agonists evoked anxiolytic effects (while higher doses lead to the opposite). The anxiolytic phenotype that we observed in tests with SGIP1$^{-/-}$ mice parallels the situation with moderately upregulated tone within ECS. This is in accordance with our hypothesis about the SGIP1 effect on CB1R signaling.
Affecting the endocannabinoid degradation and synthesis pathways also influence behavior. Increasing anandamide levels via chemical inhibition of its catabolic enzyme fatty acid amide hydrolase, or the deletion of FAAH resulted in phenotypes with behavioral aberrations comprising decreased anxiety-like behavior, as we observed in the present tests with SGIP1$^{-/-}$ mice. On the other hand, global deletion of diacylglycerol lipase (DAGL alpha), the enzyme primarily involved in the neuronal 2-AG synthesis, results in increased levels of anxiety-like behavior.

We conclude that the above-reported results of the SGIP1$^{-/-}$ mice behavioral testing resonate with mice's phenotype in which the ECS signaling was manipulated. Altered CB1R signaling in the SGIP1$^{-/-}$ mice may thus be imposed on adjacent signaling cascades.
Mice lacking beta-arrestin2 also exhibited enhanced acute responses to THC, and altered tolerance following repeated THC treatment. As pointed above, SGIP1 influences the association of CB1R with beta-arrestin2 and signaling mediated by this relationship. Also, genetic disruption of GASP1 results in reduced tolerance to cannabinoid-mediated antinociception in mice.The comparison of the results from studies with mice strains with manipulated levels of beta-arrestins, or GASP1 resonates to a high degree with our observations using SGIP1$^{-/-}$ mice and supports further our hypothesis that SGIP1 affects behavior by the modification of CB1R signaling.

*Mariana Hajj, Teresa De Vita, Claire Vol, Charlotte Renassia, Jean-Charles Bologna, Isabelle Brabet, Magali Cazade, Manuela Pastore, Jaroslav Blahos, Gilles Labesse, Jean-Philippe Pin, Laurent Prézeau*

Nonclassical Ligand-Independent Regulation of Go Protein by an Orphan Class C G-Protein-Coupled Receptor
**Molecular Pharmacology 2019** Aug;96(2):233-246. doi: 10.1124/mol.118.113019. Epub 2019 Jun 12.
The orphan G-protein-coupled receptor (GPCR) GPR158 is expressed in the brain, where it is involved in the osteocalcin effect on cognitive processes, and at the periphery, where it may contribute to glaucoma and cancers. GPR158 forms a complex with RGS7-*β*5, leading to the regulation of neighboring GPCR-induced Go protein activity. GPR158 also interacts with *α*o, although no canonical Go coupling has been reported. GPR158 displays three VCPWE motifs in its C-terminal domain that are putatively involved in G-protein regulation. Here, we addressed the scaffolding function of GPR158 and its VCPWE motifs on Go. We observed that GPR158 interacted with and stabilized the amount of RGS7-*β*5 through a 50-residue region downstream of its transmembrane domain and upstream of the VCPWE motifs. We show that two VCPWE motifs are involved in *α*o binding. Using a G*α*o-*βγ* bioluminescence resonance energy transfer (BRET) sensor, we found that GPR158 decreases the BRET signal as observed upon G-protein activation; however, no constitutive activity of GPR158 could be detected through the measurement of various G-protein-mediated downstream responses. We propose that the effect of GPR158 on Go is unlikely due to a canonical activation of Go, but rather to the trapping of G*α*o by the VCPWE motifs, possibly leading to its dissociation from *βγ* Such action of GPR158 is expected to prolong the *βγ* activity, as also observed with some activators of G-protein signaling. Taken together, our data revealed a complex functional scaffolding or signaling role for GPR158 controlling Go through an original mechanism.

**Cooperation within international research area**

Our major collaborators are; Laurent Prezeau and Jean Philippe Pin from CNRS Montpellier, France, Michel Bouvier from Montreal, Carsten Hoffman from Jena and Ken Mackie from Indianapolis.

Selected publication arising from our collaborative efforts, unless mentioned elsewhere

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Last few years we intensively collaborate with prof. Ken Mackie from Indianapolis, a renowned specialist on endocannabinoid signaling. Together with his team, we characterized the SGIP1 mice model we developed. Our common effort led to confirmation of the SGIP1 role in modification of endocannabinoid signaling, most likely via CB1R regulation.

## Research activity and characterisation of the main scientific results

During the years 2015-19 we focused on three main research activities that were all centered around the RNA splicing: 1. Formation of the splicing machinery; 2. Regulation of RNA splicing and 3. Mutation of splicing factors in retinitis pigmentosa.

### 1. Formation of the splicing machinery.

This has been a long-term interest of our team. We primarily study the formation of spliceosomal small nuclear ribonucleoprotein particles (snRNPs) and focused on a question where and when snRNP complexes form and recycle. We discovered a new quality control checkpoint, that surveillances formation of U4/U6 and U4/U6•U5 snRNPs and sequesters incomplete or defective particles in nuclear structures call the Cajal body. This discovery was published in *Novotny et al. 2015, Cell Reports*.

In a follow up work, we aimed to decipher a molecular mechanism that distinguishes between mature fully assembled snRNP and incomplete or defective complexes. We implemented a microinjection of fluorescently labelled snRNA to elucidate snRNA sequences essential for targeting and sequestration of defective snRNPs in Cajal bodies. These experiments, in combination with episomal expression of mutated snRNAs revealed that the Sm binding sequence of snRNAs and a heptameric ring of Sm proteins that is assembled around the Sm binding sequence are essential and sufficient for localization of snRNPs into Cajal bodies. This was finally confirmed by microinjection of mature and incomplete snRNPs, which was performed by Cyrille Girard from Reinhard Lührmann's laboratory. Based on these data, we proposed a model that the accessibility of the Sm ring represents the marker that signals that snRNP is not fully matured. We further suggested that an interaction between a Cajal body component and the Sm ring anchor incomplete snRNPs in Cajal bodies. These results were published in *Roithova et al. 2018, Nuc. Acids Res..*

In addition to these two seminal publications, we summarized the topic of snRNP assembly and Cajal bodies in three reviews (*Machyna et al. 2015, RNA Biol.; Stanek 2015, RNA Biol. and Stanek and Fox 2017 Curr. Op. Cell Biol.*) and published a methodological paper on snRNA microinjection *Roithova and Stanek 2019, J. Vis. Exp.*.

### 2. Regulation of RNA splicing.

Our laboratory entered the alternative splicing field in 2011 by a discovery that histone acetylation modulates alternative splicing. Since then, we have been investigating molecular details of the connection between RNA splicing and chromatin modifications. We established a system based on Transcription Activator-Like Effector domains (TALE). We fused TALE to various histone modifying enzymes and navigated them to specific exonic and intronic sequences in the genome to locally manipulate chromatin marks. We provided evidence that a local increase in H3K9 di- and trimethylation promotes inclusion of the target alternative exon, while demethylation leads to exon skipping. We further demonstrated that navigating of a H3K9 demethylase to a weak constitutive exon reduced co-transcriptional splicing. Based on these results we suggested a model that H3K9 methylation within the gene body is a factor influencing exon recognition. This work was published in *Bieberstein et al. 2016, Sci. Report.*

We further turned our attention to splicing of non-coding RNAs and aimed to elucidate a puzzling fact in the field why long non-coding RNAs (lncRNAs) are less efficiently spliced than protein coding pre-mRNAs. We disproved our original hypothesis that chromatin status dictates the difference in lncRNA and pre-mRNA splicing. Next, we turned our attention to the sequence of splice sites but did not find any difference in splice site strength. However, we discovered that splicing efficiency of lncRNAs is more dependent on the 3' splice site strength than pre-mRNAs, which suggested that lncRNA are more dependent on basic

splicing signals. Indeed, further analysis confirmed that lncRNA contain less binding site for splicing enhancers – SR proteins. We established collaboration with prof. Michaela Müller-McNicoll, an expert on iCLIP approach and confirmed that SR proteins bind much less lncRNAs than pre-mRNAs. We proposed that lncRNAs lacked the cooperative interaction network that enhances splicing, which rendered their splicing outcome more dependent on the optimality of splice sites. These results were recently published in *Krchnakova et al. 2019, Nuc. Acids. Res.*.

In addition to these two main projects developed in our laboratory, we collaborated with Igor Vorechovsky and Jana Kralovicova on alternative splicing regulation by PUF60 and RBM39 (*Kralovicova et al. 2018, Nuc. Acids. Res.*).

**3. Mutation of splicing factors in retinitis pigmentosa.**

This area of our research stemmed from our interest in biogenesis of snRNPs. We have previously shown that retinitis pigmentosa (RP)-linked mutations in Prpf31 affect its interactions with snRNP components (Huranova et al. 2009, Hum. Mol. Gen.) and that RP-associated substitutions in the splicing helicase SNRNP200 changes splicing fidelity (Cvackova et al. 2014, Hum. Mut.). We continued this research and focused on RP-linked mutation in the core splicing factor Prpf8. We established stable cell lines expressing various RP variants and monitored they localization, interaction with snRNP components and effect on splicing efficiency. We showed that 7 out of 8 mutations negatively affect formation of the U5 snRNP. More detail experiments showed that RP variants of Prpf8 interact more with the R2TP complex. We established a collaboration with Edouard Bertrand and Celine Verheggen from CNRS, Montpellier who have been extensively studying the R2TP complex. In this work, we provided evidence that the R2TP complex assisted in early steps of U5 snRNP assembly, sequestered mutated Prpf8 factors in the cytoplasm and thus acted as a surveillance factor. This collaborative work was published in *Malinova et al. 2017, J. Cell Biol.*. Our expertise in splicing and RP was recognized by editors and we were invited to write two reviews on this topic: *Ruzickova and Stanek, 2017, RNA Biol.* and *Krausova and Stanek, 2017, Sem. Cell Dev. Biol..*

**Cooperation within international research area**

Our team has a long-term collaboration with prof. Karla Neugebauer (Yale University, New Haven, USA) which is mainly focus on the Cajal body as a key structure in nuclear metabolism of snRNPs. We have a running collaborative project oriented on Cajal bodies in snRNP surveillance supported by the Czech Ministry of Education, Youth and Sport (MYES). The collaboration is also documented by a joint publication *Machyna et al. 2015, RNA Biol..*

During last five years we have established several new collaborations with internationally recognized scientists. Our team started a collaborative project on identifying snRNP chaperons with dr. Edouard Bertrand and dr. Celine Verheggen rom CNRS, Montpellier, France. This collaboration resulted in one joint publication (*Malinova et al. 2017, J. Cell Biol.)* and we are currently preparing a joint manuscript describing a function of a new snRNP chaperon.

To tackle a problem of snRNP surveillance in Cajal bodies, we joint our effort with dr. Cyrille Girard from prof. Reinhard Lührmann's lab (Max Planck Institute for Biophysical Chemistry, Göttingen, Germany). Reinhard Lührmann has been extensively studying snRNPs and the spliceosome for decades and his laboratory described composition and structure of most splicing complexes. Result of our collaboration were published in *Roithova et al. 2018, Nuc. Acids Res.*.

During our research of lncRNA splicing, we started collaboration with prof. Michaela Müller-McNicoll (Goethe University, Frankfurt, Germany) and dr. Nejc Haberman (MRC London Institute of Medical Sciences, London, UK). M. Müller-McNicoll provided us with iCLIP data

mapping interactions of SR proteins with RNAs and N. Haberman helped us with a bioinformatic analysis. Our joint publication was published in 2019 (*Krchnakova et al. 2019, Nuc. Acids. Res.*).

Finally, our expertise to map interactions among splicing factors *in situ* resulted in a collaborative publication (*Kralovicova et al. 2018, Nuc. Acids. Res.*) with Jana Kralovicova from Slovak Academy of Sciences, Centre for Biosciences, Slovak Republic and Igor Vorechovsky from University of Southampton, UK.

**HR policy of the team**

*The team will indicate the information about the management of the team, strategy of the professional development etc. complementarily with the information provided for the institute. The maximum extent is 2 pages.*

The research team is mainly comprised of students (graduate and undergraduate) supervised by the team leader. Therefore, the team has very flat management structure with minimal hierarchy.

1. Undergraduate students

We are not actively recruiting undergraduate students but they often apply to join the team to work on our projects. Undergraduate students are mainly students of Faculty of Science, Charles University but a few are also applying from the Institute of Chemical Technology. We try to support them in their research interest but due to limited available time of the team leader, we restrict the number of graduate students to three to be able to provide them with sufficient assistance and leadership. Our aim is to teach them laboratory routine, project management, independence, critical thinking and presentation skills. When they are interested in science, which they mostly are, we encourage them to continue their education in graduate programs either in the Czech Republic or abroad. During last five years three undergraduate students finished their diploma thesis in our laboratory. Two of them decided to enter PhD programs in Switzerland, one is a PhD student at the Institute Organic Chemistry and Biochemistry in Prague.

2. Graduate students

Graduate students are the main working force of the team. Therefore, the most of the team leader time is dedicated for their supervision. PhD students are chosen via a selection process organized by the PhD committee at the Institute of Molecular Genetics. Briefly, an open PhD position in the laboratory is announced on-line and PhD applicants send their applications via an on-line form. After the first selection round, which is based on information applicants provide, selected candidates are invited either to on-line or on-site interview. The aim of the process is to find the ideal match between group leaders and candidates. After joining the team, we intensively help the student to rapidly gain all necessary knowledge to be able to independently manage his/her project. During first two years, we also encourage students to attend workshops where they learn techniques and approaches important for their project. Later on, we support their active participation at meetings and conferences to learn how to present and discuss their results. Each PhD student has his/her research project and our goal is that their finish and defend their thesis within 5 years, which till now, most of them succeeded, At the end of their graduate studies, the team leader discusses with them their future professional goals and advice and support them in their career decisions. Three students finished their PhD in our lab during last five years. One is working as a research assistant in a biomedical start-up company, one is working as a postdoc in Switzerland and one is on a maternity leave.

3. Postdocs

During the last five years there have been two postdocs in the team. One postdoc was actively recruited at the beginning of the evaluation period to establish a mouse model for retinitis pigmentosa. Second postdoc was working on human cellular models of retinitis pigmentosa. The postdocs are working independently or in small units teamed up with a

graduate or undergraduate student. They are encouraged to autonomously manage their projects with rather strategical supervision from the team leader. The goal is to support their leadership skills that are necessary for their future career when they run their own team.

All team members actively participate in activities (regular lab meetings, seminars, journal clubs) where they present their projects and discuss their results and ideas. It teaches them presentation skills as well as critical thinking.

## Research activity and characterisation of the main scientific results

### A) GENERAL DESCRIPTION OF THE TEAM'S DEVELOPMENT AND ITS PURPOSE

The '*team*' to be evaluated is composed of the research department 18 (R. Sedlacek), the Laboratory of Immunological and Tumour Models (11) led by M. Reinis, and partly of the CCP represented by the Phenotyping Module (83). Altogether, CCP infrastructure has three principal Modules, the Phenotyping Module (PM), Transgenic and Archiving Module (TAM) and Animal Facility Module (AFM), out of which the PM creates the majority of publication outputs. This *'team'* was brought together for the purpose of evaluation, and also because the CCP team and the two research groups (18 and 11) evolved historically together.
Regarding the output of the team, there are three basic categories: service activities, collaborative publications based on service and expertise of the CCP team, and own publications.
The 'team' (actually rather an equivalent of a very large department with 14 Units of PM-CCP, which are partly of comparable size of the IMG's research groups with the difference that most of the PM-CCP staff consists of specialized technicians involved in the service) has dynamically developed over the years of the evaluated period with the goal to give rise to a fully-operational research infrastructure of international impact, and set up research and technology development projects. As the CCP team developed into by far the largest group at IMG, its size, complexity, and dynamics required also establishing of a professional management, administration and organization.
Majority of the staff of the 'team' to be evaluated belongs to the CCP infrastructure, whose aim is primarily not to produce its own publications. The staff principally dedicated to research comprises only several postdocs/senior researchers.

### B) CZECH CENTRE FOR PHENOGENOMICS

The **Czech Centre for Phenogenomics** (https://www.phenogenomics.cz/) is a unique research infrastructure, which not only combines genetic engineering, advanced comprehensive and standardised phenotyping and custom-tailored model characterisation, specific pathogen free (SPF) animal housing and husbandry, as well as cryopreservation and archiving, but CCP introduced in its knowledge and service portfolio also initial pipeline for preclinical evaluation and patient-derived xenograft technologies. This its activities span from the basic research and services to advanced portfolio of translational research that directly approaches patients. CCP has all these under one umbrella - in the central location at BIOCEV campus. This concentrated knowledge and technology platforms provides a unique and valuable resource for the biomedical and biotechnology research communities.

The CCP major research activity is dedicated to development of genome editing technologies and phenotype analysis with a strong focus on multidisciplinary collaboration with research community. The CCP, through its membership in INFRAFRONTIER (https://www.infrafrontier.eu/) and IMPC (https://www.mousephenotype.org/), is a partner in a collective global network that aims to comprehensively and systematically analyse the effect of loss of function of all coding genes in mouse genome. The ambitious goal of entire IMPC consortium is to fully annotate the coding mammalian genome, which is the biggest worldwide attempt to understand coding sequences logically following after sequencing the human genome. The result is ever increasing 'encyclopaedia' of gene function, that is helping to identify causative factors of human diseases as well as novel targets for therapeutic intervention. Through the adoption of standardized procedures and pipelines, and the usage of quality control measures and cross-validation, our shared goal is to produce high-quality data that will not only function as a scientific reference catalogue, but will also enable comprehensive meta-analyses that could uncover otherwise hidden disease relationships and functional interactions. By focusing expertise and resources and coordinating our efforts to avoid redundant work, we will help achieve these goals in a more cost-effective manner.

**The CCP is built based on community needs that could be characterized as follows:**

- needs to have high-standard breeding facility
- needs to have access and efficient delivery of new gene-modified rodent models
- needs to have expertise, devices, and technologies to efficiently characterized the mutants or treatments and potential therapeutic compounds (especially as a reaction to the needs of biotech and pharmaceutical organisations)
- needs to bridge basic research using animals with medical applications
- needs to systematically and comprehensively analyse experimental data and results

**Involvement RI in research projects:**

Beside service mode of CCP operations its research activities are also important. We can divide research activities in three basic modalities.

A) The most of capacity is dedicated to contribution of CCP to large international projects under umbrella of IMPC and INFRAFRONTIER. Investigation of gene function of mostly non-annotated genes based on newly generated mouse models that are part of the IMPC effort and data are freely available for research communities and public (own research).

B) Research activities, which are needed for development and validation of new technologies and methods of gene targeting, genome editing and phenotyping (imaging modalities, physiological tests and histological techniques), such activities are essential for keeping our services updated to needs of research community and thus improve service for our users in near future. The development and validation of phenotyping technologies is also critical activity for preclinical research, employing our models and expertise to define the function of selected genes and their variants and mutations in human diseases (own research).

C) The last group of research activities is based on cooperation with individual PIs on the large complex projects, which need a multimodal approach with robust technological and know-how involvements. Such research projects serve as a critical testing platform for integration of technologies established at CCP and are used for new technology development and systematic improvement of CCP service (supported research).

**Development of CCP in 2016-2019:**

CCP was built to meet the increasing demands of the Czech scientific community to study gene function using mouse models. During its development, CCP became active also internationally to satisfy also the demands of the international community using rodent models. The CCP was gradually built over the period of 2012-2016, starting at first with the transgenic core facility and animal facility that were established at the IMG new building in Krč in 2007/8. In the funding period of 2016-2019, the CCP was already a fully established and renowned centre with growing services, cooperation and increasing result uploads to a publicly available database under umbrella of IMPC.

More specifically, Transgenic and archiving module´s main goal was the increase and optimization of mouse and rat knock-out model production according to the IMPC guidelines. Major achievement was establishment of zygote electroporation, a gene delivery method that largely reduced the costs and increased the throughput of rodent model production. This, together with other technological improvements and establishment of state-of-the art protocols, led to significant increase in generation of mutant lines (16 lines in 2015 vs 337 lines in 2019). Furthermore, we established new technologies (ssODN knock-in by zygote electroporation) to allow production of sophisticated custom-tailored mutant models carrying point mutations. In close future, we plan to focus on generation of humanised rodent models, further improvements in gene-delivery methods and improvement of the genotyping process.

Development of phenotyping module was from the beginning oriented towards standardised and reproducible procedures according to the strict IMPC guidelines. In 2016, all units according to a standardized IMPC phenotyping pipeline were established in the new building:

Neurobehavioral, Cardiovascular, Lung function, Metabolism, Vision, Hearing, Embryology, Bioimaging, Histopathology, Clinical biochemistry, Immunology. All units were integrated into a comprehensive phenotyping pipeline and IMPC phenotyping efforts have been initiated. In 2017, the Bioinformatics unit was established motivated by large data acquisition and also CCP involvement in multiOMICS projects. In 2018, Unit of PDX and cancer models was established, providing unique type of services to research community based on preparation surgical orthotopic models of cancer, with aim to provide the first personalised cancer testing pipeline in the Czech Republic. In 2019, the major development was in functional integration of the CCP phenotype database with the consortial IMPC database and our data started to be publicly available. The new unit (Metabolomic units) was established to cover community needs for expanding metabolomics services and hearing unit was functionally expanded to cover more electrophysiological modalities for phenotyping. Unit was renamed to Electrophysiology unit. Development after funding period: in close future, we plan to expand our unique phenotyping procedures also towards preclinical testing pipelines with expansion of humanised mouse model of diseases and personalised PDX models. We plan also a larger involvement of AI for faster data processing and analysis.

## C) RESEARCH TOPICS

Besides the service-based activities, the '*team' of the evaluated groups* has had also its own research topics or work on diverse research topics together with specialized collaborating partners. The main topics that are investigated in our laboratories are processes and topics in metabolism, bone and tooth development, gastrointestinal tract, all of which include the physiologic and disease conditions; especial focus is the area modeling human disease including the rare diseases. Within these research fields we study selected molecules that belongs to families of serine- and metalloprotease, ubiquitin ligases, transcription and methylation factors, structural proteins and several others.

**Proteases in physiology and disease.**
The major focus here is oriented to Kallikrein Related Peptidases, shortly kallikreins (Klk). We have left the field of matrix metalloproteinases (MMPs) that we studied in previous periods (see also the gastrointestinal and immunology projects below). A new intracellular metalloproteinases Trabd2, which is regulating WNT pathway, became another focus of our research.
In the Klk-field, we generated a set of knockout mice allowing us to decipher their functional proteolytic network in the skin using TALEN or CRISPR/Cas9 technology. Altogether, we generated single or multi-deficient mutant models for **Klk5, Klk7, Klk8, Klk11, Klk13, Klk14** or related genes relevant for characterization of their pathophysiological roles (TNF RI, Caspase 14). Furthermore, we have prepared a model for a rare genetic disease Netherton Syndrome (NS) carrying a patient-derived mutation in coding sequence of **Spink5** (encoding protease inhibitor LEKTI). By combination and phenotypic analysis of abovementioned mouse models, we have elucidated molecular mechanism of NS and proposed Klk5 and Klk7 as major therapeutic targets for Netherton syndrome treatment (Kasparek et al., 2016, Kasparek et al., 2017).
As a follow up, we investigate now the Klk role in skin inflammation and other skin diseases (psoriasis, atopic dermatitis). We have generated a mouse model with Spink5 mutation carrying additional mutation in TNFRI, to explore the potential of inflammation blockers for treatment of diseases associated with Klk dysregulation. Models combining deficiency in Klk proteases together with TNFRI are now analyzed and we expect to obtain publishable data in 2021. We have also analyzed the role of Klk14 in Netherton syndrome by ablation of Klk14 gene in Klk5/7/Spink5 triple deficient mice. We have found that Klk5/7/14/Spink5 quadruple KOs are rescued from severe itch that develops in adult Klk5/7/Spink5 triple mutants, pointing to role of Klk14 in triggering itch phenotype.

Regarding the metalloproteinase **Trabd2**, we explore currently its roles in brain and head development, and in Wnt signaling, which are crucial for proper cell differentiation and proliferation during both embryonic development and adult tissue maintenance. Trabd2, a transmembrane manganese-dependent metalloproteinase, was previously described to cleave some of the Wnt proteins regardless of their involvement in canonical or non-canonical pathway. However, the precise mechanism of its action has been described only in vitro. To examine its function in vivo, we generated Trabd2-deficient mouse model using CRISPR/Cas9 technology. The Trabd2b-deficient mice display pathological effects on brain and head development whereas the level of damage differs between individual embryos. Some of the embryos remind of WT embryos, while others have hydrocephaly, and some are exencephalic lacking developed facial area. To identify the connection of Trabd2 with Wnt regulatory networks, we used Wnt signaling-challenged model of nucleoredoxin (Nxn) knockout mice (Nxn acts as a negative regulator of Wnt signaling) crossed with the Trabd2 mutants. Observed phenotypes result from defects in neural tube closure during early embryonic development, which requires proper balance of Wnt signaling activity. By model of embryonic development, we propose that Trabd2 metalloproteinase is a novel Wnt signaling regulator during mammalian development. Our work on Trabd2-Wnt pathways is linked to our cooperation with the V. Bryja group (MU Brno), in which we are responsible for generation of animal models and their characterization as documented by several projects (for instance Keiser et al., 2019, Nat.Comm.)

*Featured publications:*

- *KLK5 and KLK7 Ablation Fully Rescues Lethality of Netherton Syndrome-Like Phenotype. Kasparek P, Ileninova Z, Zbodakova O, Kanchev I, Benada O, Chalupsky K, Brattsand M, Beck IM, Sedlacek R.* ***PLoS Genet****. 2017 Jan 17;13(1):e1006566.*
- *WNT5A is transported via lipoprotein particles in the cerebrospinal fluid to regulate hindbrain morphogenesis. Kaiser K, Gyllborg D, Procházka J, Salašová A, Kompaníková P, Molina FL, Laguna-Goya R, Radaszkiewicz T, Harnoš J, Procházková M, Potěšil D, Barker RA, Casado ÁG, Zdráhal Z, Sedláček R, Arenas E, Villaescusa JC, Bryja V.* ***Nat Commun****. 2019 Apr 2;10(1):1498.*

**Molecular mechanisms of skeleton and tooth development and diseases**

In this research area, we are studying several molecules, one them is **ameloblastin (Ambn**) and mechanism of enamel formation. The teeth are unique organs among mineralized tissues combined from dentin and enamel. Whereas dentin mineralization is based on similar mechanism like in bones by deposition of hydroxyapatite crystals by protein matrix formed of collagen self-assembled filaments, the mechanism how is mineralization regulated in enamel is not clear yet. The deposition of hydroxyapatite is regulated by enamel matrix proteins (EMP). The **amelogenin (Amel)** and ameloblastin (Ambn) are the most abundant proteins from EMP. Amel and Ambn form together complex protein scaffold, which subsequently allows a highly regulated process of hydroxyapatite crystallites deposition leading to formation of tooth enamel. Our research team discovered a common essential motif for polymerization of both proteins. The presence of such motif is critical for ability of both proteins to form higher ordered protein structures, which is further essential for formation of matured enamel. The mechanism how both EMP proteins forms together mineralization scaffold is not known yet. We are proposing using advanced light microscopic methods with super-resolution ability in order to localize and describe individual protein subunits and their contribution to formation of enamel matrix scaffold in wt proteins and also in mutant form of Ambn, which is not able to form a polymeric structure. The result shall provide new insight into mechanism of formation of the hardest tissues in the body. This knowledge may also be beneficial for development of novel approaches in biomaterial science with potential biomedical applications i.e. tooth bioprinting. We have successfully developed several project and published high-impact publications - for instance, two featured publications:

- *Intrinsically disordered proteins drive enamel formation via an evolutionarily conserved self-assembly motif. Wald T, Spoutil F, Osickova A, Prochazkova M, Benada O, Kasparek P,*

*Bumba L, Klein OD, Sedlacek R, Sebo P, Prochazka J, Osicka R. Proc Natl Acad Sci U S A. 2017 Feb 28;114(9):E1641-E1650*

- *Migration of Founder Epithelial Cells Drives Proper Molar Tooth Positioning and Morphogenesis. Prochazka J, Prochazkova M, Du W, Spoutil F, Tureckova J, Hoch R, Shimogori T, Sedlacek R, Rubenstein JL, Wittmann T, Klein OD. Dev Cell. 2015 Dec 21;35(6):713-24.*

Currently, we proceed investigation in this field further und study non-canonical role of ameloblastin in regulation of physiological functions. Ambn most important common feature is ability to bind Ca2+ and phosphate ions and their intrinsically disordered structure while the molecular mechanism of Ambn function in tissues stability or regenerative processes remains unclear. We have identified strong phenotype in bone mineralization, skeletal muscle integrity, and heart muscle function in Ambn KO mice and now aim to describe Ambn functions in these organs. We are using detailed phenotyping procedures in Ambn KO and amino acid substitution mutants with monomeric version of Ambn or Ambn without interaction potential to Ca2+. We test the biological activity of Ambn peptides to assess their clinical potential in muscle integrity and regeneration.

Another example of a project focusing on biomineralization is the description of the role of Fam46a.
The **FAM46 proteins** (FAM46A, FAM46B, FAM46C, and FAM46D) are involved in many different human diseases and FAM46A is expressed in developing tooth buds and through bioinformatics, has been identified as a novel eukaryotic non-canonical poly(A) polymerase. Loss of **FAM46A** was found in patients with osteogenesis imperfecta. We have developed a Fam46a-deficient mouse model exhibiting strong phenotype in teeth development, skeleton aberrations, and metabolism abnormalities and focus now on biomineralization issues. As Fam46a is adding adenosine nucleotides to the 3'end of target mRNA as a non-canonical polyA polymerase for stabilization of mRNA, we hypothesize that the phenotype observed in Fam46a KO is the result of the downregulation of essential genes for mineralization tissue. In conclusion, in the absence of non-canonical poly A polymerase Fam46a, target mRNAs are destabilized and degraded and we observed the hypomineralization and metabolism phenotype. This study aims to characterize the molecular mechanisms that underlie the observed phenotypic changes following Fam46a ablation and determine its connection to rare human diseases.
First, we characterized the hypomineralization of the skeleton by microCT imaging. Fractures were observed in ribs and long bones and density of the skeleton was reduced. Regarding the teeth, they were hypomineralized and enamel layer was reduced suggesting a problem in amelogenesis. Analyzing the expression of enamel matrix proteins (EMP), Ambn and Amelx, in the absence of Fam46a did not significantly differ in their expression but EMP self-assembly into the extracellular matrix was impaired.
Additionally, we identified dysregulated genes in ameloblast cells with Illumina sequencing. Many of the identified genes were coding extracellular matrix proteins such as collagen, amelogenin, clusterin, biglycan. Now, we are working on identification of direct targets of Fam46a poly-A polymerase. This method is used not only to measure mRNA levels but also to measure the length of mRNA poly-A tails. Altogether, we aim to elucidate the molecular mechanisms underlying the Fam46a deficient phenotype in mice and how the absence of Fam46a leads to the observe phenotype.

**Metabolism**
Metabolism is intrinsically connected to every function organ, tissue and cell. Our group has developed a technological platform to decipher metabolic processes and characterize genes with essential metabolic functions. Thus, we participated in a study to generate and analyze metabolic phenotypes from 2,016 knockout mouse strains under the aegis of the International Mouse Phenotyping Consortium (IMPC). This study identified 974 gene knockouts with strong

metabolic phenotypes, out of which 429 genes had no previous link to metabolism and 51 genes remained functionally completely unannotated. We compared human orthologues of these uncharacterized genes in five GWAS consortia and found **23 candidate genes associated with metabolic disease** (*Rozman et al., 2018, Nat Comm*). Currently, we further proceed on analysis of several selected genes from this study.
In addition, we are starting to study a **brown adipose tissue (BAT)** that is a key player regarding the maintenance of body temperature in many mammalian species. BAT has attracted the interest of the scientific community due to its ability to transform excess ingested energy, which is mainly stored as body fat into heat that can be easily dissipated via the body surface. Stimulating BAT thermogenesis could be a way to correct positive energy balance that is typical in more and more sedentary societies with easy access to energy-rich food. For that, it is very important to better understand genetic and physiological functions of BAT thermogenesis. In a R&D project, we are setting up a monitoring system that allows measuring energy expenditure, food & water uptake, physical activity, substrate utilization in combination with dual sensor body temperature telemetry. We have successfully setup a novel in-vivo measurement of metabolic, behavioral, and physiological functions under ad libitum conditions as well as in response to a series of challenges or environmental changes, such as the type of food, room temperature and even the administration of drugs.
Another aspect of metabolism supported by a national grant is the xenobiotic metabolism - in this project we study novel intervention for the treatment of triglyceride, cholesterol, and bile acid diseases with human **CAR ligands that bind Constitutive androstane receptor (CAR)** as xenosensors in regulation of xenobiotic metabolism. Emerging evidence strongly suggests that the receptor also regulates important genes controlling glucose, lipid, cholesterol and bile acids homeostasis.
We are currently testing of CAR-mediated regulation of triglycerides, cholesterol and bile acids metabolism and homeostasis using genetically modified (humanized CAR, Insig1-/-, Abcg8-/-) mice models and primary human hepatocytes employing transcriptomic and metabolomics approaches and with novel CAR ligands.
*Featured publications:*

- *Identification of genetic elements in metabolism by high-throughput mouse phenotyping. Rozman J, et al. Nat Commun. 2018 Jan 18;9(1):288.*

**Ubiquitylation-mediated processes in health and disease; gastrointestinal tract**
Using mutant mouse models we are addressing the role of selected ubiquitin ligases whose biological roles have not been completely deciphered yet. We have begun to work in this field four years ago and the group is currently preparing several manuscripts.

One of our projects focuses on the role of the **Cullin-RING ubiquitin ligase** (CRL) complexes is the ubiquitination of substrate proteins. Altered expression of genes from CRL family has been associated with many different types of invasive cancer diseases. Even though it has been shown that many cancerogenic processes are based on misused developmental molecular programs, the role of CRLs in these processes is largely unknown with exception of the connection between CRL4 and the teratogenic potential of thalidomide during prenatal limb outgrowth via regulation of Fgf8 (Ito et al., 2010). Notably, Fgf8 expressing population is essential also for development of tooth primordia (Prochazka et al., 2015). Using explant tissue culture we discovered that inhibition of CRL complexes severely disrupt embryonic tooth development – the migratory epithelial cells fail to form the tooth primordia. We have identified a new substrate binding protein within CRL4 complex in the developing mandible via coIP followed by mass-spectrometry analysis in the mandibular protein lysates. In order to evaluate its role and also CRL4 as such in odontogenic tissue we have obtained Cop1 flox and Ddb1 flox mouse line. We now breed these lines with appropriate Cre drivers to obtain and phenotype embryos to underline the role of CRL4 in orofacial development in vivo. We also examine the ETS transcription factors, downstream effectors of Fgf pathway, as plausible targets of **CRL4/COP1 complex during odontogenesis**.

Studying the ubiquitine ligase complex **CRL4, namely cul4a**, we found that this adaptor protein resides in Paneth cells and secretory precursors down the crypt base. Its expression colocalizes with recently described ubiquitin ligase **HUWE 1** that plays a role of tumor suppressor. Our data revealed new interactions of CRL4 complex with secretory pathway proteins. Thus, our data suggest that cul4a play an important role in differentiation and maintenance of intestinal epithelia.
Another project focuses on the role of **Cullin3** which is involved in diverse cellular processes, including cell division, differentiation, and cytoskeleton remodelling or stress responses. Cul3 KO embryos exhibit early lethality by embryonic day E7.5. It is known that Cul3 is active as a heterodimer complex consisting of one neddylated and one deneddylated Cul3. Working with specific Cul3 neddylation inhibitor DI-591 disrupting this heterodimer complex we can see that tooth development is impaired. Mandible explants form E12.5 embryos treated with DI-591 show affected epithelial invagination and failed tooth formation. Tooth growth and morphogenesis is regulated by reciprocal interactions between the epithelium and the underlying mesenchyme. To study the role of Cul3 in these processes we will inactivate them in different tissues, either in neural crest derived cells and tissues by Wnt1 Cre driver, or by Fgf8 Cre driver to conditionally knock down Cul3 in migrating epithelial cells themselves.
*RNF121 project*
The RNF121 (Really interesting New gene Finger 121) gene encodes a highly conserved and ubiquitous E3 ubiquitin ligase, however, its target substrates remain largely unknown. Genome-wide association studies identified RNF121 as playing a role in cardiovascular events. To gain insights into its biological function in mammalian cells, we have generated Rnf121 knock out mice. All, the constitutive, vascular cell- and cardiomyocyte-specific ablation of Rnf121 resulted in embryonic lethality at mid-gestation (E10.5) showing internal hemorrhages, often accompanied with pericardial effusions, suggesting insufficient heart function or other blood circulation defects. In addition, high-fat diet-fed mice with reduced Rnf121 expression displayed a significantly increased body weight, plasma levels of cardiac inflammatory markers and a mild hypercholesterolemia, which are important underlying factors associated with vascular dysfunction. Altogether, our findings indicate that RNF121 has a critical role in regulation of cardiovascular homeostasis and understanding the molecular mechanism behind its function is therefore crucial.

**Mouse models of human diseases & large-scale pheno-genomics**
Using our capacity for the production of genetically modified mouse models in CCP, we have started to generate and study mouse models for Mendelian disorders and models for rare diseases. Among these disease, models for *Diamond-Blackfan anemia (DBA), Netherton Syndrome,* and also *Angelman syndrome (AS) and Prader-Willi syndrome (PWS)* project have been created and the topics studied.
The AS and PWS project is focused on deciphering molecular mechanisms of imprinting in Angelman and Prader-Willi locus in mouse models. Angelman (AS) and Prader-Willi syndomes (PWS) are caused by chromosomal microdeletion on chromosome 15 in human and chromosome 7 in mouse. Both syndromes have the same cause, uniparental disomy or chromosomal microdeletion. However, symptoms of each syndrome differ due to genomic imprinting, leading to silencing of a specific gene cluster from a single parental allele. Although AS and PWS patients remain with intact healthy allele, they lack expression from the allele due to silencing of genes in AS/PWS locus by imprinting. We sought to generate and thoroughly describe AS/PWS mouse models and eventually develop a gene therapy based on CRISPRa and CRISPRi technology delivered by AAV vectors.
The Netherton syndrome is mentioned in the section of proteases (above).
Currently, we are preparing several manuscripts that expand the findings in the Netherton syndrome, DBA and study the restoring gene functions AS NA PWS.
Regarding the '*large-scale pheno-genomics', we are continuously participating in generation of data originating from mutant mice generated in CCP and are thus co-authors of several high-impact publications – featured articles:*

- *The Deep Genome Project. Lloyd KCK, et al., Genome Biol. 2020 Feb 3;21(1):18.*
- *Human and mouse essentiality screens as a resource for disease gene discovery. Cacheiro P, et al., Genomics England Research Consortium; International Mouse Phenotyping Consortium. Nat Commun. 2020 Jan 31;11(1):655.*
- *High-throughput discovery of genetic determinants of circadian misalignment. Zhang T, et al. PLoS Genet. 2020 Jan 13;16(1):e1008577.*

**Gastrointestinal projects and Immunology topics**

Immunology topics were partially studied by the group of M. Reinis (11) and partially in cooperation with the group of Dominik Filipp, where we generated mostly the mouse models and participated in their characterization.

Here we showed the role of MMP-19 in inflammatory bowel disease (IBD) pathology. Using a DSS-induced model of colitis, we identified chemokine fractalkine (CX3CL1) as a novel substrate of MMP-19, suggesting a link between insufficient processing of CX3CL1 and inflammatory cell recruitment (Brauer et al., 2016, Mucosal Immunol).

Milan Reinis group studied dendritic cell (DC)-based vaccines and the role of immune cell subpopulations in the growth (Mikyskova at al., 2016) and rejection of TC-1/A9 tumors in a mouse strains differing in the H2-D haplotype and NKC domain (Indrova eta la 2018).

In cooperation with the group of Dominik Filipp, we participated in several projects by generation of the mouse models and their characterization *- featured article:*

- *Toll-like receptor 2 expression on c-kit+ cells tracks the emergence of embryonic definitive hematopoietic progenitors. Balounová J, Šplíchalová I, Dobešová M, Kolář M, Fišer K, Procházka J, Sedlacek R, Jurisicova A, Sung HK, Kořínek V, Alberich-Jorda M, Godin I, Filipp D. Nat Commun. 2019 Nov 15;10(1):5176.*

**Other projects: physiology and transcription factors**

There are several new projects that address new research topics, particularly new molecules that we have not studied before. However, also these new molecules are examined in the context of research fields such physiology, health and disease that we are studying - for instance:

*ZNF644.* ZNF644 is a C2H2 zinc finger gene encoding a putative transcription regulator, of which a point mutation (S672G) is associated with inherited high myopia in humans. We created several different mouse models, from which one mimics the human mutation and other to study its impact on myopia (Szczerkowska et al., 2019) and the biological meaning. Mouse with truncated form of the protein not only mimics human myopia disease phenotype but also shows sever symptoms in metabolism and female fertility. To investigate the role of Zfp644 in mouse metabolism, we perform essential examinations such as full body scanning, including: bone mineral density, tissue mineral density, lean mass and fat percentage in organism; weight measurements and calorimetry. All experiments were repeated multiple times in a period of 30 weeks. Results of this examinations suggest higher basal metabolic rate and oxygen consumption in Zfp644Δ8 males. Moreover, to reveal the impact of Zfp644 in mouse fertility we performed various experiments including expression of Zfp644 in reproductive organs, breeding performance, ovarian morphometry, estrus cycle and mammary gland growth during and after pregnancy as well as in nulliparous females. Moreover, a transplantation of ovaries from homozygous to control animal; and vice-versa was performed. We rescued homozygous ovaries transplanted to control animals. Our most recent data on influence of Zfp644 on a mouse organism show complexity of this transcription factor.

*Atf2.* **The Activating Transcription Factor 2 (ATF2)** belongs to the family of bZIP transcription factors and is described to be involved in various processes, such as cancer, inflammation, and metabolism. However, these functions were studied only in adult organisms. To decipher its role during embryonic development, we created Atf2-knockout mice. Knockout embryos show improper intestinal looping. In order to better describe the morphogenesis and to understand how such phenotype develops, we followed the looping process in various

developmental stages. We have shown that both mesentery and intestine are involved in aberrant looping morphogenesis in Atf2-knockout mice; moreover, the intestinal epithelium is affected as well. The transcription analysis we have performed revealed that the looping morphogenesis is regulated through the crosstalk between BMP and PCP signaling in an Atf2-dependent manner.

**Development of research tools**

This area interlinks CCP and the other research teams. Besides the service and supporting activities, which include consultation and education, CCP strives to develop and establish new technologies, methods and standards in its portfolio. This is essential, as we have to respond to the growing demands of our research partners and react to the latest technological developments and new cutting-edge technologies. In the past several years, we have intensively developed new technologies, especially those which include 'programmable nucleases' and phenotyping technologies to reveal new physiologic traits. Regarding the genome-editing using CRISPR/Cas systems and exceptional large production of mutant rodents in CCP, here are publications showing our development in this area- *featured articles:*

- *Reproducibility of CRISPR-Cas9 methods for generation of conditional mouse alleles: a multi-center evaluation. Gurumurthy CB, et al., Genome Biol. 2019 Aug 26;20(1):171.*
- *CRISPR/Cas9 Epigenome Editing Potential for Rare Imprinting Diseases: A Review. Syding LA, Nickl P, Kasparek P, Sedlacek R. Cells. 2020 Apr 16;9(4):993.*

# Research activity and characterization of the main scientific results

## Introduction

The Laboratory of Cell and Developmental Biology was established in January 2007 with the prospective scientific goal to elucidate the signaling mechanisms leading to cellular transformation and cancer. Two important events related to the scientific topic and laboratory personal occurred in the evaluated period. At the end of 2014, the group was strengthened by the arrival of Lucie Láníková, who after a long stay in the United States relocated to Prague. Lucie, who received funding from her own grants, brought the theme of the molecular mechanisms of erythropoiesis and the genetic bases of hematopoietic diseases. Moreover, in 2018, Tomáš Valenta, the first PhD graduate of the Laboratory, returned to the team after more than 12 years of postdoctoral stay. Tomáš also brought a new research topic focused on the cellular composition of the tissue microenvironment constituting the stem cell niche. Importantly, the topic complements well the already established research directions of the Laboratory. In the following paragraphs we present some of the results documenting our research.

We would like to mention our relationship to the BIOCEV project. This project was funded by the European Union Structural Funds and the Institute was one of the main beneficiaries of financial support. The support was used to build a complex of buildings in Vestec, a small town about 10 km from the campus of research institutes of the Academy of Sciences of the Czech Republic in Prague 4, where the main building of the Institute is located. Currently, BIOCEV hosts more than 600 researches from seven research institutions, including our Institute. Our Laboratory participated in the project and some of its employees worked in the newly established laboratories in Vestec. This part was transformed in 2016 into the Laboratory of Cancer Biology led by L. Čermák. The scientific theme of this laboratory is focused on ubiquitin ligases and their substrates involved in cancer progression, stress response, and cell cycle regulation. For "historical" reasons, the researchers of the laboratory at BIOCEV were assigned to our department. However, as the newly created laboratory has not yet had a significant publication output, we do not mention the results associated with the Laboratory of Cancer Biology in the summary below.

## The role of Wnt pathway components in healthy adult GI tissues and GI cancer

The involvement of Wnt signaling in self-renewal, regeneration and transformation of the GI epithelia make this tissue a convenient cellular system to search for novel Wnt signaling pathway components that could include epithelial stem cell markers. Indeed, in our previous study we identified the tumor necrosis factor receptor superfamily, member 19 (TNFRSF19, alternative name TROY) as a new marker of IESCs (1). In 2015, in collaboration with Tim Wang and his team from the Columbia University Medical Center (New York, US) we investigated the Troy function in the gastric epithelium. Interestingly, in contrast to the intestine, the stem cell origin of normal and neoplastic gastric glands and factors that sustain gastric stem cells are uncertain. We showed that Troy-negative stem cells in the gastric corpus and antral glands might serve as the cell-of-origin for gastric cancer. The described results were included into an article published in the prestigious Cancer Cell journal (2).

To identify genes regulated by the Wnt signaling pathway, we performed chromatin immunoprecipitation (ChIP)-on-chip assay using chromatin isolated from cultured colorectal cancer-derived cells using antibodies specific for the nuclear Wnt pathway effectors □-catenin and DNA-binding proteins of the lymphoid enhancer-binding factor/T-cell factor (LEF/TCF) family. One of the most prominent targets of the LEF/TCF/□-catenin complexes was the promoter region of the naked cuticle homolog 1 (*Nkd1*) gene. The activity of the Wnt pathway undergoes complex regulation to ensure proper functioning of this signaling mechanism during development and in adult tissues. The regulation may occur at several levels and includes both positive and negative feedback loops, and the *Nkd1* gene encodes one of the cytoplasmic negative regulators of Wnt signaling. We generated Nkd1-CreERT2 transgenic mice

expressing tamoxifen-inducible Cre enzyme inserted in frame at the translation initiation codon of the *Nkd1* gene. Subsequently, we performed lineage tracing in the intestinal epithelium of Nkd1-CreERT2/Rosa26R mice. Moreover, we employed Nkd1-CreERT2/Rosa26R mice to sort Nkd1-positive hepatocytes and perform their expression profiling. The assays showed that Nkd1 marks all cells located at the bottom part of the crypts [(i.e., stem and Paneth cells and rapidly dividing progenitors (transit amplifying cells)]. In addition, Nkd1 represents a robust marker of the perivenous hepatic regions. To gain more insight into the role of NKD1 in human neoplasia, in collaboration with colleagues from the Laboratory of Genomics and Bioinformatics [Institute of Molecular Genetics (IMG)] we performed expression and mutational analysis of colorectal and hepatocellular neoplasia. The analysis included our “experimental” collection of cancer specimens and, in addition, bioinformatic evaluation of large datasets retrieved from public resources. Importantly, high NKD1 expression levels clearly distinguished a class of tumors with deregulated Wnt signaling (3). The study not only brought interesting results, but also documented our ability to combine different experimental models and approaches that included genetically modified mice, human cancer specimens, and “in silico” analysis of publicly available datasets. Analogous combination of bioinformatic analysis of the datasets obtained from GTEx, Fantom5, and The Cancer Genome Atlas (TCGA) with RNA sequencing analysis of human colorectal cancer specimens was employed to analyze the expression patterns and isoform production of the TCF/LEF factors. Such detailed analysis of these important Wnt pathway effectors was published for the first time (4).

Subsequently, we aimed to identify and characterize the genetic program related to cell transformation induced by the loss of adenomatous polyposis coli (*Apc*) tumor suppressor gene in the mouse. The gene encodes a negative regulator of the Wnt pathway, and numerous results that include tumor cell genome sequencing clearly demonstrate that Apc inactivation is a dominant molecular mechanism initiating intestinal cancer. For the experimental design, we used a conditional *Apc* allele and expression profiling of mouse intestinal epithelial cells obtained from the intestine before and after *Apc* gene inactivation, i.e., upon Wnt pathway hyperactivation. We identified msh homeobox 1 (*Msx1*) as a gene prominently upregulated in Apc-deficient tissue. Msx1 (also known as Hox7) belongs to the muscle segment homeobox (msh) family that includes one of the most evolutionarily conserved homeobox transcription factors found in animals. Msx1 may act as a transcriptional activator and/or repressor and its function depends on the cellular context. In the mouse, the *Msx1* gene was studied especially in the development of the teeth, brain, and limbs. Nevertheless, its role in the intestine or intestinal cancer has not yet been described. In relation to our expertise we defined and characterized the functional significance of the *Msx1* gene in the mouse intestine, mouse intestinal tumors and human cancer cells. Interestingly, in transformed epithelium, the Msx1 protein was localized exclusively in ectopic crypts, i.e., in pockets of proliferating cells abnormally positioned on the villi. Moreover, tumors arising from Msx1-deficient cells displayed altered morphology reminding a villous type of adenomas. In colorectal cancer, such morphological conversion indicates tumor progression associated with elevated risk of malignancy. In summary, our work shows the previously unknown function of Msx1 in the intestinal tumorigenesis (5); the entire study was performed in our Laboratory.

In addition, we performed functional analysis of the Tcf4 transcription factor. The factor represents the key nuclear effector of canonical Wnt signaling in intestinal cells. During the course of the project, we analyzed the effect(s) of Tcf4 loss on homeostatic self-renewal and tumorigenesis of the gut tissue. In contrast to studies published previously, we used a newly generated conditional *Tcf4* allele that allows inactivation of all potential Tcf4 isoforms. Our results showed the *Tcf4* gene is not essential for embryonic intestinal development. However, Tcf4 ablation impaired adult intestinal homeostasis and suppressed tumor formation not only in the small intestine, but also in the colon (6).

In the small intestine, epithelial Paneth cells secrete Wnt ligands and thus potentially form the stem cell niche that normally could maintain the epithelial homeostasis, as apparent during the growth of small intestinal organoids that do not require external Wnt ligands in the

culture medium. Organoids are long-term intestinal cultures that fully resemble the intestinal architecture. These “mini-guts” are grown in a three-dimensional gel-like matrix (Matrigel) that simulates the native embedding of the intestinal glands. On the other hand, in the colon the identity of cells comprising the stem cell niche is unknown. In collaboration with the laboratory of K. Basler (Institute of Molecular Life Sciences, University of Zurich, Switzerland) we demonstrated that sub-epithelial mesenchymal cells expressing transcription factor Gli1 form the “enigmatic” niche for IESCs in the colon. Blocking Wnt secretion from Gli1-expressing cells abrogates colonic stem cell renewal; the stem cells are lost and as a consequence, the integrity of the colonic epithelium is corrupted, leading to lethality. External delivery of Wnt could delay and reduce the onset of the phenotype. Additionally, Gli1-expressing cells also play an important role for the maintenance of the small intestine, where they serve as a reserve Wnt source that becomes critical when Wnt secretion from Paneth cells is affected. In addition, single-cell RNA sequencing and immunohistocytochemical analyses revealed that Gli1-expressing cells represent a heterogenous cell population consisting of myofibroblasts and lipofibroblasts, some of them secreting Wnt ligands. Importantly, $Gli1^+$ cells are enriched during tissue regeneration upon intestinal damage. The study was published in Nature (7); Tomas Valenta, a member of the authors’ team, initiated and conceived the study, designed the experiments, analyzed the data, and co-wrote the manuscript. His contribution was “validated” by the first co-authorship and shared corresponding authorship.

Gut microbiota is a complex ecosystem consisting of a vast number of bacteria, viruses, and fungi that have not been fully characterized. Numerous host-microbe interactions shape the microbial community and influence the host's physiology, development of the immune system, and provoke various pathological conditions. Many multifactorial disorders occur in consequence of the disturbed mucosal barrier function or as a result of alterations in the immune response to the gut microbiota components. Moreover, imbalance in the gut microbiota composition, so-called dysbiosis, is frequently associated with intestinal cancer. In collaboration with the Laboratory of Cellular and Molecular Immunology (Institute of Microbiology of the Czech Academy of Sciences) we determined the gene expression signature of mucosa isolated from the small intestine and colon of germ-free (GF) mice and animals monoassociated with two *E. coli* strains. We also analyzed gene expression in colon organoids derived from conventionally reared, GF, and monoassociated mice. The study supported the importance of complex commensal microbiota for proper development of the mucosal immune system and metabolic functions. Additionally, a unique expression signature typical of the GF mouse-derived organoids was identified (8). Such comprehensive analysis of the intestinal mucosa and organoids derived from the monoassociated mice has not been published yet.

**New genetically-modified alleles to study Wnt signaling in mouse**

To analyze the role of the Wnt pathway in the intestine, we generated a novel mouse strain expressing a truncated TCF4 protein from the *Rosa26* locus (the strain was designated Rosa26-dnTCF4). Rosa26-dnTCF4 mice produce dominant negative TCF4 (dnTCF4) that binds the regulatory regions in Wnt-responsive genes. However, the protein is N-terminally truncated, and thus cannot bind beta-catenin and functions as a nuclear blocker of canonical Wnt signaling. In addition, dnTCF4 production is triggered only in cells expressing an active form of the Cre recombinase. This approach is fundamentally different from the methods published previously, since usage of dnTCF4 precludes any interference with additional Tcf-independent functions of □-catenin. Moreover, targeting of the heterologous *Rosa26* locus retains genes encoding the Wnt pathway components intact. The functional properties of the novel mouse strains were tested using transgenic mouse strains producing Cre in various cell lineages present in the intestinal epithelium. Intestinal stem cells expressing the transgene lost their “stemness” and did not contribute to tissue homeostasis (9).

The mice producing dnTCF4 were employed in several collaborative studies: (1) In collaboration with the Laboratory of Transcriptional Regulation (IMG) we tested the effect(s) of Wnt pathway (hyper)activation and inhibition on neural crest cell development. We showed

that suppression of the Wnt pathway is essential for the maintenance of neuroepithelial identity in the anterior neural fold. In contrast, the pathway hyperactivation in anterior neuroectoderm cells leads to their conversion into neural crest cells. Such conversion might be blocked by production of dnTCF4 in neuronal precursor cells. The study including these results was published in Development (10). (2) The canonical Wnt signaling pathway plays an important role in the establishment of neurogenic niches. It was well established that elevated Wnt/β-catenin signaling promotes differentiation of neural stem/progenitor cells (NS/PCs) towards neuroblasts. Nevertheless, it remained unclear how the differentiation program of neural progenitors is influenced by the Wnt signaling output. In collaboration with the Department of Cellular Neurophysiology (Institute of Experimental Medicine of the Czech Academy of Sciences) using transgenic mouse models including dnTCF4 mice, we identified three electrophysiologically and immunocytochemically distinct cell types whose incidence was markedly affected by Wnt signaling. In more detail, activation of the pathway suppressed gliogenesis, and promoted differentiation of NS/PCs towards a neuronal phenotype, while its inhibition led to suppressed neurogenesis and increased counts of cells of the glial phenotype. Moreover, Wnt signaling hyperactivation resulted in an increased incidence of immature neurons, while blocking the pathway led to the opposite effect. Taken together, our data indicate that the Wnt signaling pathway controls neonatal NS/PC differentiation towards cells with neuronal characteristics, which might be important for nervous tissue regeneration during central nervous system disorders (11). (3) The dnTCF4 mice were also employed to dissect the role of canonical Wnt signaling in the hematopoietic system. In collaboration with the Laboratory of Haematooncology we were the first to show that Wnt/□-catenin signaling in hematopoietic stem and progenitor cells promotes their granulocytic differentiation. Interestingly, we showed that this signaling is dispensable for the development of leukemia. In summary, our results provide novel insights into the biology of hematopoietic stem cells and additionally, they suggest that disruption of the □-catenin-TCF/LEF interaction is not a suitable strategy for leukemia treatment. The results were included in an article (Danek et al.: The □-catenin-TCF/LEF transcription-mediating complex suppresses hematopoietic stem cell self-renewal and is dispensable for leukemia development) that is in revision in the prestigious Blood journal.

Many studies have documented that in the intestinal epithelium, the canonical Wnt pathway regulates proliferation and pluripotency of IESCs. In IESCs the Wnt signaling output is potentiated by interactions of leucine-rich repeat-containing G-protein-coupled receptor 5 (LGR5) with secreted proteins R-spondins (RSPOs). Whereas LGR5 is specifically produced in IESCs, its paralog, LGR4, is broadly expressed in the proliferative compartments of the small intestinal and colonic epithelium. To study the Lgr4-dependent function(s) we generated a new mouse strain expressing a tagged variant of the Lgr4 protein. The Lgr4 locus was modified by homologous recombination in the zygote using the transcription activator-like effector nucleases (TALENs)-based technology and exogenous DNA template. The resulting allele (designated Lgr4-3HA) produces the Lgr4 protein fused with a 3HA tag at its N-terminus. The 3HA tag was placed immediately downstream of the Lgr4 sequence encoding the signal peptide of the protein. The allele is fully functional, enabling easy tracking of Lgr4 expression in adult mouse tissues. Furthermore, since the tag is localized extracellularly, expressed on the cell surface, it allows direct isolation (and analysis) of living Lgr4-positive cells obtained from the mouse organs. Additionally, the tagged protein was employed to identify Lgr4 interacting proteins using mass spectrometry (V. Kriz, in preparation).

**Hypermethylated in cancer 1 (HIC1) and its role in intestinal carcinogenesis and development**

In 2006, we identified HIC1 as an antagonist of Wnt signaling in mammalian cells (12). HIC1 represents a prototypic tumor suppressor gene frequently inactivated by DNA methylation in many types of solid tumors. The gene encodes a sequence-specific

transcriptional repressor involved in the cell cycle or stress control. In the mouse, Hic1 is essential for embryonic development and exerts an anti-tumor role in adult animals. Since Hic1-deficient mice die perinatally, to study the Hic1 function in adult tissues we generated a conditional Hic1 null allele by flanking the Hic1 coding region by loxP sites (13). The conditional knockout mice were used to study the function of Hic1 tumor suppressor in the intestine. Additionally, a gene inactivation-based screen was employed to identify genes regulated by Hic1. Interestingly, one of the genes upregulated upon Cre-mediated ablation of Hic1 encoded the toll-like receptor 2 (Tlr2) protein. Tlr2 functions as a microbial sensor initiating inflammatory and immune responses. We showed that Hic1 depletion in the intestinal epithelium resulted in increased Tlr2 expression and, in addition, Hic1 deficiency promoted NF-kappa B pathway signaling. Interestingly, in the chemical carcinogenesis model, larger and more proliferative colonic tumors develop in Hic1 mutant mice when compared to wild-type (wt) animals. In summary, our results indicated that the tumor-suppressive function of Hic1 might be related to its inhibitory action on pro-proliferative signals mediated by Tlr2 present on tumor cells (14).

Additionally, we used gene targeting to replace the sequence encoding Hic1 by citrine fluorescent protein cDNA. We demonstrated that the distribution of the Hic1-citrine fusion polypeptide corresponds to the expression pattern of wild-type Hic1 (13). The Hic1-citrine reporter mice were employed in several collaborative studies with T. M. Underhill, C. Zaph, and F. M. V. Rossi (all from the Biomedical Research Centre, University of British Columbia, Vancouver, British Columbia, Canada). The studies showed that HIC1 represents a central component of the intestinal immune system (15, 16). Very recently, we participated in the study indicating that Hic1 marks a population of cardiac stromal cells that are involved in pathological post-myocardial infarction remodeling of heart tissue (17).

**Important factors for initiation and progression of myeloproliferative disorders**

MPN are genetically very complex and heterogeneous diseases in which the acquisition of a somatic driver mutation triggers three main myeloid cytokine receptors, and phenotypically expresses as polycythemia vera (PV), essential thrombocytosis (ET), and primary myelofibrosis (PMF). The most frequently occurring gain-of-function JAK2 V617F mutation gives rise to a constitutively active JAK2 kinase, which drives the JAK/STAT signaling that leads to excessive proliferation and survival of myeloid progenitor cells and accounts for >95% of driver mutations in PV and >55% in ET and PMF. The course of the diseases may be influenced by germline predispositions, modifying mutations, their order of acquisition, and environmental factors such as aging and inflammation. Deciphering these contributory elements and their mutual interrelationships allows us to better understand the MPN pathogenesis.

A seminal study of our collaborator J.T. Prchal (University of Utah, Salt Lake City, Utah, US) analyzing a mutational “landscape” of MPN unraveled coexistence of gain-of-function JAK2 germline mutations with JAK2 V617F in PV (18). Our subsequent study, published in prestigious medical journal Blood, documented that some JAK2 germline mutations may represent a mechanism that possibly precedes acquisition of the somatic JAK2 V617F mutation. Moreover, during the PV evolution, these germline mutations may contribute to leukemic transformation of PV cell clones (19). In addition, we unraveled that oncogenic signaling of JAK2 is enhanced by the R1063H mutation and promotes neutrophilia in patients with MPN. It was also shown that germline or acquired JAK2 R1063H mutation enhances the signaling of JAK2 V617F in *cis* via coupling to dimeric myeloid cytokine receptors (20). The clinical data analysis of 390 patients in the last study was done in several hospital centers in Belgium (Brussels) and Romania (Bucharest), but the majority of molecular analyses were done in Brussels by C. Mambet and at IMG by O. Babosova (both contributed equally as first authors). Moreover, we conducted a mechanistic study how iron chelation and dioxygenase inhibition suppress cyclin D1 expression and how patients with mantle cell lymphoma (MCL)

can benefit from such treatment. Based on our data, the use of iron chelation in MCL treatment is now being considered for pre-clinical trial (21).

In collaboration with international partners, we contributed to studies that analyzed the new rearrangement of the β-globin gene cluster in individuals showing sickle cell disease phenotype (22) and association between protection against augmented erythropoiesis at high altitude and cancer (23). We also participated in a study (the majority of the results were generated at the Department of Biology, Faculty of Medicine and Dentistry, Palacky University Olomouc, Czech Republic headed by V. Divoky) showing the role of DNA damage response (DDR) in suppressing malignant progression in chronic MPN. We proposed a new concept of protective mechanisms that possibly guard myeloproliferative progenitors from cell-intrinsic and cell-extrinsic DNA damage (24).

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## Research activity and characterisation of the main scientific results

In 2015 - 2020, we focused on detailed molecular genetic analysis of the role of *Prdm9* and *Hstx2* in meiotic recombination realized as crossing-overs (COs) and noncrossovers (NCOs or gene conversions). In parallel with the results from the laboratories of Prof. Simon Myers and Peter Donnelly from University of Oxford and Camerini-Otero from NIH, Bethesda, USA, who adopted our model of mouse hybrid sterility (Davies et al. Nature 2016, Smagulova et al. Genes Dev. 2016) we tested our hypothesis (Bhattacharyya et al. PNAS 2013) on evolutionary divergence of noncoding sequence in genomes of closely related species as a cause of defective meiotic recombination and chromosome synapsis leading to hybrid sterility. Finally, in collaboration with Attila Toth from Technische Universität Dresden we contributed to identification of ANKRD31, a new DSB-promoting protein that assembles on meiotic axis.

1. Meiotic recombination between *domesticus* and *musculus* chromosomes

We studied genetic control of meiotic recombination rate in two mouse strains and chromosome substitution strains derived from them (**Balcova et al. 2016**). To assess an impact of introgressed *musculus* chromosomal intervals within the *domesticus* genome on recombination rate we quantified the foci of the class I-specific crossover component MLH1 (Mut L homolog 1, DNA mismatch repair protein) by immunofluorescence microscopy in pachytene spermatocytes of the chromosome substitution strains.We identified two autosomal and one X-linked locus which significantly modify the meiotic recombination rate. The strongest modifier, designated meiotic recombination 1, *Meir1,* was localized in a 2.7 Mb interval in chromosome X (**Lustyk et al. 2019**). A still unknown gene in the *Meir1* locus, which controls

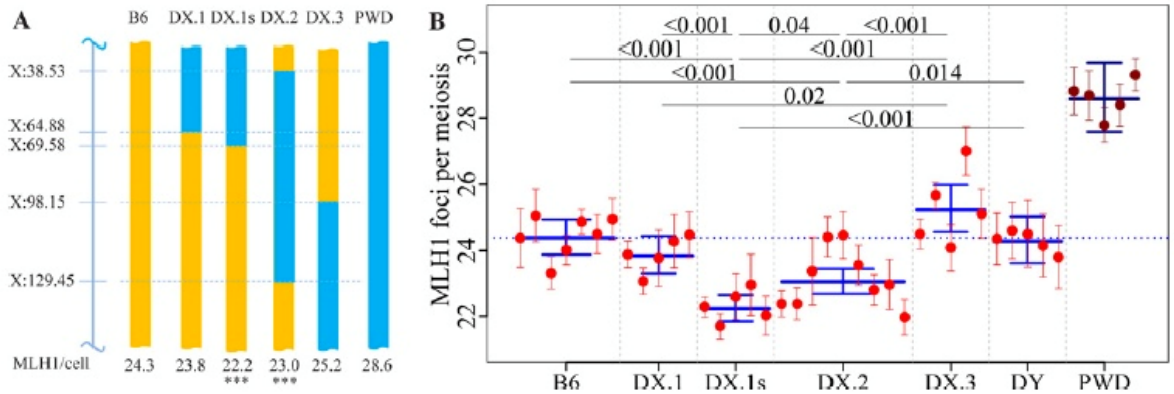



*Figure 1. Fine mapping of X-linked Meiotic recombination rate 1 (Meir1). From Balcova et al. 2016*

meiotic recombination rate is closely linked to or identical with a gene responsible for hybrid sterility within overlapping Hybrid sterility X2 locus, *Hstx2*. These findings link meiotic recombination controlled by *Prdm9* gene to hybrid sterility modified by the *Meir/Hstx2.* This work was featured in a *Perspective* by Bret Payseur (Payseur, *Genetic Links between Recombination and Speciation,* Plos Genet. 12(6): e1006066).

Using the same panel of chromosome substitution strains, previously constructed in our laboratory (**Gregorova et al. Genome. Res. 2008 18: 509-515**), we were able to identify noncrossover (gene conversion) products of meiotic DNA DSB repair **Gergelits et al. 2020**). While crossovers are easily detectable, noncrossover identification is hampered by the small size of their converted tracts and the necessity of sequence polymorphism to detect them. We identified and characterized a mouse chromosome-wide set of 94 noncrossovers by NGS sequencing of 10 mouse intersubspecific chromosome substitution strains. We determined the mean length of a conversion tract to 32 basepairs. The spatial chromosome-wide distribution of noncrossovers and crossovers significantly differed, though both sets overlapped the known H3K4me3 hotspots of PRDM9-directed histone methylation and DNA

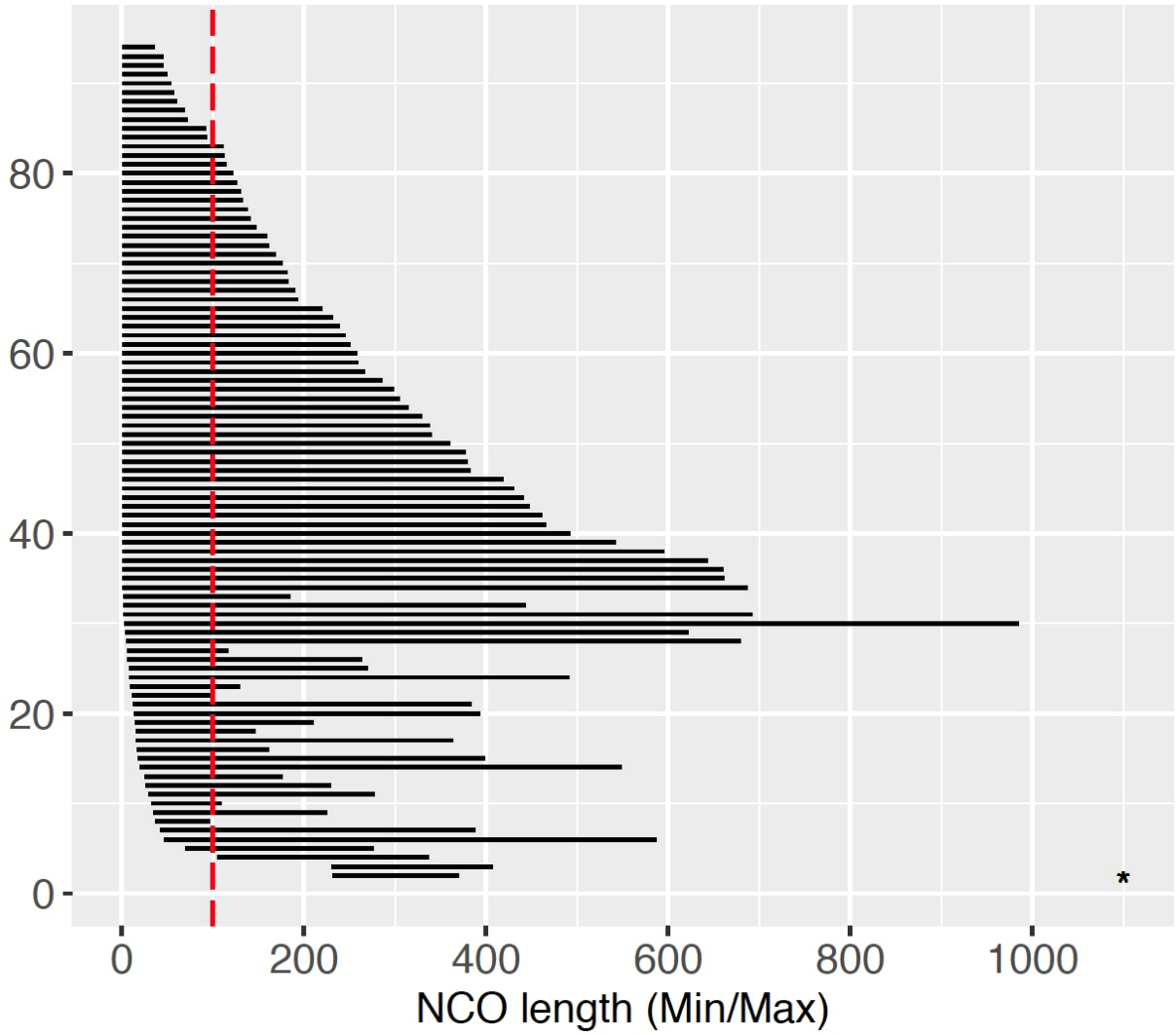



*Figure 2. Distribution of minimum and maximum length (bp) of 93 identified noncrossovers (from Gergeltis, Biorxiv 2019).*

DSBs, thus proving their origin in the standard DSB repair pathway. A significant deficit of noncrossovers descending from asymmetric DSBs proved their proposed adverse effect on meiotic recombination and pointed to sister chromatids as an alternative template for their repair. The finding has implications for the molecular mechanism of hybrid sterility in mice from crosses between closely related *Mus musculus musculus* and *Mus musculus domesticus* subspecies.

2. PRDM9 involvement in hybrid sterilty and in meiotic chromosome synapsis

In 2009 our laboratory identified *Prdm9* as the first hybrid sterility gene in vertebrates (**Mihola et al. Science 2009**). In February 2016 the group of Simon Myers published a paper (Davies et al. Nature, 2016, see **Forejt, Nature 2016** for Comment) showing that the $Cys_2His_2$ zinc-finger array representing the DNA-binding domain of mouse PRDM9 protein is decisive for hybrid sterility and demonstrated that an allelic exchange of mouse *Prdm9* DNA-binding domain for human allelic form introduced “symmetric DSBs” and thereby rescued fertility of male hybrids. The authors used our model of hybrid sterility based on the crosses of the PWD inbred strain of *musculus* origin (derived from wild mice in our laboratory, see **Gregorova and Forejt, Folia Biol. 2000**) with the laboratory mice of the B6/J strain of *domesticus* origin. Their finding put forward a possible molecular mechanism of failed pairing and synapsis of homologous chromosomes in meiosis of sterile hybrids, a phenomenon we uncovered earlier

(**Bhattacharyya et al., PNAS 2013**). To verify the idea and to investigate the relation between *Prdm9*-controlled meiotic arrest and asynapsis, we inserted random stretches of consubspecific (belonging to the same subspecies) homology on several autosomal pairs in sterile hybrids, and analyzed their ability to form synaptonemal complexes and to rescue male fertility (**Gregorova, Gergelits et al., eLife 2018**). Twenty-seven or more megabases of consubspecific homology fully restored synapsis in a given autosomal pair, and we predicted that two or more DSBs within symmetric hotspots per chromosome are necessary for successful meiosis.

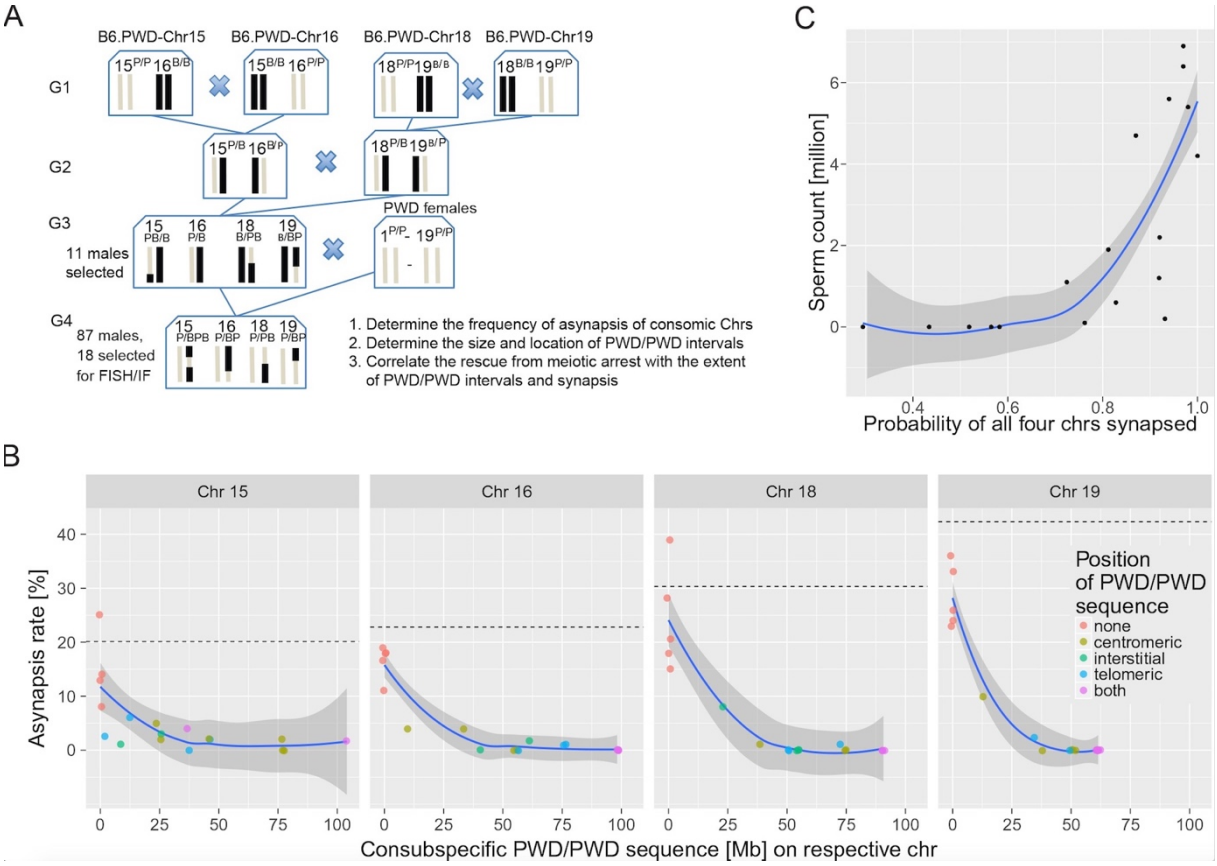



*Figure 3. The effect of consubspecific PWD/PWD stretches of genomic sequence on pachytene synapsis and meiotic progression, 4-chr cross (Gregorova, eLife 2018).*

We hypothesized that lack of symmetric DSBs and resulting impaired recombination between evolutionarily diverged chromosomes could function as one of the mechanisms of hybrid sterility occurring in various sexually reproducing species. To further test this idea we tried to increase the total number of DSBs at leptotene stage of the first meiotic prophase by introducing exogenous DNA breaks by a chemotherapeutic drug cisplatin (**Wang et al. eLIfe 2018**), known to increase meiotic recombination in mice. Indeed, a single injection of cisplatin increased frequency of DSBs determined by counting RPA and DMC1 foci at the zygotene stage of sterile hybrids, enhanced homolog recognition and increased the proportion of spermatocytes with fully synapsed homologs at pachytene.

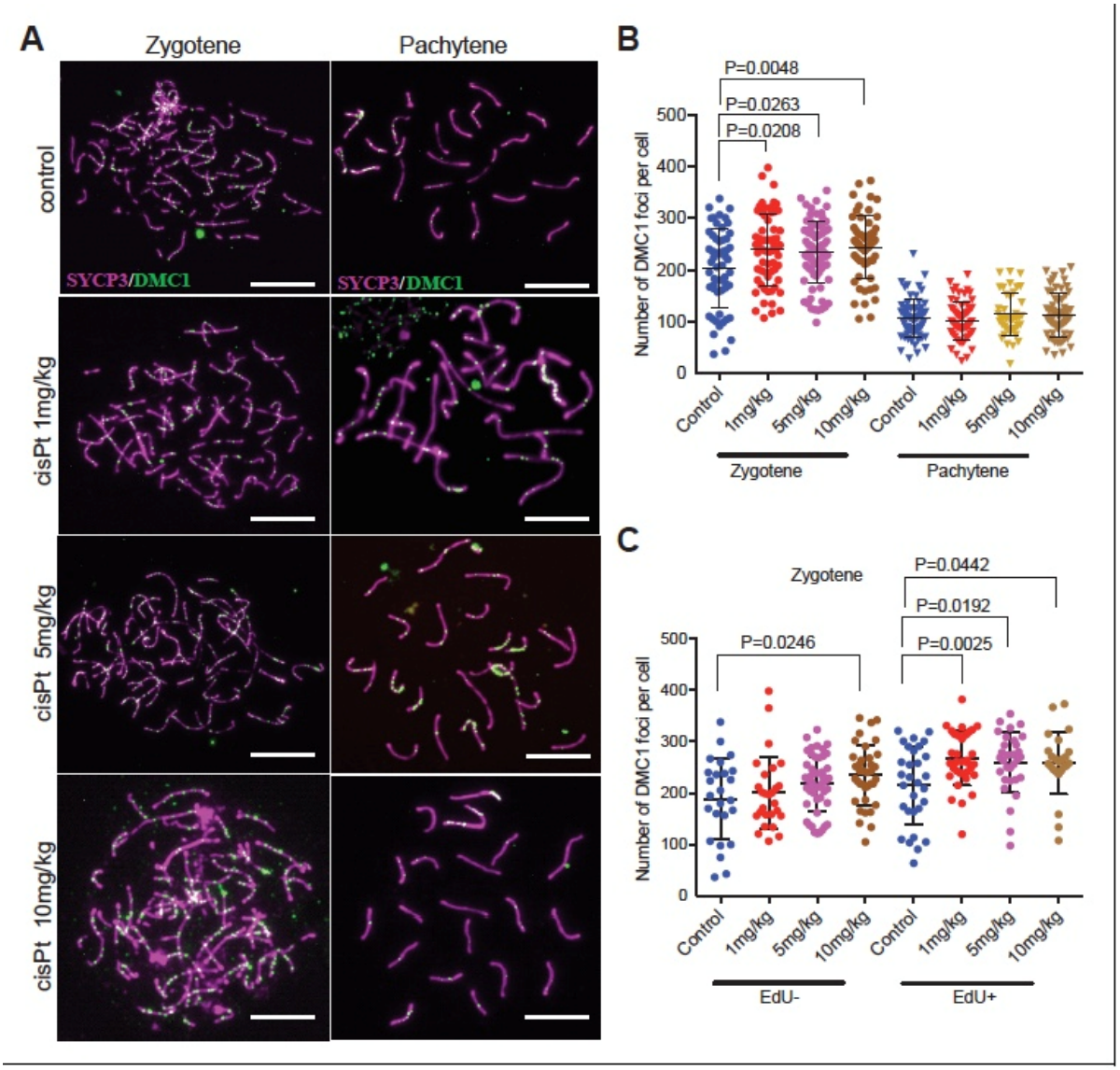



*Figure 4. CisPt increases the frequency of exogenous DSBs monitored as DMC1 foci (Wang et al. eLife 2018).*

The results bring a new evidence for a DSB-dependent mechanism of synapsis failure and infertility of intersubspecific hybrids.

3. *Prdm9* controls fertility in hybrids from natural populations of *Mus musculus*

Until recently, the unique function of PRDM9 protein in fertility of mouse intersubspecific hybrids has been studied, almost exclusively, using two mouse strains, PWD/Ph of *Mus m. musculus* origin and B6/J predominantly of *Mus m. domesticus* origin. Thus, the role of this protein as a component of reproductive barrier outside the laboratory model remained unclear. To get the first insight, we studied fertility in inter-subspecific hybrids between *Mus m. musculus* and *Mus m. domesticus* including 16 *musculus* and *domesticus* wild-derived strains. Disruption of fertility phenotypes correlated with the rate of failure of synapsis between homologous chromosomes in meiosis I and with early meiotic arrest.

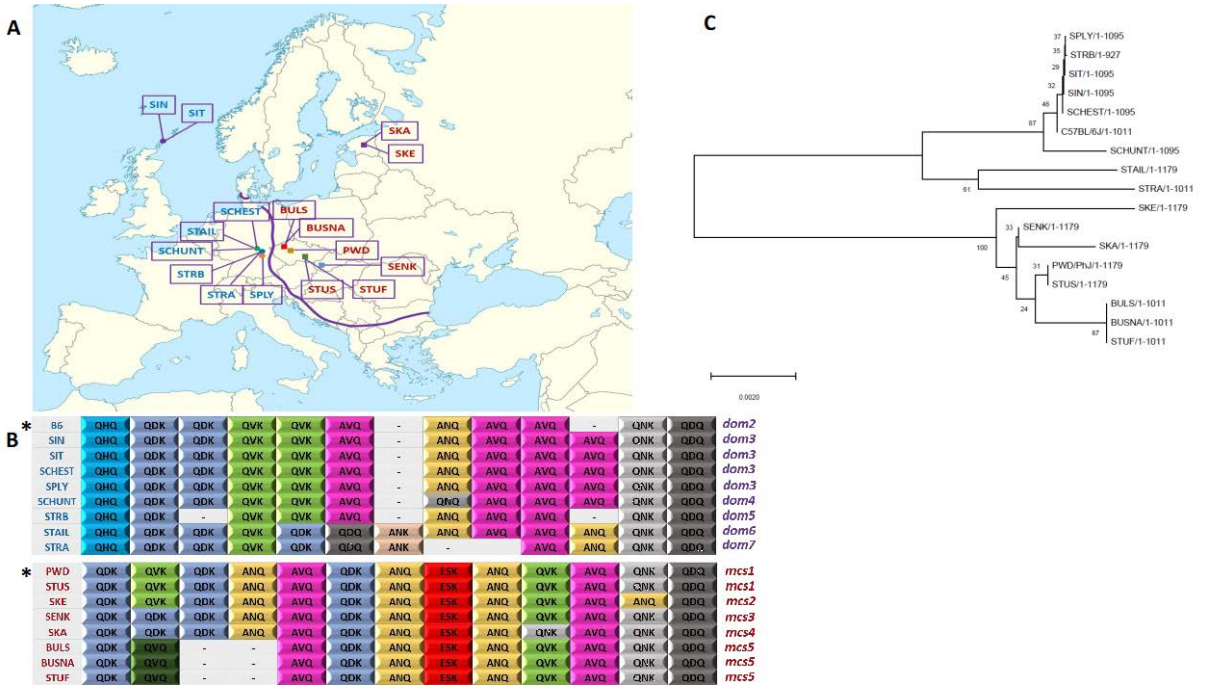



*Figure 5. Geographic distribution of founders of wild-derived strains and their Prdm9 ZnF arrays. (A) Localities of origin of eight musculus (squares) and eight domesticus (circles) strains along the hybrid zone. (B) Altogether eight Prdm9 alleles were identified by sequencing their ZnF arrays in 16 wild-derived strains. (C) Phylogenetic relationships between Prdm9 alleles estimated using the neighbour-joining method. (Mukaj et al. Molecular Biology and Evolution, in press, 2020).*

Our data showed, for the first time, the male infertility of wild-derived *musculus* and *domesticus* subspecies F1 hybrids controlled by *Prdm9* as the major hybrid sterility gene. The impairment of fertility surrogates, testes weight and sperm count, correlated with increasing difficulties of meiotic synapsis of homologous chromosomes and with meiotic arrest, which we suppose reflect the increasing asymmetry of PRDM9-dependent DNA double-strand breaks (**Mukaj et al. Molecular Biology and Evolution 2020**).

4. <u>The structure of the *Hstx2* locus on Chromosome X</u>

The impact of *Prdm9*$^{PWD/B6}$ incompatibility on infertility of intersubspecific hybrids is modulated by a genetic factor situated within the *Hstx2* locus on the PWD chromosome X (**Bhattacharyya et al. Plos Genet 2014**). To uncover the complex molecular mechanism of *Prdm9*-dependent incompatibilities in hybrid sterility we need to identify the gene behind the effect of the *Hstx2* locus. To facilitate positional cloning of this genetic factor and to overcome the recombination suppression within the 4.3 Mb encompassing the *Hstx2* locus, we designed the HSTX2-CRISPR and SPO11/Cas9 transgenes aimed to induce double-strand breaks specifically within the *Hstx2* locus by Cas9 RNA-guided DNA endonuclease DNA. One resulting PWD/B6 recombinant reduced the *Hstx2*$^{PWD}$ locus to 2.70 Mb (chromosome X: 66.51–69.21 Mb). The newly defined *Hstx2* locus still operates as the major X-linked factor of the F1 hybrid sterility, and controls meiotic chromosome synapsis and meiotic recombination rate (**Lustyk et al. Genetics 2019**. Despite extensive further crosses, the 2.70 Mb *Hstx2* interval behaved as a recombination cold spot with reduced PRDM9-mediated H3K4me3 hotspots and absence of DMC1-defined DNA double-strand-break hotspots.

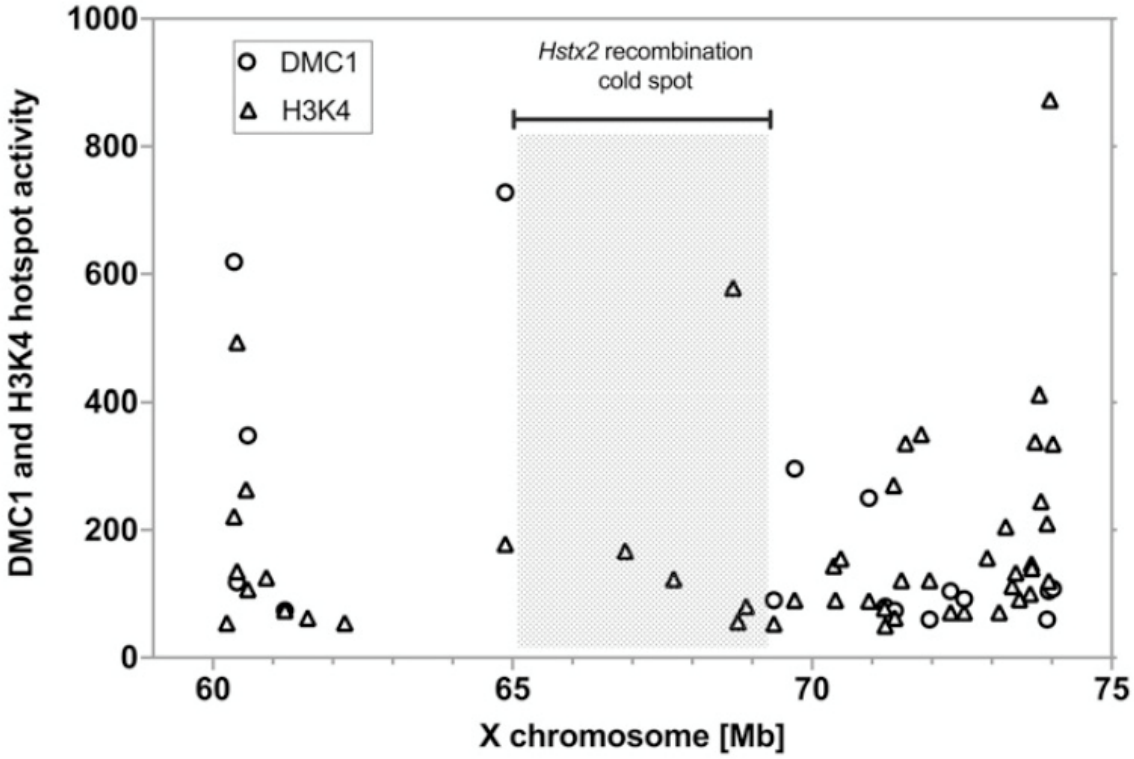



*Figure 6. Activity of PRDM9-dependent H3K4 methylation and DMC1-marked DNA DSBs in female meiosis. The DMC1 and H3K4me3 hotspots plotted within the Hstx2 locus and the adjacent regions of chromosome X (mm10 genome). The strong DMC1 hotspots coupled with H3K4 methylation lie outside the Hstx2 region (shaded), which contains only H3K4 methylation marks. Data extracted from Brick et al. (2018); visualized are hotspots with activity >50 (Lustyk et al. Genetics, 2019).*

To search for structural anomalies as a possible cause of recombination suppression, we used BIONANO optical genomic mapping and observed high incidence of subspecies-specific structural variants along the X chromosome, with a striking copy number polymorphism of the microRNA Mir465 cluster.

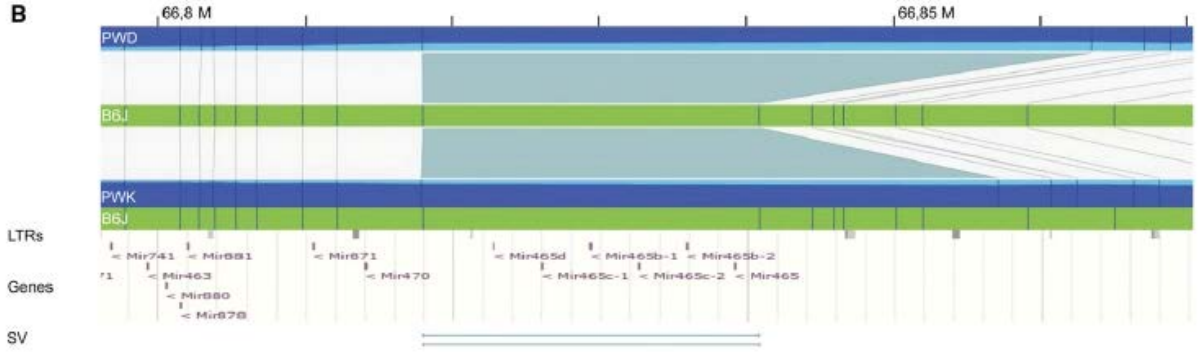


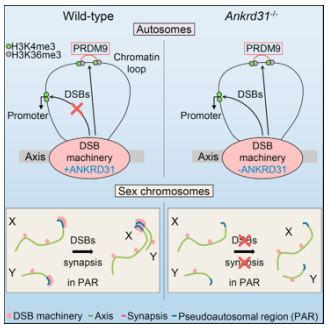

*Figure 7. Optical mapping of 80 Kb interval showing polymorphic structural variation within Hstx2 locus. PWD and PWK both bear insertions of the Mir465 cluster,compared to the orthologous region in B6/J (Lustyk et al. Genetics 2019).*

This observation together with the absence of a strong sterility phenotype in *Fmr1* neighbor (*Fmr1nb*) null mutants, also mapping to the *Hstx2* locus, support the role of microRNA Mir465 cluster as a likely candidate for *Hstx2* candidate genetic factor (**Lustyk et al. Genetics 2019**). The unique function of PRDM9 protein to initiate meiotic recombination contributed to uncover the molecular mechanism of hybrid sterility, but the role of this histone methyl transferase as

a reproductive barrier outside our laboratory model remained unclear. To fill the gap, we studied the role of *Prdm9* in inter-subspecific hybrids including 16 *musculus* and *domesticus* wild-derived strains. We found that the impairment of fertility phenotypes correlated with meiotic asynapsis rate of homologous chromosomes and all phenotypes returned to normal when the domesticus *Prdm9*$^{dom2}$ allele was substituted with its humanized form *Prdm9*$^{dom2H}$. We hypothesize that *Prdm9*-controlled F1 hybrid incompatibilities mimic an elusive initial encounter of both subspecies during their secondary contact, thus being an early cause and not a consequence of reproductive isolation. Further studies of *Prdm9*-controlled hybrid sterility may contribute to better understand the molecular mechanisms of speciation.

5. Identification of ANKRD31 DSB-promoting protein

We contributed to the discovery of ANKRD31 meiotic protein as a new component of DNA DSB-generating machinery, which initiates meiotic recombination (**Papanikos et al., including D. Lustyk, P. Jansa and J. Forejt, Mol. Cell 74, 1069–1085, 2019**).




This protein forms small foci on lepotene chromosomes and larger aggregates which persist until pachytene stage. Our contribution was to localize, using combination of immunocytochemistry and Fluorescent in Situ Hybridization (FISH) the ANKRD31 aggregates to chromosomes 4. 9 and 13 and to the pseudoautosomal region of the X and Y chromosomes. The major role of ANKRD31 is to focus the DSB machinery to PRDM9 hotspots outside the promotor and enhancer sequences. ANKRD31 is necessary for crossing-over in the PAR region and for male fertility

**Publications 2015-2020.** Authors from the **Forejt'group are in bold,** coresponding authors*, PhD students are underlined,

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## Research activity and characterization of the main scientific results

### Identification of molecular targets of pharmacological modulators suppressing FcεRI-signalosomes

Ethanol has multiple effects on biochemical events in various cell types, including FcεRI signaling in antigen-activated mast cells. However, the underlying molecular mechanisms was not known. To get a better understanding of the effect of ethanol on FcεRI-mediated signaling events, we examined the effects of short-term (15 min) treatment with non-toxic concentrations of ethanol on the biological functions of bone marrow-derived mast cells (BMMCs). We found that ethanol in a dose-dependent manner inhibited antigen-induced degranulation, calcium mobilization, production of proinflammatory cytokines (tumor necrosis factor-α, interleukin-6, and interleukin-13) and chemokines (CCL3 and CCL9), and formation of reactive oxygen species. Removal of cellular cholesterol with methyl-β-cyclodextrin MβC had a similar effect. MβC potentiated some of the inhibitory effects of ethanol, whereas cholesterol-saturated MβC had no or weak inhibitory effects. Further studies showed that exposure to ethanol or removal of cholesterol inhibited early FcεRI signaling events, including tyrosine phosphorylation of the FcεRI β and γ subunits, Lyn and Syk kinases, LAT and PAG adaptor proteins, and association of the proteins with detergent-resistant membranes. Finally, we found that ethanol at physiologically attainable concentrations inhibited passive cutaneous anaphylactic reaction, indicating that ethanol inhibits FcεRI signaling under *in vivo* conditions. The combined data suggest that ethanol inhibits the early antigen-induced signaling events in mast cells by suppressing the function of FcεRI-cholesterol signalosomes at the plasma membrane **(Draberova et al., 2015).** All authors of the study are members of the evaluated group.

Miltefosine (hexadecylphosphocholine) is a new candidate for the treatment of mast cell-driven diseases as it inhibits the activation of mast cells. It has been proposed that miltefosine acts as a lipid raft modulator through its interference with the structural organization of surface receptors in the cell plasma membrane. However, the molecular mechanism of its action was not fully understood. In our studies, we found that in antigen-activated BMMCs, miltefosine inhibited degranulation, reorganization of microtubules, and antigen-induced chemotaxis. While aggregation and tyrosine phosphorylation of IgE receptors were suppressed in activated cells pre-treated with miltefosine, overall tyrosine phosphorylation levels of Lyn and Syk kinases and $Ca^{2+}$ influx were not inhibited. In contrast, lipid raft disruptor methyl-β-cyclodextrin attenuated the $Ca^{2+}$ influx. Immunoprecipitation and *in vitro* kinase assays revealed that miltefosine inhibited $Ca^{2+}$- and diacylglycerol-regulated conventional protein kinase C (cPKC) isoforms that are important for mast cell degranulation. Inhibition of cPKCs by specific inhibitor Ly333531 affected the activation of BMMCs in the same way as miltefosine. The data obtained suggested that miltefosine modulates mast cells both at the plasma membrane and in the cytosol by inhibition of cPKCs. This alters intracellular signaling pathway(s) directed to microtubules, degranulation, and cell migration **(Rubikova et al., 2018).** This study was the result of the collaboration between our laboratory and the Laboratory of Biology of Cytoskeleton. Our laboratory performed characterization of biological properties of BMMCs, namely analysis of the calcium response.

**New regulatory roles of galectin-3 in FcεRI signaling**

To better understand the signaling pathways and genes involved in mast cell activation, we developed a high-throughput mast cell degranulation assay suitable for RNA interference experiments. Difficulties with efficient transfection have historically complicated RNAi screening in differentiated immune cells. To overcome this problem, we established a screening protocol based on lentiviral short hairpin RNA (shRNA) delivery. We used BMMCL, a mouse mast cell line that preserved high growth rate following lentiviral transduction and exhibited stable expression of mast cell surface receptors, including FcεRI and KIT. We tested 432 shRNAs specific for 144 selected genes for their effects on FcεRI-mediated mast cell degranulation and identified 15 potential regulators. In further studies, we focused on galectin-3 (Gal3), identified in this study as a negative regulator of mast cell degranulation. FcεRI-activated cells with Gal3 knockdown exhibited upregulated tyrosine phosphorylation of spleen tyrosine kinase and several other signal transduction molecules and enhanced calcium response. We showed that Gal3 promotes internalization of IgE-FcεRI complexes and targeting to LAMP1-positive endolysosomes. Furthermore, we found that Gal3 facilitates mast cell adhesion and motility on fibronectin, but negatively regulates antigen-induced chemotaxis. We also demonstrated that Gal3 is involved in regulating surface expression and internalization of β1-integrin in BMMCs. We showed that Gal3 participates in the regulation of the β1-integrin level on the cell surface in resting cells and stabilizes β1-integrin on the surface upon antigen activation. Rapid displacement of β1-integrin from the cell surface observed after antigen activation of BMMCs with Gal3 knockdown led to inefficient interactions between the plasma membrane and fibronectin-coated surfaces. We also showed that motility of the cells upon $PGE_2$ stimulation is affected by Gal3 at the level of β1-integrin activation. These defects were specific for fibronectin-mediated events, since the general ability of BMMCs with Gal3 knockdown to migrate towards chemoattractant was increased. It is possible that enhanced production of cytokines and $PGD_2$ observed in cells with Gal3 knockdown acts by autocrine mechanisms on enhanced chemotaxis toward antigen. The upregulated migration of mast cells towards supernatants from activated BMMCs with Gal3 knockdown confirmed the contribution of Gal3 to mast cell-produced mediators. The combined data indicated that Gal3 is an important regulator of mast cell surface molecules and is involved in both positive and negative regulation of FcεRI-mediated signaling events **(Bambouskova et al., 2016)**. This research paper is the result of our collaboration with the Center for Computational and Integrative Biology, Massachusetts General Hospital, Harvard Medical School, and Broad Institute of Harvard University and Massachusetts Institute of Technology, Cambridge, Massachusetts, USA. Most of the authors (64%), including the first and corresponding authors, are from the evaluated laboratory. The collaborating authors from the USA contributed by helping us with the high-throughput screening.

**Regulatory roles of ORMDL3 in the FcεRI-triggered expression of proinflammatory mediators and chemotactic response**

Single-nucleotide polymorphism studies have linked the chromosome 17q12-q21 region, where the human orosomucoid-like (*ORMDL*)*3* gene is localized, to the risk of asthma and several other inflammatory diseases **(**reviewed in **Paulenda and Draber, 2016)**. Although mast cells are involved in the development of these diseases, the contribution of ORMDL3 to mast cell physiology was unknown. In this study, we examined the role of ORMDL3 in antigen-induced activation of murine mast cells with reduced or enhanced ORMDL3 expression. Our data showed that in antigen-activated mast cells, reduced expression of the ORMDL3 protein had no effect on degranulation and calcium response, but significantly enhanced phosphorylation of AKT kinase at Ser 473 followed by enhanced phosphorylation and degradation of IκBα and translocation of the NF-κB p65 subunit into the nucleus. These events were associated with increased expression of proinflammatory cytokines (TNF-α, IL-6, and IL-13), chemokines (CCL3 and CCL4), and cyclooxygenase-2 dependent synthesis of prostaglandin D2. Antigen-mediated chemotaxis was also enhanced in ORMDL3-deficient cells, whereas spreading on fibronectin was decreased. On the other hand, increased expression of ORMDL3 had no significant effect on the studied signaling events, except for reduced antigen-mediated chemotaxis. These data were corroborated by increased IgE-antigen dependent passive cutaneous anaphylaxis in mice with locally silenced ORMDL3 using short interfering RNAs. Our data also show that antigen triggers suppression of ORMDL3 expression in the mast cells. In this study we provided evidence that downregulation of ORMDL3 expression in mast cells enhances AKT and NF-κB-directed signaling pathways and chemotaxis and contributes to the development of mast cell-mediated local inflammation *in vivo* **(Bugajev et al., 2016).** All authors of this paper are members of the evaluated team.

**New regulatory roles of C-terminal Src kinase CSK in FcεRI-mediated mast cell activation**

Previous studies showed that C-terminal Src kinase (CSK) is a major negative regulator of Src family tyrosine kinases (SFKs) that play critical roles in immunoreceptor signaling. CSK is brought in contiguity to the plasma membrane-bound SFKs via binding to transmembrane adaptor PAG (phosphoprotein associated with glycosphingolipid-enriched microdomains). Our previous findings that PAG can function as a positive regulator of the FcεRI-mediated mast cell signaling suggested that PAG and CSK have some non-overlapping regulatory functions in mast cell activation. To determine the regulatory roles of CSK in FcεRI signaling, we derived BMMCs with reduced or enhanced expression of CSK from wild-type (WT) or PAG knockout (KO) mice and analyzed their FcεRI-mediated activation events. We found that in contrast to PAG-KO cells, antigen-activated BMMCs with CSK knockdown (KD) exhibited significantly higher degranulation, calcium response, and tyrosine phosphorylation of FcεRI, SYK, and phospholipase C. Interestingly, FcεRI-mediated events in BMMCs with PAG-KO were restored upon CSK silencing. BMMCs with CSK-KD/PAG-KO resembled BMMCs with CSK-KD alone. Unexpectedly, cells with CSK-KD showed reduced kinase activity of LYN and decreased phosphorylation of transcription factor STAT5. This was accompanied by impaired production of proinflammatory cytokines and

chemokines in antigen-activated cells. In line with this, BMMCs with CSK-KD exhibited enhanced phosphorylation of protein phosphatase SHP-1, which provides a negative feedback loop for regulating phosphorylation of STAT5 and LYN kinase activity. Furthermore, we found that in WT BMMCs, SHP-1 forms complexes containing LYN, CSK, and STAT5. Altogether, our data demonstrate that in FcεRI-activated mast cells, CSK is a negative regulator of degranulation and chemotaxis, but a positive regulator of adhesion to fibronectin and production of proinflammatory cytokines. Some of these pathways are not dependent on the presence of PAG **(Potuckova et al., 2018).** All authors of this paper are members of the evaluated team**.**

**Search for the role of cytoskeletal protein 4.1R and microtubules in the FceRI signaling and mast cell chemotaxis**

Protein 4.1R, a member of the 4.1 family, functions as a bridge between cytoskeletal and plasma membrane proteins. It is expressed in T cells, where it binds to a linker for activation of T cell (LAT) family member 1 and inhibits its phosphorylation and downstream signaling events after T cell receptor triggering. The role of the 4.1R protein in cell activation through other immunoreceptors is not known. In this study, we used 4.1R-deficient (4.1R-KO) and 4.1R-WT mice and explored the role of the 4.1R protein in the FcεRI signaling in mast cells. We found that BMMCs derived from 4.1R-KO mice showed normal growth *in vitro* and expressed FcεRI and c-KIT at levels comparable to WT cells. However, 4.1R-KO cells exhibited reduced antigen-induced degranulation, calcium response, and secretion of tumor necrosis factor-α. Chemotaxis towards antigen and stem cell factor (SCF) and spreading on fibronectin were also reduced in 4.1R-KO BMMCs, whereas prostaglandin $E_2$-mediated chemotaxis was not affected. Antibody-induced aggregation of tetraspanin CD9 inhibited chemotaxis towards antigen in WT but not 4.1R-KO BMMCs, implying a CD9-4.1R protein cross-talk. Further studies documented that in the absence of 4.1R, antigen-mediated phosphorylation of FcεRI β and γ subunits was not affected, but phosphorylation of SYK and subsequent signaling events such as phosphorylation of LAT1, phospholipase Cγ1, phosphatases (SHP1 and SHIP), MAP family kinases (p38, ERK, JNK), STAT5, CBL, mTOR, and c-KIT were reduced. Immunoprecipitation studies showed the presence of both LAT1 and LAT2 (LAT, family member 2) in 4.1R immunocomplexes. The positive regulatory role of 4.1R protein in FcεRI-triggered activation was supported by *in vivo* experiments in which 4.1R-KO mice showed the normal presence of mast cells in the ear tissue and peritoneum, but exhibited impaired passive cutaneous anaphylaxis. The combined data indicate that the 4.1R protein functions as a positive regulator in the early activation events after FcεRI triggering in mast cells **(Draberova et al., 2019).** Most of the authors (86%), including the first and corresponding authors, are from the evaluated laboratory. This paper is the result of our collaboration with N. Mohandas from the Red Cell Physiology Laboratory, New York Blood Center, New York, USA, who provided 4.1R knockout mice and anti-4.1R-specific antibodies.

We also analyzed the role of microtubules in the process of FcεRI-mediated degranulation. We have demonstrated that γ-tubulin interacts with p21-activated kinase interacting exchange factor β (βPIX) and G protein-coupled receptor kinase-interacting protein (GIT) 1. Live-cell imaging disclosed that both proteins were associated with centrosomes. An enhanced level of free

cytosolic $Ca^{2+}$ affected γ-tubulin properties and stimulated the association of GIT1 and γ-tubulin complex proteins (GCPs) with γ-tubulin. Microtubule nucleation was also influenced by the $Ca^{2+}$ level. Moreover, in the activated BMMCs, γ-tubulin formed complexes with tyrosine-phosphorylated GIT1. The study was the first to provide a possible mechanism for the concerted action of tyrosine kinases, GIT1/βPIX proteins, and $Ca^{2+}$ in the propagation of signals leading to the regulation of microtubule nucleation in activated cells **(Sulimenko et al., 2015)**. This study was the result of the collaboration between our laboratory and the Laboratory of Biology of Cytoskeleton. Our laboratory performed characterization of biological properties of GIT1/βPIX-deficient and corresponding WT control mast cells.

**Reviews on the molecular mechanisms of mast cell signaling**

It is a known fact that exposure to pro-inflammatory cytokines, chemokines, bacterial and viral products, as well as some other biological products and drugs, induces mast cell transition from the basal state into a primed one, which leads to enhanced response to IgE-antigen complexes. Mast cell priming changes the threshold for antigen-mediated activation by various mechanisms, depending on the priming agent used, which alone usually do not induce mast cell degranulation. In this review, we described the priming processes induced in mast cells by various cytokines (stem cell factor, interleukins-4, -6, and -33), chemokines, other agents acting through G protein-coupled receptors (adenosine, prostaglandin $E_2$, sphingosine-1-phosphate, and β-2-adrenergic receptor agonists), toll-like receptors, and various drugs affecting the cytoskeleton. We focused on the current knowledge about the molecular mechanisms behind priming of mast cells leading to degranulation and cytokine production and discussed the biological effects of mast cell priming induced by several cytokines **(Halova et al., 2018b).** Half of the authors, including the first and corresponding authors, are from the evaluated laboratory. This paper is the result of our collaboration with the Immunology and Allergy Unit, Department of Medicine, Karolinska Institutet, Clinical Immunology and Transfusion Medicine, Karolinska University Hospital, Stockholm, Sweden, Alberta Respiratory Center and Department of Medicine, University of Alberta, Edmonton, AB, Canada, and Department of Medical Sciences, Uppsala University, Uppsala, Sweden.

We also reviewed recent data suggesting that an intricate network of inhibitory and activating receptors, specific signaling pathways, and adaptor proteins governs mast cell responsiveness to stimuli. We discussed the biological and clinical relevance of negative and positive signaling modalities that control mast cell activation, with emphasis on novel FcεRI regulators, IgE-independent pathways [e.g., Mas-related G protein-coupled receptor X2 (MRGPRX2)], tetraspanins, and the CD300 family of inhibitory and activating receptors **(Bulfone-Paus et al., 2017)**. This paper is the result of our collaboration with several leaders in the field of mast cell signaling: Division of Musculoskeletal and Dermatological Sciences, Faculty of Biology, Medicine and Health, University of Manchester, Manchester, UK; Immunology and Allergy unit, Department of Medicine, Karolinska Institutet and Karolinska University Hospital, Stockholm, Sweden; Department of Medical Sciences, Uppsala University, Uppsala, Sweden; INSERM U1149, Centre de Recherche sur l'Inflammation, Paris, France; CNRS ERL8252, Paris, France;

Université Paris Diderot, Sorbonne Paris Cité, Faculté de Médecine, Site Xavier Bichat, Inflamex Laboratory of Excellence, Paris, France; Pharmacology and Experimental Therapeutics Unit, School of Pharmacy, Institute for Drug Research, Faculty of Medicine, Hebrew University of Jerusalem, Jerusalem, Israel. We contributed mainly by writing the whole chapter on the tetraspanin family in mast cell signaling.

Signal transduction and chemotaxis with focus on new findings in FcεRI and KIT receptor signaling was subject of another review. In this review we summarized all data related to the effect of gene knockout of key signaling molecules on SCF- or antigen-mediated chemotaxis, degranulation, $Ca^{2+}$ response, passive cutaneous anaphylaxis, and passive systematic anaphylaxis **(Draber et al., 2016)**. Most of the authors (75%), including the first and corresponding author, are from the evaluated laboratory. This paper is the result of our collaboration with T. Kawakami, Division of Cell Biology, La Jolla Institute for Allergy and Immunology, 9420 Athena Circle La Jolla, CA 92037, USA, Laboratory for Allergic Disease and RIKEN Center for Integrative Medical Sciences (IMS-RCAI), Yokohama 230-0045, Japan.

We also wrote a review focused on recent biophysical, microscopic, and functional studies indicating that tetraspanin-enriched domains (TEMs) and transmembrane adaptor protein (TRAP)-enriched domains are involved in compartmentalization of physicochemical events of such important processes as immunoreceptor signal transduction and chemotaxis. We presented data based on analysis of selected mast cell-expressed tetraspanins [cluster of differentiation (CD)9, CD53, CD63, CD81, CD151)] or TRAPs [linker for activation of T cells (LAT), non-T cell activation linker (NTAL), and phosphoprotein associated with glycosphingolipid-enriched membrane microdomains (PAG)] pointing to a diversity within these two families and brought evidence of the important roles of these molecules in signaling events. We pointed out that although our understanding of TEMs and TRAP-enriched domains is far from complete, pharmaceutical applications of the knowledge about these domains are under way **(Halova and Draber, 2016)**. All authors of this paper are members of the evaluated team**.**

Finally, we wrote a review on the role of ORMDL proteins as guardians of cellular sphingolipids in asthma. This review is based on genome-wide association studies, which linked expression of ORMDL3 protein to asthma in humans. It has been found that ORMDL proteins are key regulators of serine palmitoyltransferase, an enzyme catalyzing the initial step of sphingolipid biosynthesis. Sphingolipids play prominent roles in cell signaling and response to stress, and they affect the mechanistic properties of cellular membranes. Deregulation of sphingolipid biosynthesis and their recycling has been proven to support and even cause several diseases including allergy, inflammation, and asthma. We discuss that ORMDL3, the most extensively studied member of the ORMDL family, has been shown to be important for endoplasmic reticulum homeostasis by regulating the unfolded protein response and calcium response. In immune cells, ORMDL3 is involved in migration and in the production of proinflammatory cytokines. Furthermore, changes in the expression level of ORMDL3 are important in allergen-induced asthma pathologies. The combined data show that ORMDL family proteins may serve as new therapeutic targets for the

treatment of asthma and several other life-threatening diseases **(Paulenda and Draber, 2016).** All authors of this paper are members of the evaluated team.

**New research tools and methods**

The activation of mast cells initiates signaling events leading to a rapid release of preformed inflammatory mediators from secretory granules and overall cell morphology changes. Mast cell activation also causes reorganization of cytoskeletal components that are associated with membrane ruffling, spreading, and migration. We described methods used for visualization of the mast cell cytoskeleton, focusing on its two major components, microfilaments and microtubules, and their changes after cell triggering **(Draber and Draber, 2015).** This methodological study was performed in two collaborating laboratories at IMG. In our laboratory, we focused on the visualization of microfilaments and their interactions with the plasma membrane, whereas in the Laboratory of Biology of Cytoskeleton, researchers focused on visualization of microtubules and their dynamic changes in activated mast cells.

Genotyping and gene expression analyses by polymerase chain reaction (PCR) have been extensively used on a daily basis in our laboratory. To make the assays more robust, we previously introduced new enhancers, which increased the specificity and yield of DNA amplicons (Shaik et al., 2008; Horáková et al., 2011). We extended these experiments and found that the real-time PCR setup with a buffer supplemented with 1M 1,2-propanediol, 0.2M trehalose, and SYBR green I provides a reliable technique for qPCR genotyping from murine or human whole blood and detection of single-nucleotide polymorphisms. Elimination of DNA extraction and the use of the standard Taq DNA polymerase and DNA dye brought substantial savings in labor and cost **(Utekal et al., 2015)**. At present, the PCR mix containing trehalose and 1,2-propanediol is commercially available from the Top-Bio company (http://www.top-bio.cz).

Furthermore, we developed a new method for the detection of microRNA. The principle of the method is based on mixing the examined sample containg isolated RNA with a synthetic single-stranded template DNA, which is designed so that it binds complementarily to the studied miRNA. When miRNA is present, it hybridizes to the template DNA (free in solution or immobilized on a surface of magnetic or other particles) to form a hybrid double-stranded DNA-RNA structures that are resistant to exonuclease I. However, if the miRNA is not present (or is unable to bind complementarily to the template DNA), a double-stranded structure does not form, the template DNA is degraded in the presence of exonuclease I and cannot serve as a template for subsequent qRT-PCR with added oligonucleotide primers. The method could be used for miRNA detection and quantification, relative (comparing the amount of miRNA in different samples) or absolute (determining the absolute amount of miRNA in the original sample). The method was patented **(Tumova et al., 2017)**. The method was developed in the evaluated team (2 authors), and one author was from the commercial sector.

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## Research activity in the MCI Laboratory

### Defining important regulators and mechanisms of leishmaniasis

In the Laboratory of MCI, a genome-wide search was performed, which led to the discovery of a network-like structure of genes that regulate the dissemination of the parasite inside a mammalian organism. Host genes revealed a wide variety of heterogeneous effects that included distinct organ-specific control, single-gene effects, gene-gene interactions, and sex-dependent control **(Kobets et al., 2019).** The researchers also analyzed eosinophilia - a phenotype that is, in some cases, associated with leishmaniasis. They present the first identification of sex-dependent autosomal loci controlling eosinophilic infiltration. The positive correlation between eosinophil infiltration and parasite load in males suggests that this sex-dependent eosinophilic infiltration reflects ineffective inflammation **(Slapnickova et al., 2016)**. They also described novel potential mechanisms influencing *L. major* infection – interferon-induced GTPases. They observed upregulation of GBP2b and GBP5 in mice infected with *L. major* parasites. Co-localization of the GBP2b protein with most *L. major* parasites in the skin of resistant and intermediate strains, but not in highly susceptible BALB/c mice, suggested that this molecule might play a role in the defense against leishmaniasis **(Sohrabi et al., 2018)**. They also wrote a spotlight on the role of the mannose receptor in non-healing *Leishmania major* infection **(Sohrabi and Lipoldova, 2018)**.

#### Shared components of leishmaniasis and other complex diseases

This project was based on the prediction that data from one biological system can help to identify genes and mechanisms in other systems. Using approaches developed in the leishmaniasis analysis, they mapped a novel locus on chromosome 7 controlling survival after infection with tick-borne encephalitis virus. Its bioinformatics analysis led to the detection of nine potential candidate genes. One of them, *Cd33*, carried a nonsense mutation in the STS strain. Interestingly, this locus co-localizes with locus *Lmr21* that controls susceptibility to *L. major* **(Palus et al., 2018)**. They were also involved in the work of COST Action BM1404 Mye-EUNITER on critical evaluations of the assays available for the functional analysis of human and murine MRCs **(Bruger et al., 2019)**.

#### Development of translational applications

Leishmaniasis initially occurred in the intertropical zones of America and Africa and spread into the temperate zone of South America, Asia, and southern Europe. Currently, it spreads to northern Europe and USA. An effective vaccine against the infection does not exist, and the drugs in use have many undesirable side effects. New drugs are therefore highly needed. The MCI laboratory analyzed the leishmanicidal activity and the mechanism of action of calcium ionophore calcimycin and was the first to demonstrate that calcimycin has a direct leishmanicidal effect on *L. major*. They also showed that constitutive $Ca^{2+}$/calmodulin-dependent nitric oxide synthase is involved in the parasite death **(Grekov et al., 2017)**. Furthermore, they performed screening of a 2448-chemotype library for leishmanicidal activity and found a novel compound, diphenyleneiodonium, which demonstrates potent leishmanicidal activity *in vitro* and *in vivo*. They patented this compound both in EU **(Grekov et al. EP3054941, 2017)** and USA **(Grekov et al. US 10350176).**

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## Research activity and characterisation of the main scientific results

Here we describe the principal published data during the evaluated period in four main directions. For some additional details, please see also the part “Assessment of the activity plan of the team for the period of 2015-2019”.
All the papers discussed were developed with original ideas and performed in our laboratory, occasionally with technical help from other departments. The only exception was the paper by Kitamura et al., developed mostly in M. Harata’s laboratory, where we participated mostly in the microscopy part.

**1) Phosphoinositides, NMI, nuclear compartmentalization and regulation of gene expression**

Phosphoinositides (PIs) are glycerol-based phospholipids containing hydrophilic inositol ring. The inositol ring is mono-, bis-, or tris-phosphorylated yielding seven PIs members. Ample evidence shows that PIs localize both to the cytoplasm and to the nucleus. However, tools for direct visualization of nuclear PIs are limited and many studies thus employ indirect approaches, such as staining of their metabolic enzymes. Since localization and mobility of PIs differ from their metabolic enzymes, these approaches may result in incomplete data. We decided before going in deeper studies to chech all the existing tools and tested commercially available PIs antibodies by light microscopy on fixed cells, tested their specificity using protein-lipid overlay assay and blocking assay, and compared their staining patterns. Additionally, we prepared recombinant PIs-binding domains and tested them on both fixed and live cells by light microscopy. The results provide a useful overview of usability of the tools tested and stress that the selection of adequate tools is critical. Knowing the localization of individual PIs in various functional compartments enabled us to better understand the roles of PIs in the cell nucleus.
We then continued to our principal topic – roles of phosphoinositides in the cell nucleus, and described a novel type of nuclear structure - nuclear lipid islets (NLIs). They are of 40-100 nm with a lipidic interior, and phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P2] molecules comprise a significant part of their surface. Most of NLIs have RNA at the periphery. Consistent with that, RNA is required for their integrity. The NLI periphery is associated with Pol II transcription machinery, including the largest Pol II subunit, transcription factors and NM1 (also known as NMI). The PtdIns(4,5)P2-NM1 interaction is important for Pol II transcription, since NM1 knockdown reduces the Pol II
transcription level, and the overexpression of wild-type NM1 [but not NM1 mutated in the PtdIns(4,5)P2-binding site] rescues the transcription. Importantly, Pol II transcription is dependent on NLI integrity, because an enzymatic reduction of the PtdIns(4,5)P2 level results in a decrease of the Pol II transcription level. Furthermore, about half of nascent transcripts localise to NLIs, and transcriptionally active transgene loci preferentially colocalise with NLIs. We hypothesized that NLIs serve as a structural platform that facilitates the formation of Pol II transcription factories, thus participating in the formation of nuclear architecture competent for transcription.
As we previoiusly studied PIP2 involvement in transcription of rRNA, we further contributed to our understanding of the regulation of rRNA genes transcription by PIP2 epigenetical repression of rRNA genes transcription via interaction with PHF8. We showed that PIP2 directly interacts with histone lysine demethylase PHF8 (PHD finger protein 8) and represses demethylation of H3K9me2 through this interaction. We identify the C-terminal K/R-rich motif as PIP2-binding site within PHF8, and address the function of this PIP2-PHF8 complex. PIP2-binding mutant of PHF8 has increased the activity of rDNA promoter (20%) and expression of pre-rRNA genes (47S-100%; 45S-66%). Furthermore, trypsin digestion reveals a potential conformational change of PHF8 upon PIP2 binding. These observations identified new function of nuclear PIP2in the fine-tuning of rDNA transcription.

Published original manuscripts:
Tools for visualization of phosphoinositides in the cell nucleus.
Kalasova I, Fáberová V, Kalendová A, Yildirim S, Uličná L, Venit T, Hozák P.
Histochem Cell Biol. 2016, 145:485-96. doi: 10.1007/s00418-016-1409-8.

Nuclear phosphatidylinositol 4,5-bisphosphate islets contribute to efficient RNA polymerase II-dependent transcription.
Sobol M, Krausová A, Yildirim S, Kalasová I, Fáberová V, Vrkoslav V, Philimonenko V, Marášek P, Pastorek L, Čapek M, Lubovská Z, Uličná L, Tsuji T, Lísa M, Cvačka J, Fujimoto T, Hozak P
J Cell Sci. 2018, 131:jcs211094. doi: 10.1242/jcs.211094.

PIP2 epigenetically represses rRNA genes transcription interacting with PHF8.
Ulicna L, Kalendova A, Kalasova I, Vacik T, Hozák P.
Biochim Biophys Acta Mol Cell Biol Lipids. 2018, 1863:266-275. doi: 10.1016/j.bbalip.2017.12.008.

The many functions of phosphoinositides in cytosolic signaling were extensively studied; however, their activities in the cell nucleus are much less clear. Based on our data and current literature, we summarized our views and produced three reviews.
We discussed the molecular mechanisms via which nuclear phosphoinositides, in particular phosphatidylinositol (4,5)-bisphosphate (PI(4,5)P2), modulate nuclear processes. We focused on PI(4,5)P2's role in the modulation of RNA polymerase I activity, and functions of the nuclear lipid islets-recently described nucleoplasmic PI(4,5)P2-rich compartment involved in RNA polymerase II transcription.
Further on, we discussed how the phase separation is involved in the formation of various nuclear compartments and molecular condensates separated from surrounding environment. The surface of such structures spatiotemporally coordinates formation of protein complexes. PI(4,5)P2 (PIP2) integration into phase-separated structures might provide an additional step in their spatial diversification by attracting certain proteins with affinity to PIP2. Our laboratory has recently identified novel membrane-free PIP2-containing structures, so called Nuclear Lipid Islets (NLIs). We provided here also an evidence that these structures are evolutionary conserved in different organisms, and hypothesized that NLIs serve as a scaffolding platform which facilitates the formation of transcription factories, thus participating in the formation of nuclear architecture competent for transcription, and speculate on a possible role of NLIs in the integration of various processes linked to RNAPII transcription, chromatin remodeling, actin-myosin
interaction, alternative splicing and lamin structures.

Published reviews:
Nuclear Phosphoinositides-Versatile Regulators of Genome Functions.
Castano E, Yildirim S, Fáberová V, Krausová A, Uličná L, Paprčková D, Sztacho M, Hozák P
Cells. 2019, 8:649. doi: 10.3390/cells8070649.

Nuclear phosphoinositides and phase separation: Important players in nuclear compartmentalization.
Sztacho M, Sobol M, Balaban C, Escudeiro Lopes SE, Hozák P.
Adv Biol Regul. 2019,71:111-117. doi: 10.1016/j.jbior.2018.09.009.

Phospholipids and inositol phosphates linked to the epigenome.
Uličná L, Paprčková D, Fáberová V, Hozák P.
Histochem Cell Biol. 2018, 150:245-253. doi: 10.1007/s00418-018-1690-9.

**2) Lamin complexes in nuclear organization**

The nuclear periphery (NP) plays a substantial role in chromatin organization. Heterochromatin at the NP is interspersed with active chromatin surrounding nuclear pore complexes (NPCs); however, details of the peripheral chromatin organization are missing. To discern the distribution of epigenetic marks at the NP of HeLa nuclei, we used structured illumination microscopy combined with a new MATLAB software tool for automatic NP and NPC detection, measurements of fluorescent intensity and statistical analysis of measured data. Our results show that marks for both active and non-active chromatin associate differentially with NPCs. The incidence of heterochromatin marks, such as H3K27me2 and H3K9me2, was significantly lower around NPCs. In contrast, the presence of marks of active chromatin such as H3K4me2 was only decreased very
slightly around the NPCs or not at all (H3K9Ac). Interestingly, the histone demethylases LSD1 (also known as KDM1A) and KDM2A were enriched within the NPCs, suggesting that there was a chromatin-modifying mechanism at the NPCs. Inhibition of transcription resulted in a larger drop in the distribution of H1, H3K9me2 and H3K23me2, which implies that transcription has a role in the organization of heterochromatin at the NP.
We then continued to characterize these findings in more detail and showed that TPR, the protein located preferentially within the nuclear baskets of NPCs, associates with lamin B1. The depletion of TPR affects the organization of lamin B1 but not lamin A/C within the nuclear lamina as shown by stimulated emission depletion microscopy. Finally, reduction of TPR affects the distribution of NPCs within the nuclear envelope and the effect can be reversed by simultaneous knock-down of lamin A/C or the overexpression of lamin B1. Our work suggests a novel role for the TPR at the nuclear periphery: the TPR contributes to the organization of the nuclear lamina and in cooperation with lamins guards the interphase assembly of nuclear pore complexes.

Published original manusripts:

Chromatin organization at the nuclear periphery as revealed by image analysis of structured illumination microscopy data.
Fišerová J, Efenberková M, Sieger T, Maninová M, Uhlířová J, Hozák P.
J Cell Sci. 2017, 130:2066-2077. doi: 10.1242/jcs.198424.

Nuclear pore protein TPR associates with lamin B1 and affects nuclear lamina organization and nuclear pore distribution.
Fišerová J, Maninová M, Sieger T, Uhlířová J, Šebestová L, Efenberková M, Čapek M, Fišer K, Hozák P.
Cell Mol Life Sci. 2019,76:2199-2216. doi: 10.1007/s00018-019-03037-0.

**3) Involvement of actin-binding and actin-related proteins in nuclear regulatory processes.**

Paxillin (PXN) is a well-known focal adhesion protein that has been implicated in signal transduction from the extracellular matrix. Recently, it has been shown to shuttle between the cytoplasm and the nucleus. When inside the nucleus, paxillin promotes cell proliferation. Here, we introduce paxillin as a transcriptional regulator of IGF2 and H19 genes. It does not affect the allelic expression of the two genes; rather, it regulates long-range chromosomal interactions between the IGF2 or H19 promoter and a shared distal enhancer on an active allele. Specifically, paxillin stimulates the interaction between the enhancer and the IGF2 promoter, thus activating IGF2 gene transcription, whereas it restrains the interaction between the enhancer and the H19 promoter, downregulating the H19 gene. We found that paxillin interacts with cohesin and the mediator complex,
which have been shown to mediate long-range chromosomal looping. We propose that
these interactions occur at the IGF2 and H19 gene cluster and are involved in the formation of loops between the IGF2 and H19 promoters and the enhancer, and thus the expression of the

corresponding genes. These observations contribute to a mechanistic explanation of the role of paxillin in proliferation and fetal development.

The actin family members, consisting of actin and actin-related proteins (ARPs), are essential components of chromatin remodeling complexes. ARP6, one of the nuclear ARPs, is part of the Snf-2-related CREB-binding protein activator protein (SRCAP) chromatin remodeling complex, which promotes the deposition of the histone variant H2A.Z into the chromatin. In this study, we showed that ARP6 influences the structure and the function of the nucleolus. ARP6 is localized in the central region of the nucleolus, and its knockdown induced a morphological change in the nucleolus. We also found that in the presence of high concentrations of glucose ARP6 contributed to the maintenance of active ribosomal DNA (rDNA) transcription by placing H2A.Z into the chromatin. In contrast, under starvation, ARP6 was required for cell survival through the repression of rDNA transcription independently of H2A.Z. These findings reveal novel pleiotropic roles for the actin family in nuclear organization and metabolic homeostasis.

Published manuscripts:
Paxillin-dependent regulation of IGF2 and H19 gene cluster expression.
Marášek P, Dzijak R, Studenyak I, Fišerová J, Uličná L, Novák P, Hozák P.
J Cell Sci. 2015, 128:3106-16. doi: 10.1242/jcs.170985.

The actin family protein ARP6 contributes to the structure and the function of the nucleolus.
Kitamura H, Matsumori H, Kalendova A, Hozak P, Goldberg IG, Nakao M, Saitoh N, Harata M.
Biochem Biophys Res Commun. 2015, 464:554-60. doi: 10.1016/j.bbrc.2015.07.005.
In this manuscript, we p

5) Development of methods

Artefact-free microscopic images represent a key requirement of multi-parametric image analysis in modern biomedical research. Holography microscopy (HM) is one of the quantitative phase imaging techniques, which has been finding new applications in life science, especially in morphological screening, cell migration, and cancer research. Rather than the classical imaging of absorbing (typically stained) specimens by bright-field microscopy, the information about the light-wave's phase shifts induced by the biological sample is employed for final image reconstruction. In this comparative study, we investigated the usability and the reported advantage of the holography imaging. The claimed halo-free imaging was analyzed compared to the widely used Zernike phase-contrast microscopy. The intensity and phase cross-membrane profiles at the periphery of the cell were quantified. The intensity profile for cells in the phase-contrast images suffers from the significant increase in intensity values around the cell border. On the contrary, no distorted profile is present outside the cell membrane in holography images. The gradual increase in phase shift values is present in the internal part of the cell body projection in holography image. This increase may be related to the increase in the cell internal material according to the dry mass theory. Our experimental data proved the halo-free nature of the holography imaging, which is an important prerequisite of the correct thresholding and cell segmentation, nowadays frequently required in high-content screening and other image-based analysis. Consequently, HM is a method of choice whenever the image analysis relies on the accurate data on cell boundaries.

In biomedical studies, the colocalization is commonly understood as the overlap between distinctive labelings in images. This term is usually associated especially with quantitative evaluation of the immunostaining in fluorescence microscopy. On the other hand, the

evaluation of the immunolabeling colocalization in the electron microscopy images is still under-investigated and biased by the subjective and non-quantitative interpretation of the image data. We introduce a novel computational technique for quantifying the level of colocalization in pointed patterns. Our approach follows the idea included in the widely used Manders' colocalization coefficients in fluorescence microscopy and represents its counterpart for electron microscopy. In presented
methodology, colocalization is understood as the product of the spatial interactions at the single-particle (single-molecule) level. Our approach extends the current significance testing in the immunoelectron microscopy images and establishes the descriptive colocalization coefficients. To demonstrate the performance of the proposed coefficients, we investigated the level of spatial interactions of phosphatidylinositol 4,5-bisphosphate with fibrillarin in nucleoli. We compared the electron microscopy colocalization coefficients with Manders' colocalization coefficients for confocal microscopy and super-resolution structured illumination microscopy. The similar tendency of the values obtained using different colocalization approaches suggests the biological validity of the scientific conclusions. The presented methodology represents a good basis for further development of the quantitative analysis of immunoelectron microscopy data and can be used for studying molecular interactions at the ultrastructural level. Moreover, this methodology can be applied also to the other super-resolution microscopy techniques focused on characterization of discrete pointed structures.

Published original manuscripts:

Holography microscopy as an artifact-free alternative to phase-contrast.
Pastorek L, Venit T, Hozák P.
Histochem Cell Biol. 2018, 149:179-186. doi: 10.1007/s00418-017-1610-4.

Colocalization coefficients evaluating the distribution of molecular targets in microscopy methods based on pointed patterns.
Pastorek L(1)(2), Sobol M(1)(2), Hozák P.
Histochem Cell Biol. 2016, 146:391-406. doi: 10.1007/s00418-016-1467-y.

## Research activity and characterisation of the main scientific results

### Regulation of microtubule nucleation and organization

Activation of mast cells by aggregation of the high-affinity IgE receptors (FcεRI) initiates signaling events leading to the release of inflammatory and allergic mediators stored in cytoplasmic granules. A vital role in this process plays the activation of tyrosine kinases and changes in concentrations of intracellular $Ca^{2+}$. Although microtubules are involved in the process leading to degranulation, the molecular mechanisms that control microtubule rearrangement during activation are mostly unknown. We initially found that activation of bone marrow-derived mast cells (BMMCs) resulted in the generation of protrusions containing microtubules (microtubule protrusions; Hájková et al., J. Immunol. 186: 913, 2011). We have newly demonstrated that γ-tubulin interacts with p21-activated kinase interacting exchange factor β (βPIX) and G protein-coupled receptor kinase-interacting protein (GIT) 1. Live-cell imaging disclosed that both proteins were associated with centrosomes. An enhanced level of free cytosolic $Ca^{2+}$ affected γ-tubulin properties and stimulated the association of GIT1 and γ-tubulin complex proteins (GCPs) with γ-tubulin. Microtubule nucleation was also influenced by $Ca^{2+}$ level. Moreover, in the activated BMMCs, γ-tubulin formed complexes with tyrosine-phosphorylated GIT1. The study provided for the first time, a possible mechanism for the concerted action of tyrosine kinases, GIT1/βPIX proteins, and $Ca^{2+}$ in the propagation of signals leading to the regulation of microtubule nucleation in activated cells (**Sulimenko et al., 2015**). The study was performed primarily in our laboratory. Laboratory of Signal Transduction at IMG helped with the characterization of GIT1/βPIX-deficient mast cells. Association of GIT1 and βPIX with centrosomes was not unique for BMMCs, as it was observed in various cell types. Moreover, p21 protein (Cdc42/Rac)-activated kinase 1 (PAK1) was also associated with interphase centrosomes in different cell types. While depletion of βPIX stimulated microtubule nucleation, depletion of GIT1 or PAK1 resulted in decreased nucleation. The importance of PAK1 for microtubule nucleation was corroborated by inhibition of its kinase activity. GIT1 with PAK1 thus represent positive regulators, and βPIX is a negative regulator of microtubule nucleation. Importantly, the regulatory roles of these signaling proteins in microtubule nucleation correlated with the recruitment of γ-tubulin to the centrosome. GIT1/βPIX signaling proteins were phosphorylated by PAK1 and directly interacted with γ-tubulin. The binding site for γ-tubulin on GIT1 was located in its N-terminal domain targeting GIT1 to the centrosome. Altogether, based on these data, we suggested a novel regulatory mechanism of microtubule formation in interphase cells, in which GIT1 and βPIX signaling proteins, phosphorylated by the PAK1 kinase, modulate microtubule nucleation (**Černohorská et al., 2016**). This work was performed exclusively in our laboratory.

The tyrosine phosphorylation of numerous substrates in activated BMMCs is only transient and returns to baseline levels several minutes after receptor triggering. It is well accepted that protein tyrosine kinases are essential during mast cell signaling, but the exact function of protein tyrosine phosphatases is less understood. We identified SH2 domain-containing protein tyrosine phosphatase 1 (SHP-1), forming complexes with γ-tubulin complex proteins, as a negative regulator of microtubule nucleation from the centrosomes of BMMCs. The regulation is due to the changes in γ-tubulin accumulation. During antigen-induced activation, SHP-1 modulates the activity of the Syk kinase, which is located to centrosomes and affects the organization of microtubules. Deletion of SHP-1 results in a substantial increase of microtubule protrusions in the course of activation. Our data suggested a novel mechanism for attenuation of microtubule formation in later stages of mast cell activation (**Klebanovych et al., 2019**). The study was performed primarily in our laboratory. Light Microscopy Core Facility at IMG helped with the development of macro for image processing.

Besides protein tyrosine kinases and phosphatases, other signaling molecules might modulate the organization of microtubules during the activation of BMMCs. We have found that miltefosine (hexadecylphosphocholine), a new candidate for the treatment of mast cell-driven diseases, inhibited the reorganization of microtubules, degranulation and antigen-induced chemotaxis in the activated cells. While aggregation and tyrosine phosphorylation of IgE receptors were suppressed in activated cells pre-treated with miltefosine, the $Ca^{2+}$ influx was not affected. Tagged-miltefosine rapidly localized into the cell interior and inhibited the movement of intracellular granules. Miltefosine inhibited $Ca^{2+}$- and diacylglycerol-regulated conventional protein kinase C (cPKC) isoforms that are important for mast cell degranulation. Collectively, our data revealed that miltefosine modulates mast cells at multiple sites. This alters the intracellular signaling pathway(s) directed to microtubules, degranulation, and migration (**Rubíková et al.**, **2018**). The study was performed primarily in our laboratory in collaboration with the Laboratory of Signal Transduction at IMG, that helped with degranulation assay

It has been well established that microtubules are implicated in actin-dependent cellular processes such as focal adhesion turnover, the establishment of cell polarity, and migration. We have found that profilin, controlling actin nucleation and assembly processes in eukaryotic cells, associates with microtubules via formins. Our study showed that profilin dynamically associates with microtubules, and this profilin fraction contributes to balancing actin assembly during cell growth and affects the microtubule dynamics. The depletion of profilin resulted in faster growth of microtubules. On the other hand, stimulation of actin dynamics reduced the profilin association along the microtubules. The regulatory role of profilin in microtubule plus-end dynamics further support the conjecture of a close actin-microtubule interrelationship and underscoring profilin as a unique regulator of force generation and cellular behavior in eukaryotes (**Nejedlá et al., 2016**). In this collaborative study with Stockholm University, Sweden, we performed „fishing experiments“ of profilin on stabilized microtubules and analyzed the effect of profilin on tubulin polymerization.

**New functions for γ-tubulins**

Mammals possess two genes encoding γ-tubulin. The studies on the functions of γ-tubulin isotypes were hampered by the unavailability of antibodies that are capable of distinguishing between highly conserved γ-tubulin-1 and γ-tubulin-2. We provided evidence that discrimination between human γ-tubulin isotypes is possible, based on their different electrophoretic properties and immunoreactivity with antibodies. Specifically, monoclonal antibody TU-30, prepared in our laboratory, recognizes a unique epitope on human γ-tubulin-1. We demonstrated enhanced expression of γ-tubulin-2 during neuronal differentiation of human neuroblastoma cells in the face of unchanged levels of γ-tubulin-1. We have found that despite a significant accumulation of γ-tubulin-2 in the adult human brain, γ-tubulin-1 is the dominant isotype and is constitutively expressed in mature neurons. We were also the first to show that enhanced expression of γ-tubulin-2 in neuroblastoma cells was triggered by oxidative stress induced by mitochondrial inhibitors and that γ-tubulins associate with mitochondria. These data indicate that in the face of predominant γ-tubulin-1 expression, the accumulation of γ-tubulin-2 in mature neurons and neuroblastoma cells during oxidative stress may denote a prosurvival role of γ-tubulin-2 in neurons (**Dráberová et al., 2017**) The study was performed primarily in our laboratory in collaboration with Drexel University College of Medicine, Philadelphia, USA (histochemical analysis), the Laboratory of the Nucleus at IMG (immunoelectron microscopy) and University Hospital Brno (clinical samples for biochemical analysis).

Over the past decade, genetic studies have identified numerous causative mutations in centrosomal proteins in subjects with malformation of cortical development (MCD). We

investigated the consequences of four human MCD-related *TUBG1* variants (Tyr92Cys, Ser259Leu, Thr331Pro, and Leu387Pro) on the cortical development by using *in-utero* electroporation and a knock-in *Tubg1*$^{Y92C/+}$ mouse model. We showed that pathogenic *TUBG1* variants affected neuronal positioning by disrupting neuronal migration without a significant effect on progenitor proliferation. Our results suggested that disease-related *TUBG1* variants exert their pathogenicity by changing microtubule dynamics rather than centrosomal positioning or nucleation ability. Furthermore, *Tubg1*$^{Y92C/+}$ animals showed neuroanatomical and behavioral defects and increased epileptic cortical activity. We demonstrated that *Tubg1*$^{Y92C/+}$ mice partially mimicked the human phenotype, and therefore represent a relevant model for further investigations of the physiopathology of MCD (**Ivanova et al., 2019)**. In this large collaborative study, we established cell lines expressing tagged mutated γ-tubulins and performed immunoprecipitation experiments.

The high abundance of γ-tubulin in *Arabidopsis* cells facilitated purification and characterization of the large molecular species of γ-tubulin. Their analysis by various microscopic techniques revealed the presence of linear filaments with a double protofilament substructure, filament bundles, and aggregates. Filament formation from highly purified γ-tubulin free of GCPs was demonstrated for both plant and human γ-tubulin. Super-resolution microscopy of *Arabidopsis* cells revealed fine, short γ-tubulin fibrillar structures enriched on mitotic microtubular arrays that accumulated at the poles of spindles, outer nuclear envelope, and were also present in the nuclei. Our findings that γ-tubulin preserves the capability of prokaryotic tubulins to self-organize into filaments assembling by lateral interaction into bundles suggest that besides microtubule nucleation, γ-tubulin may also have scaffolding or sequestration functions (**Chumová et al., 2018**). In this work, we performed immunopurification of human γ-tubulin, fluorescence microscopy of isolated γ-tubulin, and electrophoretic analysis of tubulin oligomers.

**Dysregulation of microtubule proteins in cancer cells**

Based on our original findings that overexpression and ectopic γ-tubulin cellular distribution is linked with tumor progression in gliomas, the most prevalent brain tumors (Katsetos et al., J. Neuropathol. Exp. Neurol. 65: 465, 2006; Hořejší et al., J. Cell Physiol. 227: 367, 2012), we have examined expression and distribution of GCP2 and GCP3 proteins in glioblastoma cells. GCP2 and GCP3 formed complexes with γ-tubulin in the nucleoli, as confirmed by reciprocal immunoprecipitation experiments and immunoelectron microscopy. GCP2 and GCP3 depletion caused the accumulation of cells in G2/M and mitotic delay but did not affect nucleolar integrity. Similarly, as in the case of γ-tubulin overexpression, the overexpression of GCP2 antagonized the inhibitory effect of tumor suppressor CDK5RAP3 on the DNA damage G2/M checkpoint activity. Immunoreactivity for GCP2 and GCP3 was significantly increased in glioblastoma tissue samples when compared to normal brains. These findings suggest that dysregulation of γ-tubulin complex proteins in glioblastomas may be linked to altered transcriptional checkpoint activity or interaction with signaling pathways associated with a malignant phenotype (**Dráberová et al., 2015**). The study was performed primarily in our laboratory in collaboration with Drexel University College of Medicine (histochemical analysis), Laboratory of the Nucleus at IMG (immunoelectron microscopy), and University Hospital Brno (clinical samples for biochemistry). Laboratories from Temple University, Bergen University, and General Hospital Athens provided clinical samples for histochemistry. We also participated in the immunohistochemical analysis of γ-tubulin expression in non-small cell lung cancer (NSCLC) and effect γ-tubulin expression on patient survival. Our findings suggest that a high level of γ-tubulin expression may have an impact on better clinical response to postoperative chemotherapy at more advanced NSCLC stages (**Maounis et al., 2019**). In this collaborative work with General Hospital Athens, we secured the production of monoclonal antibodies to γ-tubulin and helped with data analysis.

Microtubules are the primary targets for chemotherapeutic agents (tubulin binding agents, TBAs). Within the long list of synthetic or naturally occurring compounds interacting with microtubules, small molecules with steroid structure represent an important class. We have found that in the panel of newly synthesized steroidal dimers based on estradiol, testosterone, or pregnenolone, the estradiol dimer had the most prominent cytotoxic, anti-mitotic and anti-tubulin activities. It thus represents a new structural type of steroid inhibitor, which suppresses tubulin polymerization in cancer cells (**Jurášek et al., 2018**). Four members from our laboratory participated in this collaborative work with the University of Chemistry and Technology in Prague and Palacky University in Olomouc. We performed an analysis of tubulin polymerization, measurements of microtubule dynamics, and immunofluorescence experiments. In search of new anti-cancer TBAs, we also participated in the characterization of new noscapine derivatives that exhibited cytostatic activities (**Ghaly et al., 2017**). This study was a joint project between Departments of Chemistry and Oncology, University of Alberta, Edmonton, Canada, and our laboratory. We performed immunofluorescence experiments and measurement of microtubule dynamics.

**Modulation of microtubules by nanosecond electropulses**

Although TBAs are essential components in the treatment of many cancers, the potential dose-dependent toxic side effects, as well as the development of resistance, can limit TBAs clinical applications. A perspective modulation strategy to overcome the above-mentioned limitations is targeting of tubulin with electromagnetic fields. We have reported a novel strategy for controlling tubulin self-assembly by nanosecond electropulses (nsEPs). The polymerization was dependent on nsEPs dosage. The kinetics of microtubule formation was tightly linked to the nsEPs effects on structural properties of tubulin and tubulin solvent interface. Moreover, nsEPs affected the overall size of the tubulin dimers. We suggest that nsEPs-induced conformational changes in tubulin C-terminal domains alter tubulin polymerization **(Chafai et al., 2019**). In this collaborative work, on the border of scientific disciplines (cell biology and physics) on which participated teams from IMG CAS, Institute of Photonics and Electronics CAS, Institute of Physiology CAS, we isolated critical components for this study (highly purified polymerization-competent tubulin). The chip for nsEPs delivery to samples evaluated by super-resolution microscopy (SIM, structured illumination microscopy) was designed, fabricated, and used in the following work. We showed that nsEPs change in cells microtubule plus ends marked by EB3 protein (**Havelka et al., 2019**). We prepared a mast cell line expressing EB3-mNeonGreen and performed live-cell imaging.

**New research tools and methods**

The Refractive index of tubulin is an important parameter underlying fundamental electromagnetic and biophysical properties of microtubules. Yet, the only experimental data available in the current literature show values of tubulin refractive index (n=2.36 - 2.90), which are much higher than established theories predict based on the weighted contribution of the polarizability of individual amino acids constituting tubulin. To resolve this controversy, we reported by rigorous experimental analysis that the refractive index of purified tubulin dimer is n=1.64 (at 589 nm, 25°C), which is much closer to the values predicted by established theories (**Krivosudský et al., 2017**). For this work, we provided highly purified tubulin.

Activation of mast cells initiates signaling events leading to a rapid release of preformed inflammatory mediators from secretory granules, and overall changes in cell morphology. Mast cell activation also causes reorganization of cytoskeletal components, which are associated with membrane ruffling, spreading, and migration. We described methods used for

visualization of the mast cell cytoskeleton, focusing on its two major components, microfilaments and microtubules, and their changes after cell triggering. (**Dráber P. and Dráber Pe, 2015**). This methodical study was performed in our laboratory (visualization of microtubules and their dynamic changes) and in the Laboratory of Signal Transduction at IMG (visualization of microfilaments and their interactions with plasma membrane).

An integral part of our research is the production of unique probes (recombinant proteins and monoclonal antibodies) recognizing microtubule components. For example, we generated hybridomas producing first monoclonal antibodies specific for GCP2 (**Dráberová et al., 2015**). Based on business agreements, the hybridoma cell lines were transferred to a spin-off company of IMG, Exbio, a.s., for commercial use of the antibodies. We have also developed a sensitive ELISA test for the quantification of γ-tubulin. As used antibodies to γ-tubulin recognize phylogenetically highly conserved epitopes, it is expected that the ELISA test can be applied for quantification of γ-tubulin of different species.

**Review publications**

Most publications of our laboratory prepared in the evaluated period were research papers. However, we also published two review articles. In a review on microtubule targets in glioma therapy we focused on (1) the aberrant over-expression of βIII-tubulin; (2) the ectopic overexpression of γ-tubulin; (3) the microtubule-severing ATPase spastin; and (4) the modulating role of posttranslational modifications of tubulin in the context of interaction of microtubules with motor proteins (**Katsetos et al., 2015**) The review was prepared in collaboration with Drexel University College of Medicine and University of Alberta. Two co-authors were from our laboratory. We also published a review on the regulation of microtubule nucleation mediated by γ-tubulin complexes, in which we critically evaluated recent works on the factors and regulatory mechanisms that are involved in centrosomal and non-centrosomal microtubule nucleation **(Sulimenko et al., 2017**). This review was prepared in our laboratory.

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(Authors and/or co-authors of the evaluated team are underlined)

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## Research activity and characterisation of the main scientific results

Research activity of the team has always been determined by (unpredictable) funding sources. Luckily, funding of the team during 2015-2019 was excellent and allowed to go into depth of some of the studied questions. This included producing unique mammalian models, whose analyses did not appear in the publication record yet.

Team's research during 2015-2019 covered three overlapping research areas:

I. Analysis gene expression during mouse oocyte-to-embryo transition (OET)

II. Long non-coding RNAs in mouse oocytes and zygotes

III. Mammalian small RNA pathways.

Research activity in these three research areas is described in detail below:

### I. Analysis gene expression during mouse oocyte-to-embryo transition

Analysis of gene expression and its control during OET has been traditional research interest of the lab although it was pushed aside a bit during 2015-2019 as the main work effort has been concentrated on small RNA pathways. Nonetheless, there were significant contributions to this traditional research interest of the group.

First, the team leader was involved in international collaboration focused on dissecting the earliest events in the mammalian zygotic genome activation. This included laboratory of Fugaku Aoki (U. of Tokyo), who generated an excellent RNA-sequencing (RNA-seq) dataset covering oocyte-to-embryo transition and provided expertise concerning gene expression analysis of the genome activation, Kristian Vlahovicek (Zagreb U.), whose laboratory provided bioinformatics expertise, and Richard M. Schultz (UCSC Davis) and the team leader, who provided expertise on gene expression in oocytes and early embryos, the team leader also on mammalian transcriptome analysis by RNA-sequencing. The effort succeeded to provide the first direct insight into nature and scale of the earliest phase of the zygotic genome activation (ZGA) when genes in the zygotic genome become first transcribed. In a comprehensive article published in EMBO Journal, it was shown that ZGA has a form of global promiscuous low-level transcription, which rarely produces functional gene products because of inefficient post-transcriptional processing (splicing and polyadenylation) of polymerase II transcripts.

Abe K, Yamamoto R, Franke V, Cao M, Suzuki Y, Suzuki MG, Vlahovicek K, Svoboda P, Schultz RM, Aoki F. (2015) The first murine zygotic transcription is promiscuous and uncoupled from splicing and 3' processing. EMBO J.;34(11):1523-37. doi: 10.15252/embj.201490648

In this publication, the team leader is a co-corresponding author and he made a major contribution to the data analysis and characterization of the phenomenon, wrote the draft of the article, and managed the publication process.

In the meantime, team's effort focused on annotation of long non-coding RNAs (lncRNAs, see the next section) and comparative analysis of mammalian maternal transcriptomes with a particular focus on contribution of retrotransposons to gene expression in the germline and its evolution. These analyses were lead by the team leader and were done in collaboration with group of Kristian Vlahovicek. This work yielded perhaps the most significant article of the group, which identified the largest contribution of a single retrotransposon family to gene expression remodelling and gene evolution discovered so far:

Franke V, Ganesh S, Karlic R, Malik R, Pasulka J, Horvat F, Kuzman M, Fulka H, Cernohorska M, Urbanova J, Svobodova E, Ma J, Suzuki Y, Aoki F, Schultz RM, Vlahovicek K, Svoboda P. (2017) Long terminal repeats power evolution of genes and gene expression programs in mammalian oocytes and zygotes. Genome Res.;27(8):1384-1394. doi: 10.1101/gr.216150.116.

The publication brings up several textbook examples how evolution remodels existing and generates new genes. It reports remodelling and rewiring of >1000 mouse genes by a single non-autonomous retrotransposon family known as MaLR. It shows how stochastically but stereotypically retrotransposons shape gene expression during OET during evolution. This work also describes evolution of new mammalian genes, including lncRNAs and even characterizes a new protein-coding gene expressed in mouse oocytes, which evolved *de novo* from a random sequence, which first became a long non-coding RNA, which then acquired protein-coding capacity.

The final experimental work concerning OET that was published during the evaluation period focused on the role of the CCR4-NOT deadenylase complex in maternal RNA degradation. The team produced TALEN-induced knock-out of *Cnot6l*, which encodes one of the catalytical subunits of CCR4-NOT and which was an interesting candidate for differential mRNA degradation observed in earlier OET transcriptome studies.

Horvat F, Fulka H, Jankele R, Malik R, Jun M, Solcova K, Sedlacek R, Vlahovicek K, Schultz RM, Svoboda P. (2018) Role of *Cnot6l* in maternal mRNA turnover. Life Sci Alliance. 2018 Jul 16;1(4):e201800084. doi: 10.26508/lsa.201800084.

We could show that loss of *Cnot6l* affects mRNA degradation mainly after fertilization and that transcriptome disturbance caused by its loss negatively affects development.

Finally, we spent the entire reporting period studying the role of terminal RNA transferase *Papd7* in conditional and CRISPR-mediated knock-out mouse models. This work revealed complex relationship between phenotype and underlying genetic makeup and has not been published yet. The complexity of the phenotype and dissection of associated molecular mechanisms just takes time it takes. In this case, it turned out that the gene encodes additional isoforms and that different types of knock-out yield phenotypes, which apparently reflect different roles of PAPD7 domains and isoforms. In the end, we used three different alleles to dissect PAPD7 function in vivo, particularly in the male germline, where it became apparent that it is somehow associated with retrotransposon repression.

This work is still in progress but should be finally completed during the next reporting period. That will make analysis the longest lab project because the first conditional *Papd7* mice were obtained ten years ago. The project turned into a nightmare when a microdeletion in a supposedly validated conditional construct ruined the first two years of work. It took another year to validate and produce correctly modified mice, expand the colony and start producing recombined alleles. Then it turned out that a critical exon deletion had a weaker phenotype than a LacZ fusion allele. This revealed existence of a truncated protein isoform translated after the critical exon. This lead to analysis of PAPD7 protein variants that could be produced under different conditions, which lead to a decision to produce a large CRISPR-mediated deletion of the gene to finally sort the issue … so much about stereotypical analysis of gene function *in vivo*.

Radek Malik, a senior researcher in the group also contributed to work on analysis of gene expression in oocytes conducted at the Institute of Animal Physiology and Genetics of the ASCR in Libechov, so additional two publications may appear on team's publication list. However, they are not included in this summary of group 28 as their intellectual ownership belongs to the Libechov team. Because of that, these papers should not be credited to group 28. The same applies to publications of Helena Fulkova that were not co-authored by the team leader petr Svoboda.

In addition to the experimental articles above, the team leader wrote or contributed to review articles covering this research topic, namely:

Svoboda P, Franke V, Schultz RM. (2015) Sculpting the Transcriptome During the Oocyte-to-Embryo Transition in Mouse. *Curr Top Dev Biol*.;113:305-49. doi:10.1016/bs.ctdb.2015.06.004.

Svoboda P, Fulka H, Malik R. (2017) Clearance of Parental Products. *Adv Exp Med Biol*.;953:489-535. DOI: 10.1007/978-3-319-46095-6_10

Svoboda P. Mammalian zygotic genome activation. (2018) *Semin Cell Dev Biol*. 84:118-126. doi: 10.1016/j.semcdb.2017.12.006.

Schultz RM, Stein P, Svoboda P. (2018) The oocyte-to-embryo transition in mouse: past, present, and future. *Biol Reprod*. 2018 Jul 1;99(1):160-174. doi: 10.1093/biolre/ioy013.

### II. Long non-coding RNAs in mouse oocytes and zygotes

During the reviewed period, the team was a member of a group of teams, which obtained Marie Curie Initial Training Network (FP7) funding. The team could recruit one PhD student who would study lncRNAs in oocytes and early embryos. This student, Sravya Ganesh, coordinated annotation and analyzed annotated lncRNAs in oocytes and early embryos (in collaboration with group of Kristian Vlahovicek). She also performed functional analysis of five lncRNAs using CRISPR-mediated knock-out. This work, mainly capitalizing on above-mentioned RNA-seq data produced by Fugaku Aoki's lab, yielded a comprehensive catalogue of 1400 most expressed OET lncRNAs.

Karlic R*, Ganesh S*, Franke V, Svobodova E, Urbanova J, Suzuki Y, Aoki F, Vlahovicek K, Svoboda P. (2017) Long non-coding RNA exchange during the oocyte-to-embryo transition in mice. *DNA Res*.;24(2):129-141. doi: 10.1093/dnares/dsw058. Erratum in: DNA Res.;24(2):219-220

*shared first authorship

This work identified an oocyte-specific class of lncRNAs that function as substrates for production of mRNA-targeting small RNAs in endogenous RNA interference (RNAi) in mouse oocytes. It also contains functional analysis of two maternal lncRNAs using CRISPR-based knock-outs – with weak or no phenotypes (a stereotypical pain of functional studies on lncRNAs). Research on maternal lncRNAs continued, three other lncRNAs were investigated but analysis of two was put on hold and most efforts focused on one lncRNA, named *Sirena1*, which is the most abundant lncRNA found in mouse oocytes. The article was accepted in 2019 but appeared in press in January 2020, so technically is not a part of the evaluation period although most of the work on it was done 2015-2019.

Ganesh S, Horvat F, Drutovic D, Efenberkova M, Pinkas D, Jindrova A, Pasulka J, Iyyappan R, Malik R, Susor A, Vlahovicek K, Solc P, Svoboda P. (2020) The most abundant maternal lncRNA Sirena1 acts post-transcriptionally and impacts mitochondrial distribution. Nucleic Acids Res. 2020 Jan 20. pii: gkz1239. doi:10.1093/nar/gkz1239.

*Sirena1* exemplifies evolution of a maternal lncRNA serving as a substrate for RNAi but it is unique because of its extreme abundance and apparent regulation by cytoplasmic polyadenylation – adoption of this mechanism by an lncRNA is unprecedented as this mechanism was studied for decades as a key mechanism controlling translation of dormant maternal mRNAs.

After expiration of ITN funding, lncRNA research was funded in part by Czech Science Foundation grant CERNA supporting a consortium of Czech RNA groups, and after that by NPU I program Biomodels4Health. In the end, the group has been saturated by frustrating work on lncRNAs without strong phenotypes to the point that it does not plan to invest into this RNA category more time, funds, and energy..

As it seemed advisable to keep funding bodies happy with team's publication output, two reviews focused on lncRNAs were also published:

Svoboda P. Long and small noncoding RNAs during oocyte-to-embryo transition in mammals (2017). *Biochem Soc Trans*. 45(5):1117-1124. doi: 10.1042/BST20170033.

Ganesh S, Svoboda P. (2016) Retrotransposon-associated long non-coding RNAs in mice and men. *Pflugers Arch.* 468(6):1049-60. doi: 10.1007/s00424-016-1818-5.

Arguably the most significant outcome on the work on lncRNAs in the germline was discovery that lncRNAs readily emerge thanks to retrotransposon-derived promoters & first exons. This supports a notion that, albeit spliced and polyadenylated, most lncRNAs are irrelevant opportunistic transcripts. This is well in line with evolutionary theory – most lncRNAs likely represent a stochastic transcriptional noise exposed to natural selection, which gives rise to a minority of functionally relevant lncRNAs.

### III. Mammalian small RNA pathways.

Most of team's effort & funding during the evaluation period was invested into work on small RNAs. The team set out to clarify misconceptions and experimental artifacts accompanying mammalian RNA for years.

Small RNA pathways in mammals are fascinating. Somatic cells employ essentially just one pathway, the microRNA (miRNA) pathway, where small RNAs selectively suppress translation and destabilize mRNAs. The key factor producing miRNAs from small hairpin precursors is RNase III Dicer. Mammals have only one Dicer, which is well adapted for miRNA production. And it seem it has been adapting for this role during vertebrate evolution (>500 million years). RNAi interference (RNAi), sequence-specific mRNA degradation induced by long dsRNA is mainly a defense pathway employed in plants and invertebrates as an innate immunity response. In mammals, RNAi pathway is vestigial with exception of mouse oocytes, where it is essential and suppresses retrotransposons as well as controls endogenous genes. Beyond oocytes is endogenous mammalian RNAi essentially physiologically irrelevant, despite a reports making the opposite claim. The third mammalian small RNA pathway is so-called piRNA pathway, which is a germline defense system against retrotransposons.

Remarkably, mouse oocytes are the only mammalian cell type where small RNAs from all three pathways are unambiguously present. Notably, unlike RNAi, miRNA and piRNA pathways are non-essential for mouse oocytes. The team investigated the molecular mechanisms underlying these observations and examined crosstalks between the three pathways, their common features and mouse-specific adaptations.

Although this work had low experimental paper output, so far, it has been the most exciting and interesting part of team's research. Small RNA research focused on three complementary objectives, which overlap with three work packages (WP) of an awarded ERC Consolidator grant *Dicer*-Dependent Defense in Mammals (D-FENS, 6/2015-6/2020) as follows:

WP1 tested how well is RNAi, the aforementioned ancient innate immunity system, tolerated *in vivo* and whether it improves antiviral defense in mice. The plan was to re-engineer RNAi in cultured somatic cells and in the mouse *in vivo*.

WP2 defined common and species-specific features of mammalian RNAi and miRNA pathways in order to understand co-existence of these pathways.

WP3 examined whether RNAi functionally complements the piRNA pathway, the key germline genome defense system against mobile elements, in suppression of mobile elements mice.

#### WP1 Dicer DExD domain & antiviral potential of RNAi

WP1 objective was exploring consequences of hyperactive RNAi *in vivo*. While RNAi is the main invertebrate antiviral pathway, its antiviral role in mammals is unclear. The team approached the issue by producing a "super RNAi" mouse model where the endogenous *Dicer* gene was modified to express an equivalent of Dicer$^{O}$, a truncated Dicer isoform naturally appearing in mouse oocytes. Dicer$^{O}$ is responsible for high RNAi activity in the oocyte; this was reported by the team in 2013 in a paper by Flemr et al. (10.1016/j.cell.2013.10.001). The

key person on this part of the project was Eliska Svobodova/Taborska, who is currently preparing her PhD thesis for defense.

Ectopic expression of Dicer$^{O}$ in embryonic stem cells (ESCs) suggested that a modified *Dicer* allele (denoted *Dicer$^{X}$*) might be sufficient to boost RNAi. During 2015 and 2016, the team explored constraints for highly active RNAi in cultured cells, in order to determine conditions for inducing effective RNAi *in vivo*. In mouse ESCs and fibroblasts (NIH 3T3 cell line), effects of different *Dicer* alleles, dsRNA binding proteins, suppression of sequence-independent effects of dsRNA, and different types of dsRNA substrates were tested. In cultured cells, RNAi could be boosted with genetic modifications ~100-fold. In 2019, the work on constraints of RNAi came out in an article in Life Science Alliance journal.

Demeter T, et al. (2019) Main constraints for RNAi induced by expressed long dsRNA in mouse cells. *Life Sci Alliance*. 2(1) pii: e201800289).

While in a (yet) non-impacted journal, this work is important because clarifies a decade of misconceptions about endogenous mammalian RNAi and provides a framework for explaining occasional detection of RNAi effects and commonly observed negative results, which had been discussed at scientific meetings but almost never published. As a part of WP3, this work shows that what is often presented as RNAi is hijacking of the mammalian miRNA pathway and most of these instances represent rather miRNA-like and non-canonical miRNA regulations rather than canonical RNAi.

The team currently works on a publication describing effects of RNAi reintroduced to mammalian cells through genetic modification *Dicer* in cell culture and in mice *in vivo*. The cell lines created during this work were examined for antiviral immunity *in vitro* in collaboration with Ulrich Elling from IMBA Institute in Vienna with negative results, so far. Similarly to ectopic expression experiments, endogenous *Dicer* modification in ESCs increases siRNA production ~10-fold. This makes RNAi activity still rather inefficient, unless a component of the interferon response is mutated. The team does that by eliminating protein kinase R (PKR). Combination of *Pkr* knock-out and Dicer modification boosts siRNA production ~100 fold and yields robust RNAi activity.

The team also published a review on mammalian Dicer function:

Svobodova E, Kubikova J, Svoboda P. (2016) Production of small RNAs by mammalian Dicer. *Pflugers Arch*.;468(6):1089-102. doi: 10.1007/s00424-016-1817-6

***Production of Dicer$^{X}$ mice with ubiquitously enhanced siRNA production***

The main aim of WP1 was production of mice expressing Dicer$^{X}$ in order to produce an *in vivo* model to test hypotheses concerning evolution of RNAi and its antiviral role in mammals. The team struggled with technical difficulties for several years during which two simpler *Dicer* alleles in mice were produced (denoted *Dicer$^{X1}$* and *Dicer$^{X2}$*), but they did not work as expected. The team also worked on a third modified allele (*Dicer$^{X3}$*), which employed a more complicated genetic engineering strategy (two guided nucleases & homologous recombination in ESCs followed by production of chimeric mice and germline transmission). In the end, it took four consecutive attempts with different engineered ESC clones of different background until a chimeric founder was obtained that finally transmitted the *Dicer$^{X3}$* allele into F1, which transmitted it to F2. This breakthrough came at the end of 2017. These animals have been in breeding and analysis since. For simplicity, *Dicer$^{X3}$* allele will be referred to as *Dicer$^{X}$* hereafter.

As the outlook for success for producing *Dicer$^{X}$* mice looked bleak during 2014-2017, the team also worked on a contingency plan - an inducible *Dicer$^{X}$* transgene. The team successfully produced mice carrying the transgene at the same time as it finally succeeded with the *Dicer$^{X}$* mouse model. The transgene model could have some advantages in terms of tissue-specific RNAi activation but the whole-animal *Dicer$^{X}$* overexpression is apparently lethal (consistent

with *Dicer*$^{X/X}$ phenotype). The transgene was thus put on hold (archived) to save team's bleeding budget.

The team also produced a control mouse for *Dicer*$^{X}$. It is a re-engineered wild-type *Dicer* where introns 2-6 were removed and replaced with a spliced cDNA segment. This control animal denoted *Dicer*$^{SOM}$ allows for distinguishing phenotypes caused by the truncated *Dicer* from phenotypes coming from elimination of intronic sequences (which could contain enhancers or other regulatory sequences). This mouse control model also became a valuable experimental model for WP3.

### *Analysis of Dicer*$^{X}$ *mice with ubiquitously enhanced siRNA production*

Mice can tolerate a single *Dicer*$^{X}$ allele but animals homozygous for *Dicer*$^{X}$ die perinataly, which suggests that the allele is functionally modified and not a simple lack-of-function allele (*Dicer* null embryos die early post-implantation). *Dicer*$^{X/+}$ animals have been further analyzed for RNAi activity and viral resistance (see the next section).

The phenotype of *Dicer*$^{X/X}$ animals was analyzed during 2019 with aim to obtain insights into the cause of their perinatal lethality. In collaboration with the Czech Centre for Phenogenomics (IMG infrastructure), the team completed analysis of embryo growth, obtained micro CT scans of embryos, and analyzed their small RNA transcriptome. Results show that *Dicer*$^{X/X}$ embryos undergo growth retardation between day E12.5 and E14.5 and exhibit dysregulation of many miRNAs. The molecular basis of miRNA expression changes is unclear but it is not a problem of miRNA cleavage fidelity and it is likely linked with relaxed substrate specificity and increased Dicer activity. This analysis continues beyond 2019.

Perinatal lethality of *Dicer*$^{X/X}$ demonstrates that the short Dicer isoform from oocytes reduces animal fitness when becoming the sole Dicer. This conflict with the miRNA pathway likely represents a barrier for reactivation of RNAi in mammals *in toto*. Mouse oocytes can employ endogenous RNAi because their miRNA pathway is non-essential.

### *Antiviral defense by hyperactive RNAi*

Analysis of antiviral effects *in vivo* was done in collaboration with Shubhada Bopegamage from the Slovak Medical University (SMU). Antiviral response in *Dicer*$^{X/+}$ animals is being tested using a coxsackie virus B3 model (+ssRNA virus from Enterovirus family). During 2018 and 2019, three infection experiments on two groups of mice transported to SMU from Prague were performed. The first group of mice was ~two weeks younger (transported to Bratislava before the third week) and yielded results suggesting a positive effect of *Dicer*$^{X}$ allele on fitness of infected mice.

qPCR data showed that the number of viral RNA copies was significantly higher in wild type mice than in *Dicer*$^{X/+}$ mice. Wild type mice also lost more weight than *Dicer*$^{X/+}$ mice and showed worse histopathology. Taken together, the infection was significantly milder in *Dicer*$^{X/+}$ mice in terms of histopathological changes and virus titres or RNA copies. The recovery was faster and complete in the Dicer mice at day 5.

The second mouse group, which was analyzed in two subsequent experiments in 2019, showed weaker infection and while *Dicer*$^{X/+}$ mice seemed to fare better, the difference was not as strong in the first experiment. Small RNAs sequencing from several infected tissues was inconclusive as putative viral siRNA levels were too low to draw any conclusions. Additional sequencing of samples with highest viral titters is in progress. We also cross mice with dsRNA-expressing mice and *Pkr*$^{-/-}$ mice to be able to evaluate changes in siRNA production in different genotypes.

To sum up, issues challenging the work plan of WP3 were solved. The team produced the *Dicer*$^{X}$ mouse model, which undergoes phenotype assessment and analysis of viral response.

It should be pointed out that the *Dicer*$^{X}$ model was being developed from 2014 until end of 2017. Then a large part of 2018 took bringing the model onto a pure B6 background. We are writing up the first publication from this work at the moment. That's how long some of team's projects take and the team leader hopes that the evaluation committee will understand and convey this to CAS. The team leader has had enough of CAS criticism of high costs and low productivity at IMG.

**WP2 Revising the role of RNAi in the female germline in mammals.**

WP2 aims at understanding the roles of RNAi and miRNA pathways during mammalian OET. In particular, WP2 addresses relationship between RNAi, retrotransposon suppression, and control of gene expression in mammalian oocytes. The objective is to find common and species-specific functions and adaptation of RNAi. WP2 objectives were: 1) Explore evolutionary and functional aspects of maternal mRNA degradation by RNAi with a particular focus on MT- and pseudogene-derived endo-siRNAs and 2) Examine functional co-existence of RNAi and miRNA pathways in mammalian oocytes.

***Evolutionary and functional aspects of maternal mRNA degradation by RNAi***

We performed a systematic and thorough analysis of RNA-seq data from oocytes and early embryos from mouse, hamster, human, and cow. This work was published in Genome Research as mentioned above.

Franke V, Ganesh S, Karlic R, Malik R, Pasulka J, Horvat F, Kuzman M, Fulka H, Cernohorska M, Urbanova J, Svobodova E, Ma J, Suzuki Y, Aoki F, Schultz RM, Vlahovicek K, Svoboda P. (2017) Long terminal repeats power evolution of genes and gene expression programs in mammalian oocytes and zygotes. *Genome Res.*;27(8):1384-1394. doi: 10.1101/gr.216150.116.

A significant portion of this work was identification of lncRNAs carrying antisense pseudogene sequences, which produce regulatory siRNAs. Their evolution was in part fuelled by LTR insertions, thus providing an interesting nexus between retrotransposon repression by endogenous RNAi and evolution of RNAi targeting repertoire through retrotransposon insertions.

More recently, analysis of loss-of-RNAi phenotype was completed, which included time lapse microscopy and rescue experiments of RNAi phenotype (in collaboration with Petr Solc from the Institute of Animal Physiology and Genetics of CAS). In addition, thanks to development of GMO rat systems at the Czech Center for Phenogenomics, infrastructure of IMG, one of the genome modification experiment selected for trial production of genetically modified rats with RNAi-deficient oocytes. These rats were produced and their phenotype analysis will be performed in May 2020. This will finally answer the question whether the essential role of endogenous RNAi evolved in rodents earlier or it is a mouse-specific phenomenon.

***Functional co-existence of RNAi and miRNA pathways in mammalian oocytes***

In terms of analysis of miRNA and RNAi pathways in mammalian oocytes, a significant progress was made in analysis of RNAi and miRNA pathway capacity in bovine and porcine oocytes. These data helped to understand the molecular mechanism behind inactivity of the miRNA pathway in mouse oocytes, which was co-reported by the group in 2010 (10.1016/j.cub.2009.12.042). Current results suggests that miRNA inactivity in oocytes is likely a common case for mammalian if not most oocytes. miRNAs simply do not adapt their expression to the increasing cytoplasmic volume of a growing oocyte and become diluted to the point where their activity does not make a significant impact on the expanded maternal transcriptome. A manuscript by Kataruka et al. Low miRNA abundance disables microRNA pathway in mammalian oocytes (early preprint: https://doi.org/10.1101/757153) is being revised after the first round of review now.

Additional work on miRNAs during 2015-2019 included collaboration with Petr Bartunek's group on high-throughput screening for miRNA pathway inhibitors, which revealed how problematic is a common luciferase assay for miRNA activity when it is used for high throughput screening:

Brustikova K, Sedlak D, Kubikova J, Skuta C, Solcova K, Malik R, Bartunek P, Svoboda P. (2018) Cell-Based Reporter System for High-Throughput Screening of MicroRNA Pathway Inhibitors and Its Limitations. *Front Genet*. 9:45. doi:10.3389/fgene.2018.00045

Strategies for analyzing miRNAs were summarized in a review:

Svoboda P. (2015) A toolbox for miRNA analysis. *FEBS Lett*. 589(14):1694-701. doi: 10.1016/j.febslet.2015.04.054.

**WP3 Relationship of RNAi & piRNA pathways in the germline.**

WP3 studied maintenance of genome stability and control of interspersed mobile elements in the germline. It should yield original insights into functional redundancy between RNAi and piRNA pathways, allowing for defining general and species-specific features of piRNA and endo-siRNA origins and functions. The WP3 objective was to examine a possible functional overlap of RNAi and piRNA pathways. Specific objectives were: 1) Producing a hamster knock-out model to test whether piRNAs are important for mammalian oocytes naturally lacking significant expression of *Dicer*$^{O}$. 2) Investigating whether RNAi compensates lack of piRNAs in mouse oocytes.

***piRNA pathway knock-out in golden hamster***

The hamster knock-out, which started in 2015, has been another challenging long term project. The team successfully produced the hamster piRNA knock-out (CRISPR-induced *Mov10l1* exon 4 deletion) and have been breeding the animals since March 2018. Given their reproductive parameters (animals reach reproductive age in three months and their mating is way more complicated than mouse mating), the first homozygous knock-outs for analysis were obtained in 2019.

So far, it was established that both sexes of *Mov10l1* hamster homozygous mutants are sterile. This is quite surprising because mice lacking the pathways are fully fertile. Male knock-outs phenocopy *Mov10l1* knock-out in mice. Female sterility was confirmed upon repeated mating of seven null mutants. We also obtained ovarian histology and maternal transcriptomes from mutants. Off note is that transcriptome analysis in hamsters is a major bioinformatics challenge as the golden hamster genome is poorly assembled (not even available among the UCSC browseable genomes), and proper retrotransposon annotation is virtually non-existent.

Female sterility did not manifest as any apparent ovarian phenotype; maternal transcriptomes, except of retrotransposon transcripts, appear almost normal. This suggested that sterility would be caused by a meiotic or post-zygotic defect, which would manifest post-ovulation. While exciting, this complicates the analysis because there is no reliable *in vitro* culture protocol for meiotic maturation, fertilization, and cultivation of zygotes. Instead, one has to rely on producing zygotes *in vivo* upon superovulation, which is another obstacle when comparing that to superovulation in mice. Luckily, an excellent PhD student Zuzana Loubalova managed to establish a superovulation and mating system, which enabled examining meiotic maturation and zygotic genome activation in Spring 2020.

Sterility of piRNA knockout females is highly significant because it completely changes the view of significance of the piRNA pathway in mammals as well as interpretation of mouse data (see also the next section). Next to the *Dicer*$^{X}$ model, production of the hamster knock-out model has been a major milestone, state-of-the-art, high-risk etc. element of the D-FENS project. We hope to complete a publication reporting this part of the project in 2020 or 2021, depending how the publication process will go.

***piRNA & RNAi pathway redundancy in in mouse oocytes***

For analyzing redundancy of RNAi and piRNA pathways, we initially wanted to generate mice carrying a combination of piRNA knock-out (Mili-/-) and RNAi knock-out (MT-/- mice lacking *Dicer*$^{O}$, which were produced in the lab in 2013). While double-mutants did not show any additional phenotype, detailed analysis of MT$^{-/-}$ mice showed that there is residual Dicer activity, partially coming from a second, previously not recognized, Dicer$^{O}$ isoform driven by a recent MT insertion (this result was published in the 2017 Genome Research paper). The analysis was then halted until generation of *Dicer*$^{SOM}$, the new RNAi-deficient mouse model (the control mouse from WP1). The mouse colony was then expanded to obtain *Dicer*$^{SOM}$ homozygotes, which were crossed with piRNA-defficient *Mili*$^{-/-}$ mice. During 2018-2019, double mutants were analyzed and results were published in PLOS Genetics.

Taborska E, Pasulka J, Malik R, Horvat F, Jenickova I, Jelić Matošević Z, Svoboda P. (2019) Restricted and non-essential redundancy of RNAi and piRNA pathways in mouse oocytes. *PLoS Genet*. 15(12):e1008261. doi:10.1371/journal.pgen.1008261

This work demonstrates redundancy of piRNA and RNAi pathway in suppression of L1 retrotransposons in the female germline while no additional ovarian phenotype has been found double mutants. This is actually consistent with hamster data and leaves open possibility that RNAi masks a post-zygotic phenotype, which could not be found in mice because loss of RNAi causes meiotic spindle defect preventing further analysis.

## Research activity and characterisation of the main scientific results

*For result achieved in cooperation with other teams, in which the first AND corresponding author is not from our team, the team's contribution to the result is specified. This statement applies to 9 out of 31 publication outputs.*

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Our team performed transgenesis in amphioxus which was essential for evolutionary conclusions

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We provided essential tool for inactivation of genes in mouse retina an interpreted the data.

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## Research activity and characterisation of the main scientific results

### Structure-based design of carborane-based inhibitors of human carbonic anhydrase IX

In this project we focused on design of novel and original inhibitors targeting therapeutically relevant isoenzyme of human carbonic anhydrase (CA).
Human CAs are metalloenzymes with important roles in various physiological and pathological processes (e.g., tumorigenicity, obesity, and epilepsy). There are 14 CA isoforms in humans, several of which are established diagnostic and therapeutic targets. In particular, there is significant interest in the development of selective inhibitors targeting CA isoform IX (CAIX). CAIX is a tumor-associated transmembrane isoenzyme, the overexpression of which is induced by hypoxia. CAIX serves as a tumor marker and as a prognostic factor for several human cancers and thus represents a valuable target for antitumor therapy.
Design of a novel generation of selective inhibitors is the current challenge in the development of new therapeutic agents able to inhibit specific CA isoenzymes. Currently used CA inhibitors lack selectivity and cause numerous unwanted side effects. Conventional CA inhibitors contain a sulfonamide, sulfamate or sulfamide group connected to an organic moiety usually composed of an aromatic ring or conjugated ring system. In our structure-assisted design of CA inhibitors, we focused on designing inhibitors containing substituted boron clusters instead of the organic ring system. Various strategies have emerged to overcome the specificity problem and we have contributed to this effort by introducing dicarbarborane (with the broadly used trivial name carboranes) clusters as three-dimensional pharmacophores in sulfonamide and sulfamide CA inhibitors. Unlike planar compounds, carboranes possess a three-dimensional pharmacophore providing necessary hydrophobic interactions for filling hydrophobic cavities, and their role in the design of pharmacologically relevant molecules has been firmly established. Carboranes are non-toxic abiotic compounds that can increase interaction energy and have good *in vivo* stability and bioavailability.
The idea to use boron clusters were conceived from the analysis of X-ray structures of CAII in complex with isoquinoline-based inhibitors that inhibitors inhibited CAIX with $K_i$ values in the nanomolar range. Crystal structures of the enzyme-inhibitor complexes revealed two different binding modes within the active site of CAII and engagement of two opposite sides of the active site cavity (Figure 2A). Following this analysis, we hypothesized that the binding space within the enzyme active site cavity could be effectively filled by an inhibitor bearing a three-dimensional, hydrophobic scaffold (Figure 2B).

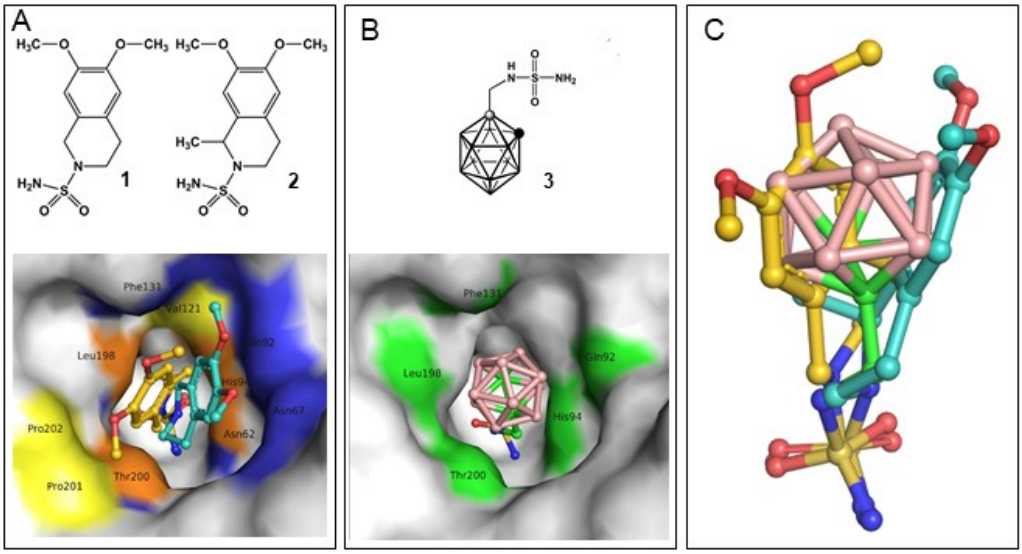


**Figure 1:** *Structure-inspired design of carborane inhibitors of human CA isoenzymes.*
**A.** Structural formulas of two isoquinoline-based compounds that inspired design of carborane sulfonamide inhibitors are shown along with a detailed overview of their binding to CAII (PDB codes 3IGP and 3PO6). The active site of CAII is shown in surface representation; residues interacting with **1** and **2** are colored yellow and blue, respectively. Residues interacting with both compounds are highlighted in orange. **B.** Structural formula of the lead carborane sulfonamide compound is shown along with its interactions with the CAII active site (PDB code 3MDG). **C.** Superposition of **1**-**3** as they bind to the CAII active site.

The crystal structure of CAII in complex with lead carborane sulphonamide compound confirmed binding to the enzyme active site in the predicted pose and revealed key interactions responsible for inhibitor binding and enzyme inhibition (Figure 1B. C). Subsequently, we designed and investigated a series of compounds containing *closo* and *nido* carborane clusters and further established that selectivity towards cancer-specific CAIX can be achieved.
During the evaluated period we used structure-assisted design and developed a series of more than 70 sulfamides incorporating carborane clusters. The lead compounds from this series inhibited CAIX with $K_i$ values in the low nanomolar or subnanomolar range, with some inhibitors being more than 1000-fold more selective for tumor-specific CAIX than CAII present in normal tissue[1]. The compounds demonstrated favorable *in vitro* toxicology and pharmacokinetics profiles and reduced tumor size in mice[1].This series of compounds also became the subject of successful patent applications (EP12786800.8, 16/3/2016; US9,290,529B, 22/3/2016).
This project was carried out in collaboration with Institute of inorganic Chemistry and Institute of Organic Chemistry and Biochemistry CAS and Institute of Molecular and Translational Medicine, Olomouc, CR.

**Structure-based design of inhibitors of human nucleotidases**

5′-nucleotidases are ubiquitous enzymes that catalyze the dephosphorylation of nucleoside monophosphates and thus help regulate cellular pools of nucleotides and nucleosides. Besides this role, 5′-nucleotidases affect the pharmacological efficacy of nucleoside analogs used as anti-viral and anti-cancer drugs since these compounds must be at first phosphorylated to exert a therapeutic effect. Intracellular 5'-nucleotidases are capable to dephosphorylate these nucleotide analogs and affect their therapeutic action. Therefore, the development of potent 5'-nucleotidase inhibitors may reverse drug resistance and increase the efficacy of clinically used nucleoside analogs.

Two human 5′-nucleotidases, cytosolic 5'(3')-deoxyribonucleotidase (cdN) and mitochondrial 5'(3')-deoxyribonucleotidase (mdN), were subjects of our structural studies. We have previously explored inhibition of cdN and mdN by *(S)*-1-[2-deoxy-3,5-*O*-(phosphono-benzylidene)-β-D-threo-pentofuranosyl]thymine substituted in the *para* position of the benzylidene moiety with various substituents. As a second approach to design specific mdN and cdN inhibitors, we modified the base moiety of lead compound with a second phosphonate group (Figure 2). Addition of the phosphonate group was intended to mimic the position of the phosphate ion found in the active site of mdN in several crystal structures we have determined. This phosphate ion, originating from the crystallization solution, interacts with the catalytic residues and magnesium ion and mimics the position of the substrate 3'-phosphate in the active site (Figure 2A). Thus, we proposed attaching the phosphonate group to C5 of the thymine base *via* various linkers, giving rise to a series of bisphosphonate compounds (Figure 2B).

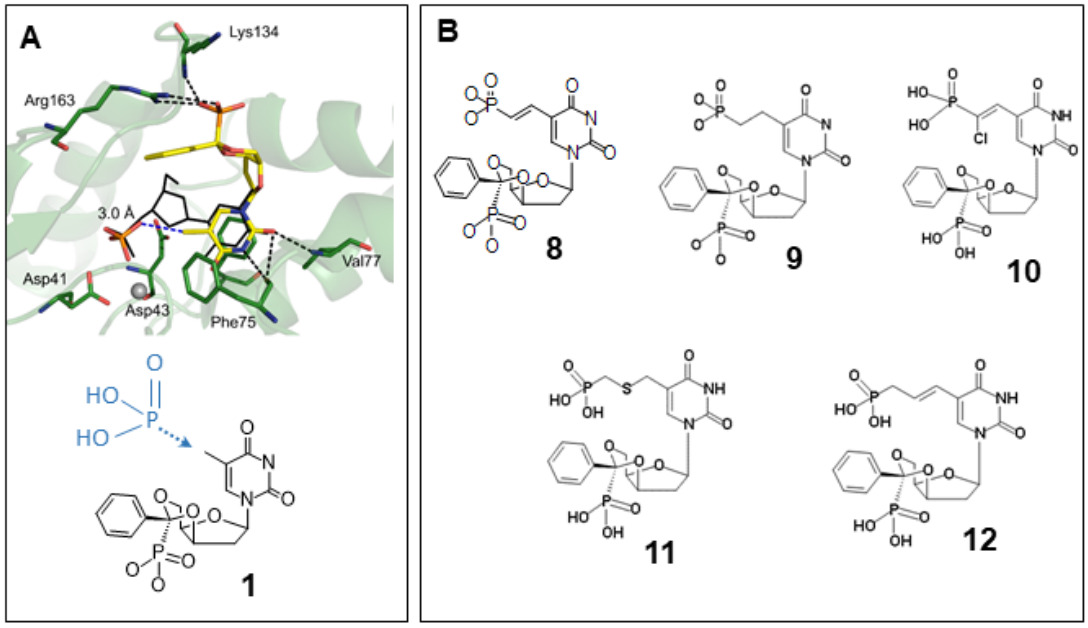


**Figure 2:** *Structure-inspired design of bisphosphonate human 5'(3')-deoxynucleotidase inhibitors.*
**A.** Structure of mdN (green) in complex with **1** (yellow sticks, PDB code 1Q91) superimposed with the position of dTMP from its complex with an inactive mdN mutant (solid black lines, PDB code 1Z4L). The dashed blue line indicates the distance (3 Å) between the base methyl and the phosphate oxygen, which was used to design attachment of a second phosphonate group to the base. **B.** Bisphosphonate compounds designed to explore the optimal linker connecting the phosphonate to the base.

The addition of a second phosphonate group substantially improved the inhibitory properties and also increased selectivity towards cdN. The strongest compound inhibited both enzymes at concentrations in the nanomolar range, making it the most potent inhibitor of these enzymes reported to date. In addition, some compounds showed selectivity for the cdN variant. Crystal structures solved for several inhibitors in complex with mdN or cdN provided a structural basis for understanding the inhibition profile of bisphosphonate compounds [2, 3].
In conclusion, during the evaluated period, we thus succeeded in rational design of conformationally constrained nucleoside phosphonic acid inhibitors of mitochondrial and cytosolic 5‘(3‘)-nucleotidases. We obtained high-resolution structural information about the interactions of inhibitors with cdN and mdN and uncovered two possible binding modes. Structural information about interactions with the mdN and cdN active sites can be applied to future inhibitor design efforts.

This project was carried out in collaboration with Institute of Organic Chemistry and Biochemisty CAS.

**Antibody fragments of potential diagnostic and therapeutic use**

We have employed several approaches of engineering recombinant antibody fragments, such as a recombinant single chain fragments (scFv) aiming at their use in structural studies and use in diagnostics and therapy.
We constructed scFv of MEM-85 antibody targeting hyaluronate receptor CD44 and used it as a tool for structural characterization of the CD44–antibody complex and identified epitope residues by NMR spectroscopy. We also built a rigid body model of the CD44–scFv MEM-85 complex using a low-resolution structure obtained by small-angle X-ray scattering [4]. This study provided detailed insight into the mechanism of antibody action. This project was carried out in collaboration with Institute of Organic Chemistry and Biochemisty CAS and EMBL Hamburg, Germany.
We also participated in design of polymers carrying toxic payloads or fluorescence tags that are specifically targeted by scFv fragments to cancer cells, in collaboration with Institute of Macromolecular Chemistry CAS. Two alternative approaches were used. In the first approach, the contact of the scFv with the polymer is mediated by the interaction of two peptides forming coiled-coil interface. Such interaction is specific and does not require any other chemistry for antibody-polymer conjugation. We have optimized the structure of targeted macromolecular therapeutic (with improved structure of the coiled-coil heterodimer between the HPMA based polymer and the scFv fragment targeting BCL1 leukemia and introducing pirarubicin instead of doxorubicin as a cytostatic drug) and evaluated its activity *in vivo* using a BCL1 leukemia model [5]. In the second approach, highly specific interaction between bungarotoxin covalently linked to the polymer and the recombinant scFv modified with a C-terminal bungarotoxin-binding peptide was used for attachement. By this approach, water-soluble polymer cancerostatic actively targeted against cancer cells expressing a disialoganglioside antigen GD2 was designed, synthesized and characterized. A polymer conjugate of an antitumor drug doxorubicin with a HPMA-based copolymer was specifically targeted against GD2 antigen-positive tumor cells using a recombinant scFv of an anti-GD2 monoclonal antibody [6].

**LEDGF/p75 interactome**

We contributed to structural analysis of important interactions of lens epithelium-derived growth factor LEDGF/p75 in collaboration with Institute of Organic Chemistry and Biochemisty CAS and KU Leven, Belgioum. LEDGF/p75 is a transcriptional co-activator that contributes to regulation of gene expression by tethering other factors to actively transcribed genes on chromatin. Its chromatin tethering activity is hijacked in two important disease settings: HIV and mixed lineage leukemia. The basis for the biological regulation of LEDGF/p75's interaction to binding partners has remained unknown.
During the evaluated period, we uncovered molecular features crucial for interaction with LEDGF/p75 with several binding partners. We found that multiple cellular proteins interact with LEDGF/p75 through a conserved partially unstructured consensus motif, as illustrated in Figure 3. This allowed us to identify previously unknown direct interactions between LEDGF/p75 and major transcriptional regulatory factors, such as IWS1 and Mediator complex [7].

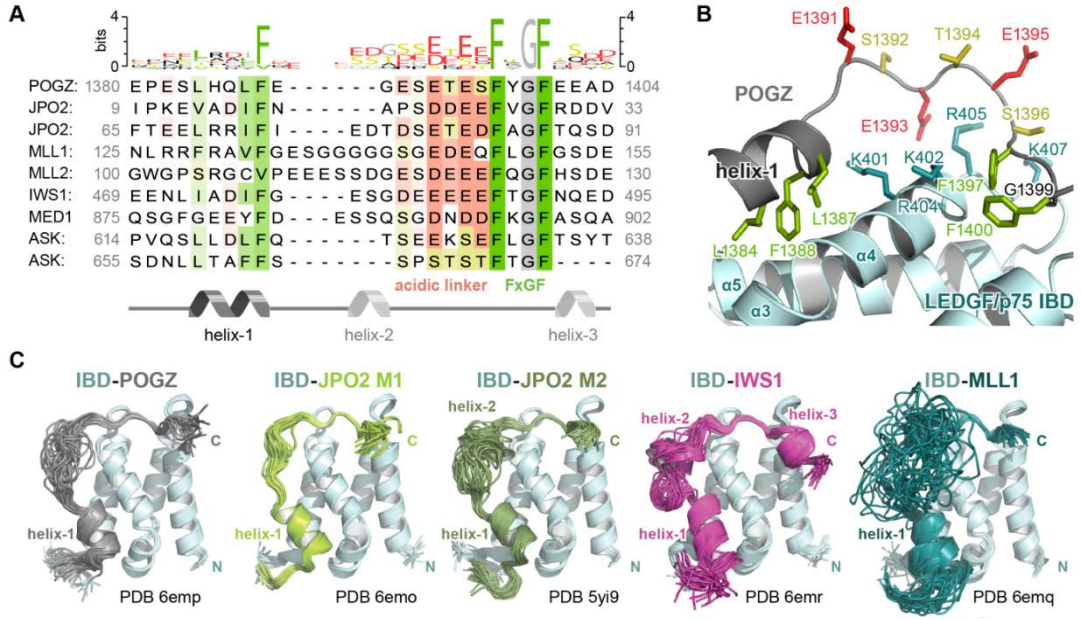


**Figure 3: LEDGF/p75 IBD binding partners interact in a structurally conserved manner.** (A) Multiple sequence alignment illustrating the conserved LEDGF/p75 IBD binding motif (IBM). (B) Detail of the POGZ IBM-IBD interface from the obtained solution structure. The linker region stabilizes the interaction by non-specific electrostatic contacts with the positively charged patch on the IBD surface. (C) Solution structures of the IBD in complex with the binding motifs from POGZ, JPO2 motif 1 and 2 (M1, M2), IWS1 and MLL1 determined by NMR spectroscopy.

## Other projects and collaborations

The team participated in several collaborative projects, where we contributed by our expertise in structural biology or in preparation of antibody fragments and recombinant proteins.
We collaborated on structural studies of enzymes with biotechnological potential [8-10] (collaboartin with Institute of Microbiology CAS) or with medicinal relevance [11-13] (collaboration with Institute of Organic Chemistry and Biochemstry CAS). We prepared antibody fragments for potential diagnostics and therapeutic applications [14-18] (collaboration with University of Chemical technology Prague and Charles university Prague). During years 2016-2019 the team were also involved in design of biosensors in collaboration with University of Chemical technology, Prague for detection of bacteria in drinking water (utility model 33021, Electrochemical cell for determining bacterial drinking water contamination).

## References:

(Authors and/or co-authors of the evaluated team are **in bold**; corresponding author from the evaluated team is marked by asterisk)
Complete list of publications: https://www.img.cas.cz/research/pavlina-rezacova/publications/

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2. Pachl, P., Simak, O., Budesinsky, M., **Brynda, J.,** Rosenberg, I. & **Rezacova, P.*** (2018) Structure-Based Optimization of Bisphosphonate Nucleoside Inhibitors of Human 5(3)-deoxyribonucleotidases, *Eur J Org Chem*, 5144-5153.

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13. Man, P., **Fabry, M., Sieglova, I**., Kavan, D., Novak, P. & Hnizda, A. (2019) Thiopurine intolerance-causing mutations in NUDT15 induce temperature-dependent destabilization of the catalytic site, *Biochim Biophys Acta Proteins Proteom.* **1867**, 376-381.

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## Research activity and characterisation of the main scientific results

The research activity of the Group can be roughly divided into two fields: cancer transcriptomics and bioinformatics, databases and genomics. Below we detail the research areas and provide related references.

*Cancer transcriptomics*. We have been involved in a long term-project focused on identification of specific genetic markers in cancer tissue isolates from patients with head and neck squamous cell carcinomas and skin cancers. We use Illumina microarray and sequencing technology and, in collaboration, immunohistochemistry for detection of appropriate gene sets. We concentrate on tumour microenvironment interactions between cancer associated fibroblasts and tumour cells. Recently we have published a summary of this collaborative work [3]. In the same period, we have collected a large data set of patient samples (>100 patients with triplicate samples) and cancer associated fibroblasts that are being analysed in the recently awarded project Center for Tumor Ecology - Research of the Cancer Microenvironment Supporting Cancer Growth and Spread supported by the Operational Programme Research, Development and Education. In collaboration with other groups of the Institute, members of the Laboratory became involved in research on transcriptional profiles of several models of malignant [8, 11] and immune diseases [4, 5] with focus on identification of new biomarkers or progressive molecular characteristics of the diseases that will be usable for diagnosis, prognosis or development of novel therapies.

*Bioinformatics and databases, genomics.* Together with data production by high-throughput technologies, we analyse the data for intramural users and develop new scientific software. The laboratory forms the local node of the Pan-European ELIXIR bioinformatics research infrastructure. We developed a pipeline called Scrimer that automates multiple steps for design of locus-specific PCR and genotyping primers from transcriptome data [10]. We curate and maintain the database of mitochondrial sequences coming from the ancient DNA samples AmtDB [1], which we use in our studies in paleogenomics, ancient DNA, human population genetics and history of populations in central Europe in Neolithic, Bronze Age and Iron Age and their connections to modern-day populations, Next, we developed and maintain the database of human endogenous retroviruses HERVd. In collaboration with the Laboratory of Viral and Cellular Genetics we study endogenous retroviruses also in other species [7, 9] and search for GC-rich genes deemed to be missing in avian genomes [6]. In partnership with other intramural and external groups we participated in a genomic study of evolutionary diversification of photoreceptor genes [12] and applied the methods of metagenomics and bioinformatics to estimate the genetic potential of microorganisms present in contaminated soil samples in order to disentangle the role of the plants and fertilization on their decontamination [2]. Recently, we started to collaborate with the laboratory of Radka Reifová (Charles University in Prague) on the study of closely related species of songbirds. Members of our team have already determined the genome sequence of common nightingale and we are currently sequencing the genome of another closely related species – thrush nightingale.

*Below, we provide the list of twelve most important publications of our team. Authors from our group are emphasized in bold. Between 2015 and 2019, we published or participated on altogether 80 scientific publications in impacted journals.*

1. **Ehler E, Novotný J**, Juras A, Chylenski M, **Moravcík O, Paces J**: AmtDB: a database of ancient human mitochondrial genomes. Nucleic Acids Res 2019 47(D1): D29-D32. [https://academic.oup.com/nar/article/47/D1/D29/5106144]

This article introduces our open database of ancient mitochondrial genomes. It is the most comprehensive published database focused entirely on human populations from prehistoric

and early historic times, which brings the full mitogenome sequences together with extensive metadata, together with advanced search and mapping tools. It is used by aDNA geneticists, anthropologists, archaeologists and other researchers from all over the world, having over 10,000 unique users in the last year.

This study was performed together with our colleagues from the Institute of Anthropology, UAM, Poznan, Poland. Vast majority of the work was performed by our part of the team, including study design, database development and administration, and article preparation, which is documented by both first (and second) author and corresponding authors of the article being from our laboratory.

2. **Ridl J, Kolar M**, Strejcek M, **Strnad H**, Stursa P, **Paces J**, Macek T, Uhlik O: Plants Rather than Mineral Fertilization Shape Microbial Community Structure and Functional Potential in Legacy Contaminated Soil. Front Microbiol 2016 7: 995. [https://www.frontiersin.org/articles/10.3389/fmicb.2016.00995/full]

Plant-microbe interactions are of particular importance in polluted soils. This study determined how selected plants and fertilization shape the structure and functional potential of microbial communities in contaminated soils. We combined shotgun and 16S rRNA amplicon sequencing followed by an innovative bioinformatical approach. Our results elucidate plant-microbe relationships in a contaminated environment with possible implications for the phyto/rhizoremediation of contaminated areas.

This manuscript was made in collaboration with the University of Chemistry and Technology, Prague. Members of our laboratory participated on the experimental design of the study and performed all the computational analyses of the sequencing data. M. Kolář developed bioinformatical methods, J. Rídl determined all DNA sequences and performed bioinformatical analyses. J. Rídl, the first author of the paper, and O. Uhlík (from UCT) wrote the manuscript together.

3. Kodet O, Lacina L, Krejčí E, Dvořánková B, Grim M, Štork J, Kodetová D, **Vlček Č, Šáchová J, Kolář M, Strnad H**, Smetana K Jr: Melanoma cells influence the differentiation pattern of human epidermal keratinocytes. Molecular Cancer 2015 14: Art. No. 1

Nodular melanoma is one of the most life threatening tumours with poor therapeutic outcome. Permissive microenvironment is essential for its progression. Features of this microenvironment are arising from the molecular crosstalk between the melanoma cells (MC) and the surrounding cell populations here represented by human primary keratinocytes (HPK). Using transcriptome profiling, we studied the effect of MC on HPK and observed induction of markers of a low differentiation state.

The clinical samples were collected by our long term collaborators from Charles University in Prague. RNA extraction, microarray analysis, bioinformatical and statistical analyses were performed in the Laboratory of Genomics and Bioinformatics, IMG, CAS. The resulting data were biologically interpreted in collaboration with our colleagues from Charles University in Prague and jointly published.

4. Plzák J, Bouček J, Bandúrová V, **Kolář M, Hradilová, M**, Szabo P, Lacina L, Chovanec M, Smetana, K Jr.: The Head and Neck Squamous Cell Carcinoma Microenvironment as a Potential Target for Cancer Therapy. Cancers 2019 11(4):440 [https://doi.org/10.3390/cancers11040440]

The tumour microenvironment participates in the control of local aggressiveness of cancer cells, their growth, and their consequent migration to lymph nodes and distant organs during

metastatic spread. In this review, we focus on the specificity of the microenvironment of head and neck cancer with emphasis on the mechanisms of intercellular crosstalk, their manipulation and potential therapeutic application.

This is a review article in which we summarize results of our long term collaboration with Charles University in Prague. It is based on original research articles in which the Laboratory of Genomics and Bioinformatics, IMG, CAS, performed RNA extraction, microarray analysis, bioinformatical and statistical analyses.

5. Balounová J, Šplíchalová I, Dobešová M, **Kolář M**, Fišer K, Procházka J, Sedlacek R, Jurisicova A, Sung HK, Kořínek V, Alberich-Jorda M, Godin I, Filipp D: Toll-like receptor 2 expression on c-kit cells tracks the emergence of embryonic definitive hematopoietic progenitors. Nat Commun 2019 10(1): 5176.

Hematopoiesis in mammalian embryos proceeds through three successive waves of hematopoietic progenitors. We show that toll-like receptors (TLRs) are expressed during early mouse embryogenesis and provide phenotypic and functional evidence that the expression of TLR2 marks the emergence of precursors of erythro-myeloid progenitors (EMPs). We demonstrate that the activation of the Tlr2 locus tracks the earliest events in the process of EMP and hematopoietic stem cells specification.

Transcriptomic and bioinformatical analyses were performed by M. Kolář from the Laboratory of Genomics and Bioinformatics, IMG, CAS. Experiment design, data interpretation and manuscript writing were performed mainly by the Laboratory of Immunobiology, IMG, CAS, within a long-term collaboration.

6. Yamano T, Dobeš J, Vobořil M, Steinert M, Brabec T, Ziętara N, Dobešová M, Ohnmacht C, Laan M, Peterson P, Benes V, Sedláček R, Hanayama R, **Kolář M**, Klein L, Filipp D: Aire-expressing ILC3-like cells in the lymph node display potent APC features. J Exp Med 2019 216(5): 1027-1037.

The autoimmune regulator (Aire) serves an essential function for T cell tolerance by promoting the “promiscuous” expression of tissue antigens in thymic epithelial cells. Aire is also detected in rare cells in peripheral lymphoid organs, but the identity of these cells is poorly understood. Here, we report that Aire protein–expressing cells in lymph nodes exhibit typical group 3 innate lymphoid cell characteristics that probably serve in the control of T cell responses.

Transcriptomic and bioinformatical analyses were performed by M. Kolář from the Laboratory of Genomics and Bioinformatics, IMG, CAS. Experiment design, data interpretation and manuscript writing were performed mainly by the Laboratory of Immunobiology, IMG, CAS, within a long-term collaboration.

7. Hron T, Pajer P, **Pačes J**, Bartůněk P, Elleder D: Hidden genes in birds. Genome Biol 2015 16: 164.

Bird genomes include regions with high GC content that in many cases make assembly of the sequenced genomes problematic or impossible. Here, we were able to identify many GC-rich genes that were previously claimed as missing in assembled bird genomes while present in the raw sequencing data.

Database development, bioinformatical analyses, and supervision of the bioinformatic part were performed by J. Pačes from the Laboratory of Genomics and Bioinformatics, IMG, CAS. Experiment design, data interpretation and manuscript writing were performed mainly by the Laboratory of Viral and Cellular Genetics, IMG, CAS within a long-term collaboration.

8. Hron T, Farkašová H, Padhi A, **Pačes J**, Elleder D: Life history of the oldest lentivirus: characterization of ELVgv integrations in the dermopteran genome. Mol Biol Evol 2016 33(10): 2659-69.

Endogenous retroviral elements belong to genomic repetitive nucleotide sequences. Here, we identified and described the so far oldest endogenous lentivirus. Contrary to other viral clades, endogenous lentiviruses are very rare and thus not easy to find. Specific bioinformatical approaches are necessary to identify them.

Database development, bioinformatical analyses, and supervision of the bioinformatic part were performed by J. Pačes from the Laboratory of Genomics and Bioinformatics, IMG, CAS. Experiment design, data interpretation and manuscript writing were performed mainly by the Laboratory of Viral and Cellular Genetics, IMG, CAS within a long-term collaboration.

9. Hrckulak D, **Kolar M, Strnad H**, Korinek V: TCF/LEF Transcription Factors: An Update from the Internet Resources. Cancers (Basel) 2016 8(7).

The mammalian TCF/LEF family comprises four nuclear factors that display common structural features and are often expressed in overlapping patterns implying their redundancy. Such redundancy was indeed observed in gene targeting studies; however, individual family members also exhibit unique features that are not recapitulated by the related proteins. In this review, we summarized current knowledge about the specific features of individual TCFs.

Bioinformatical analyses of publicly available databases and data mining of public web tools were performed by M. Kolář and H. Strnad from the Laboratory of Genomics and Bioinformatics, IMG, CAS. Data interpretation and manuscript writing were performed mainly by the Laboratory of Cell and Developmental Biology, IMG, CAS, within a long-term collaboration.

10. **Farkašová H**, Hron T, **Pačes J**, Hulva P, Benda P, Gifford RJ, Elleder D: Discovery of an endogenous Deltaretrovirus in the genome of long-fingered bats (Chiroptera: Miniopteridae). Proc Natl Acad Sci U S A 2017 114(12): 3145-3150.

Endogenous retroviral elements belong to genomic repetitive nucleotide sequences. Here, we discovered and described the first endogenous deltaretrovirus. Contrary to other viral clades, there were no known endogenous delta retroviruses prior to this article. Specific bioinformatical approaches were necessary for its identification.

Database development, bioinformatical analyses, and supervision of the bioinformatic part were performed by J. Pačes from the Laboratory of Genomics and Bioinformatics, IMG, CAS. Experiment design, data interpretation and manuscript writing were performed mainly by the Laboratory of Viral and Cellular Genetics, IMG, CAS within a long-term collaboration.

11. Mořkovský L, **Pačes J, Rídl J**, Reifová R: Scrimer: designing primers from transcriptome data. Mol Ecol Resour 2015 15(6): 1415-20.

With the next-generation sequencing methods, it has become possible to obtain genome-wide sequence data even for non-model species. Currently, there is no available software for automated design of PCR and genotyping primers from next-generation sequence data. We developed the Scrimer pipeline that automates multiple steps: adaptor removal, read mapping, selection of SNPs and multiple primer design from transcriptome data. The primers can be used in conjunction with several genotyping methods.

This work was accomplished in collaboration with the Faculty of Science, Charles University in Prague. J. Rídl did the library preparations and sequencing of the testing data set. J. Pačes contributed initial data processing and participated on the pipeline design. Our colleagues from R. Reifová laboratory designed the study, developed the Scrimer software, wrote the Scrimer documentation and prepared the manuscript.

12. Hubackova S, Pribyl M, Kyjacova L, Moudra A, Dzijak R, Salovska B, **Strnad H**, Tambor V, Imrichova T, Svec J, Vodicka P, Vaclavikova R, Rob L, Bartek J, Hodny Z: Interferon-regulated suprabasin is essential for stress-induced stem-like cell conversion and therapy resistance of human malignancies. Mol Oncol 2019.

To elucidate the mechanisms of resistance to radio- and chemotherapy, we exposed human cancer cell lines to clinically relevant doses of 5-azacytidine or radiation and compared the transcriptomes of surviving cell populations, including low-adherent stem-like cells. Mobilisation of these cells was prevented by knockdown of Erk 1/2 and interferon responsible factor 1, indicating that loss of adhesion and anoikis resistance required an active Erk pathway interlinked with interferon signalling.

Transcriptomic and bioinformatical analyses were performed by H. Strnad from the Laboratory of Genomics and Bioinformatics, IMG, CAS. Experiment design, data interpretation and manuscript writing were performed mainly by the Laboratory of Genome Integrity, IMG, CAS.

# Research activity and characterisation of the main scientific results

## 1. Studies on cellular senescence

Cellular senescence, a specific phenotype of proliferating cells evoked by persistent activation of cell cycle checkpoints, is implicated in fundamental (patho)physiological processes such as fetal development, wound healing, aging and aging-associated diseases including cancer and as such is the main research topic of the laboratory. Our involvement in investigation of cellular senescence spans several yet unclear topics such as 1) the nature of irreparable/persistent DNA damage response (DDR) driving development of senescence, 2) the mechanism of senescence induction and understanding of senescent phenotype, namely 3) the adverse effects of cellular senescence mediated by the patho/physiological actions of secreted factors and molecular mechanisms behind them including the role of senescent cells in modulating tissue microenvironment and malignant traits of cancer cells including therapy resistance, and 4) development of new pharmacological approaches specifically targeting senescent cells (i.e., senolytics and senostatics). Our main achievements in this area are shortly described further.

### *1.1. Mechanisms of cytokine-induced cellular senescence*

At the beginning of evaluated period we continued to explore our concept of DNA damage response-mediated vicious cycle (Bartek et al., 2008) and published a study (Hubackova et al., 2015), in which we uncovered the missing part of the circle – the molecular mechanisms linking the DNA damage response with genotoxic effects of cytokine-driven signaling pathways. Specifically, we showed that two cytokines TNF□ and IFN□□ shown to induce senescence in mouse pancreatic □-cancer cells, induce NADPH oxidases Nox4 and Nox1, reactive oxygen species (ROS), DDR and senescence in human normal and cancerous cells. The expression of Nox4 required unperturbed JAK/STAT signaling and, importantly, the effect was mediated by downstream activation of TGF□/Smad pathway. Additionally, the expression of ANT2 was suppressed by IFN□ contributing to cytokine-induced genotoxicity. Our data also revealed some unexpected differences between cytokine effects in mouse and human cells, and mechanistically implicated the TGF□/SMAD pathway, via induction of NADPH oxidases and suppression of ANT2, as key mediators of IFN□/TNF□-evoked genotoxicity.
In the follow up study performed in collaboration with Prof. Jiri Neuzil's laboratory (Hubackova et al., 2019a), we extended our 20 years long involvement in investigation of the ANT2 gene (Barath et al., 1999) to show that ANT2 protects cancer cells against anticancer agent mitochondria-targeted tamoxifen MitoTam. Importantly, besides potent anticancer effects, MitoTam has been identified in this study as a very efficient senolytic drug, four times more effective then current inhibitors of Bcl2 protein family such as ABT263 (Navitoclax). The sensitivity of senescent cells to MitoTam is caused by low levels of ANT2 inherent to senescent state.

### *1.2. Unraveling phenotype and specific markers of senescent cells*

Currently, a determination of senescent state relies on a set of unspecific markers. To allow to specifically target senescent cells with senolytic therapies, we aimed to identify new cell-surface proteins differentially expressed on human senescent cells (Mrazkova et al., 2018). Employing differential SILAC proteomic analysis of cell surface proteins, we identified more than 70 proteins specifically enriched on the surface of senescent cells including neural adhesion molecule L1CAM, a potent oncogene, which presence in human senescent cancer cells is associated with increased oxidative stress and altered cell migration properties. We showed that L1CAM is controlled by a number of cell growth- and metabolism-related pathways during development of senescent cells. Functionally, senescent cells with enhanced surface L1CAM showed increased adhesion to extracellular matrix and migrated faster.

To understand dynamics of senescent phenotype development on proteome level and also to assess effects of oxidative stress on protein thiol residue oxidation and protein function (manuscript in preparation) we developed a new analytical method capable of simultaneously assessing cellular protein expression and cysteine oxidation (Vajrychova et al., 2019). Using this method, we revealed a specific pattern of redox changes in peroxiredoxins in a short time-interval of cell exposure to hydrogen peroxide.

### *1.3. Role of senescent secretome in tumor biology*

In two collaborative studies with Laboratory of Immunological and Tumour Models, IMG, we studied the effect of senescent cells on tumor growth of cancer cells in syngeneic mouse model (Simova et al., 2016). Here we showed that observed acceleration of tumor growth was attributable to a protumorigenic environment created by the co-injected senescent and proliferating cancer cells rather than to escape of the docetaxel-treated cells from senescence. Importantly, accelerated tumor growth was effectively inhibited by cell immunotherapy using irradiated TC-1 cells engineered to produce interleukin IL-12. Collectively, our data document that immunotherapy, such as the IL-12 treatment, can provide an effective strategy for elimination of the detrimental effects caused by bystander senescent tumor cells in vivo.
In the second study (Sapega et al., 2018), we characterized two different types of senescent cancer cells in relation to the nature of senescence inductors and secretory phenotype and its capability to induce secondary 'bystander' senescence. The described cell line- and treatment-associated differences in the phenotypes of senescent cells may be relevant in optimization of cancer chemo- and immunotherapy.

Altogether, these studies provide mechanistic insights into senescence of human and mouse cells and pave new ways for development of novel strategies for the remedy of human diseases where senescent cells play a crucial pathogenetic component.

## 2. Mechanisms of DNA damage response and repair

In our effort to understand cellular responses to DNA damage we continued to study multiple facets of this phenomenon utilizing different approaches and experimental models.

One of our long term interests is an understanding a role of promyelocytic leukemia protein (PML) and PML nuclear bodies in irreparable/persistent DNA damage response. Co-associations of PML nuclear bodies with sites of persistent DNA damage foci is characteristic feature of senescent cells (Hubackova et al., 2012; Hubackova et al., 2010; Janderova-Rossmeislova et al., 2007). In follow up of these studies we showed that PML is involved in modulation of DNA repair (Vancurova et al., 2019). In this study, we unraveled that an accrual of the PML to persistent DNA damage lesions is dependent on RNF168/53BP1 axis of DNA repair machinery. Importantly, we found that PML-deficient cells are more prone to low-dose of ionizing radiation and to DNA damaging agents causing such damage that must be repaired by homologous recombination-directed DNA repair. Clinical relevance of these findings is supported by the fact that PML is downregulated in up to two thirds of human solid tumors. Link between PML and HDR repair may help to extend the concept of synthetic lethality between PARP inhibition and HR defects.
Furthermore, in continuation to our previous study (Janderova-Rossmeislova et al., 2007) we investigated associations of PML with nucleolar surface (termed PML-nucleolar associations; PNAs) formed during nucleolar stress induced, besides others, by topoisomerase inhibitor doxorubicin (Imrichova et al., 2019). Using the state-of-the-art microscopic techniques including super-resolution and time lapse life microscopy we described very complex spatiotemporal dynamics and three-dimensional structure of PNAs. The most important results of this study demonstrate continuous transition between individual stages of PNAs and their

spatial reorganization in relation to changes of activity of RNA polymerase I (RNAPI; inhibited and reactivated in response to onset and recovery nucleolar stress, respectively). The other findings of this study also indicate that PML-nucleolar compartment can be involved in repair of genes encoding ribosomal DNA (rDNA) or in sorting of unrepaired rDNA.
We also identified the signals and analyzed PML domains responsible for PNAs formation (Imrichova at al., manuscript in preparation). Importantly, we defined conditions at which PNAs form with the highest probability, i.e. the combination of topoisomerase and RNAPI inhibition. Deletion analysis and mutagenesis of PML revealed SUMO-interacting motif regulated by CK2-dependent phosphorylation of adjacent serine rich region together with PML exon8B to be involved in interaction of PML with nucleolus. Finally, we proved a presence of SUMO signal on rDNA during formation of PNAs. Thus, the SUMOylation of proteins binding rDNA could be involved in interaction of PML with nucleolus.

In collaboration with our colleagues from Department of Genome Integrity in Olomouc, we explored new therapeutic options based on clinically available drugs with anticancer effects, including inhibitors of PARP1 enzyme (PARPi), and histone deacetylases (vorinostat), respectively, and disulfiram (DSF, known as alcohol-abuse drug Antabuse) and its copper-chelating metabolite CuET that inhibit protein turnover (Majera et al., 2019).

Transcription-replication conflicts (TRCs) represent a significant source of genomic instability in cells experiencing DNA replication stress e.g. in precancerous lesions. Although there is a great deal of knowledge about the strategies that cells evolved to avoid TRCs, understanding of how a replication fork restarts DNA synthesis upon a TRC remains elusive. We have found that RECQ5 helicase binds to RNA polymerases I and II and suppresses transcription-associated genomic instability by promoting resolution of conflicts between transcription and replication machineries. We have shown that RECQ5 promotes replication fork progression through actively transcribed genes and prevents accumulation of RAD51 recombinase at sites of transcription-replication interference (Urban et al., 2016). Recent studies have shown that head-on TRCs promote the formation of R-loops, which represent a potent block to replication fork progression. R-loops are generated by invasion of the nascent RNA transcript into the DNA duplex behind the RNA polymerase, leading to the formation of an RNA:DNA hybrid, with extruded single-strand DNA (ssDNA). We have developed a cellular system for isolation and detection of R-loops and used this tool to study the cellular factors contributing to replication stress sensitivity. We have identified the pre-mRNA cleavage factor WDR33 as regulator of replication stress resilience and demonstrated that, when WDR33 function is impaired, unreleased nascent transcripts and genomic loci re-localize toward the nuclear periphery, where they cause replication stress and DNA damage (Teloni et al., 2019). Our recent studies have shown that replication restart upon R-loop-mediated TRCs relies on MUS81 endonuclease, RAD52 single-strand annealing protein and the DNA ligase 4/XRCC4 complex (Chappidi et al., 2020). Moreover, our studies provided insights into the molecular mechanism underlying the activation of ATR kinase, a master regulator of the cellular response to DNA damage. Specifically, we have identified the mismatch-binding protein MutSβ as a new DNA damage sensor in the process of ATR activation by replication-associated DSBs. Our work has demonstrated that MutSβ binds to hairpin loops persisting in RPA-coated single-stranded DNA (ssDNA) at sites of DNA damage and mediates the recruitment of the ATR-ATRIP complex, a prerequisite for ATR activation by TOPBP1. Based on these findings, we have proposed a model wherein the formation of hairpin loops in ssDNA generated at sites of DNA damage could signal lack of free RPA in the cell and serve as a trigger for ATR activation in a process mediated by MutSβ (Burdova et al., 2015).

Our research uncovers molecular mechanisms that enforce genomic stability under conditions of replication stress. Understanding the cellular responses to replication stress is crucial to the understanding of tumorigenesis and for development of new approaches of therapeutic interventions and diagnostic tools in early stages of cancer.

Next three studies upon DNA damage response were also performed in collaboration and focused on 1) the role of protein DAXX, a regular component of PML nuclear bodies, in DDR (Brazina et al., 2015), identification of components of ubiquitylation machinery involved in DNA repair (Frankum et al., 2015), and 3) distinct dynamics of cellular responses (oxidative and DNA replication stress) to oncogenic activation c-Myc and H-Ras (Maya-Mendoza et al., 2015).

**3. Mechanisms of cancer resistance**

Among the most important results obtained in this evaluation period is the contribution to mechanisms of therapeutic resistance of cancer cells. In two related studies we investigated mechanisms of radioresistance of prostate and breast carcinoma cell lines (Hubackova et al., 2019b; Kyjacova et al., 2015). We have found that population of cancer cells surviving fractionated ionizing radiation contains, besides adherent senescent cells, also low-adherent anoikis-resistant subpopulation of cells with stem cell-like phenotype (Kyjacova et al., 2015). This is the first report linking cellular senescence with cancer stem cell properties. Based on genome profiling comparing all radioresistant subpopulations with parental cells (Hubackova et al., 2019b) we found next that anoikis- and radio-resistant fraction of cancer cells can be mobilized also by IFNgamma signaling and its anoikis-resistant survival is in part dependent on IFNgamma pathway-mediated Erk signaling. We identified a new gene - a novel protooncogene suprabasin (SBSN), taking part in IFNgamma/Erk-mediated anoikis resistance. The main significance of this study is the finding that stress-activated interferon response is a common denominator regulating the anoikis-resistance of radio- and chemo-resistant survivors via Erk pathway. Moreover, SBSN broadens out the family of genes involved in therapy-resistance protecting the stem-like cancer cells against anoikis. Importantly, we found the expression of SBSN in human samples of colorectal and ovary carcinomas. Using mouse model we demonstrated that its expression is associated with metastatic process.
One of the unexpected findings is the expression of SBSN in bone marrow isolated from myelodysplastic syndrome (MDS) patients, where the level of SBSN correlates with disease stage (Pribyl et al., accepted).

**4. Role of DNA damage and DNA damage response in human diseases**

Besides the basic research described above we investigated the role of DNA damage response in pathogenesis of human hemato-oncologic diseases.

***4.1. Myelodysplastic syndrome***

There is emerging evidence that both primary and secondary MDS is associated with DNA damaging activity in bone marrow. Based on our in vitro studies that 5-azacytidine can induce cellular senescence and proinflammatory cytokine secretion we investigated the levels of eleven proinflammatory cytokines in bone marrow plasma of MDS patients during progression of the disease and in relation to 5-azacytidine therapy (Moudra et al., 2016). Overall, we have found that all forms of MDS feature a deregulated proinflammatory cytokine landscape in the bone marrow and such alterations are further augmented by therapy of MDS patients with 5-AC.

NAD(P)H quinone dehydrogenase 1 (NQO1) is an enzyme that detoxifies quinones and reduces oxidative stress. The naturally occurring germline polymorphism of NQO1 (NQO1*2) with a cytosine-to-thymidine substitution at nucleotide position 609 of the NQO1 cDNA results in the loss of NQO1 activity and rapid degradation of the protein encoded by NQO1*2 due to an unstable structure. The loss of NQO1 activity can result into disease susceptibility and therapeutic response (Fagerholm et al., 2008). Examining a cohort of 187 MDS patients we studied how the presence of NQO1*2 polymorphism affects the development and progression of MDS and assessed the correlation between NQO1*2 and the overall survival (OS, calculated from the date of diagnosis), presence of karyotypic abnormalities, and response to

hypomethylating therapy in MDS patients (Moudra et al., 2019). We found that the presence of NQO1*2 alleles is associated with higher probability of the development of MDS, faster disease progression, sensitivity to blood transfusion-provoked iron overload, and shorter expected OS.

### *4.2. Polycythemia vera*

Polycythemia vera (PV) is another myeloproliferative preleukemic disease with oncogenic signaling and inflammatory component. Despite the high risk and DNA-damaging inflammatory microenvironment, PV progenitors tend to preserve their genomic stability over decades until their progression to post-PV myelofibrosis/acute myeloid leukemia. In collaboration with the group of V. Divoky (Faculty of Medicine and Dentistry, Palacky University, Olomouc, Czech Republic) we studied the mechanisms behind this paradox. Using induced pluripotent stem cells-derived CD34+ progenitor-enriched cultures from JAK2V617F+ PV patient and from JAK2 wild-type healthy control, and patients' bone marrow sections from different disease stages, we demonstrate that JAK2V617F induces an intrinsic IFNγ- and NF-κB-associated inflammatory program, while suppressing inflammation-evoked DNA damage both in vitro and in vivo by overexpression the dual-specificity phosphatase 1 (Stetka et al., 2019). We contributed to this study with our expertise in DNA damage response and cytokine signaling (J. Bartek and Z. Hodny).

## 5. Development of new therapeutic strategies

### *5.1. Photothermal therapy of cancer with gold nanorods*

In previous evaluation period we initiated studies aiming to develop novel nanotechnology-based approaches for cancer treatment. We continued to investigate various aspects of so called photothermal therapy using gold nanorods. In our first published study on this topic (Zarska et al., 2016) we described the cellular uptake and subcellular fate of cationic gold nanorods stabilized by quaternary ammonium compound (16-mercaptohexadecyl) trimethylammonium bromide (MTAB) as a two-step process composed of passive adhesion to cell membrane sulfated proteoglycans and active transmembrane/intracellular transport via clathrin-mediated endocytosis and macropinocytosis. This specific kind of gold nanorods features very high cellular uptake suitable for photothermal therapy. In following study studying biological response of the (16 mercaptohexadecyl)trimethylammonium bromide-modified cationic gold nanorods (MTAB-GNRs) (Zarska et al., 2018) we reported that MTAB-GNRs) do not disturb the cellular homeostasis and do not induce severe stress response and genotoxicity in vitro and acute toxicity in vivo in the mouse animal model despite their outstandingly high accumulation in cells and retention in the spleen after intravenous administration. MTAB-GNRs are thus a promising tool for future evaluation of photothermal therapy in preclinical trials.

In collaboration with organic chemists from University of Hradec Kralove and physicists from Czech Technical University in Prague we developed new surfactants for coating of gold nanorods to study the effects of surfactant chemistry on biophysical (thermal) and biological (cell uptake and toxicity) properties (manuscript in preparation). In frame of this research we developed a novel highly hydrophilic cationic surfactant composed of the quaternary ammonium group and ethylene glycol chain that reduces the compound cytotoxicity in the free state while allowing the preparation of stable nanorods with high cellular uptake and lysosomal localization (Salajkova et al., 2019).
In collaboration with 2nd Faculty of Medicine, Charles University, Prague, we started to develop experimental approaches of photothermal therapy for treatment of glioblastoma using cranial window technique in mouse.

**6. Reviews:**

In the evaluated period we were invited to write reviews and commentaries in the area of our expertise (Andrs et al., 2016) (Hodny et al., 2016) (Urban et al., 2017) and one book chapter (Hubackova et al., 2016).

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## Research activity and characterisation of the main scientific results

### 1.1 Neural stem cells

Neural stem cells (NSCs) are defined by their dual ability to self-renew through mitotic cell division or differentiate into the varied neural cell types of the central nervous system. DISP3/PTCHD2 is a sterol-sensing domain-containing protein, highly expressed in neural tissues, whose expression is regulated by thyroid hormone. We used a mouse NSC line to investigate what effect DISP3 may have on the self-renewal and/or differentiation potential of the cells. We demonstrated that NSC differentiation triggered a reduction in DISP3 expression. Moreover, when DISP3 expression was disrupted, the NSC “stemness” was suppressed, leading to a larger population of cells undergoing spontaneous differentiation, while DISP3 overexpression primed cells to proliferate faster. Together, our findings showed that in NSCs, DISP3 is important regulator of the transition from the self-renewal to the differentiation state (Konirova et al. 2017).

Cranial irradiation is a useful tool for the treatment of primary and metastatic brain tumors; however, the use of such therapy is accompanied by devastating side effects. The impact of radiation-induced damage to the brain is multifactorial, but the damage to NSC populations seems to play a role. We examined the effects of irradiation on NSCs isolated from the ventricular-subventricular zone of mouse brain. Our findings describe the increased transcriptional activity of p53 targets and proliferative arrest after irradiation. Moreover, we showed that most cells do not undergo apoptosis after irradiation but rather cease proliferation and start a differentiation program, which may represent a mechanism whereby damaged NSCs eliminate potentially hazardous cells and circumvent the debilitating consequences of cumulative DNA damage (Konirova et al. 2019, Michaelidesova et al. 2019).

### 1.2 Modifiers of epigenetic silencing

Endogenous retroviruses and other repetitive sequences represent an ever present source of risk, either from retroviral reactivation or as a cause of genomic recombination. Cells mitigate this risk by enveloping these elements in tightly packed constitutive heterochromatin, a process that is mediated by specific epigenetic mediators. Several years ago a forward genetics screen was established in the Emma Whitelaw laboratory to identify epigenetic factors that contribute to the deposition of constitutive heterochromatin in mice. These factors were termed modifiers of murine metastable epialleles (*Momme*) and our group has continued to study the role of a few of the genes identified in this screen. We have focused mainly on the mouse lines *MommeD6* and *MommeD20* which were found to contain point mutations in the gene *Fam208a/Tasor*, a gene that until recently has been largely uncharacterized.

The *MommeD6* and *MommeD20* mouse lines are both homozygous lethal. A detailed embryological analysis of these lines showed that the embryos were capable of initiating gastrulation but exhibited a fitness defect that limited their developmental progression. We showed that the fitness defect caused activation of p53 signalling and importantly, could be partially rescued by p53 inactivation. Transcriptional profiling of mutant embryos before the onset of developmental phenotypes, identified significant activation of genes associated with p53 signalling as well as a subclass of long non-coding RNAs termed the pluripotency-associated transcripts (Platr). These results indicated a delay in epigenetic silencing of specific loci already in the pluripotent cells of the epiblast, preceding the onset of gastrulation (Bhargava et al. 2017).

### 1.3 Zebrafish as a model organism

#### 1.3.1 Development of erythroid cells in vertebrates

Our focus in studying mechanisms of erythropoiesis led us to investigate the relationship between erythroid and thrombocytic differentiation. Based on our previous research (Svoboda et al. 2014), in our review (Svoboda and Bartunek 2015) we describe the ontology of erythro-

thrombopoiesis during adult hematopoiesis with focus on the phylogenetic origin of mammalian erythrocytes and thrombocytes. Although the evolutionary relationship between mammalian and non-mammalian erythroid cells is clear, the appearance of mammalian megakaryocytes is less so. In our work, we discuss recent data indicating that non-mammalian thrombocytes and megakaryocytes are homologs. Finally, we hypothesize that erythroid and thrombocytic differentiation evolved from a single ancestral lineage, which would explain the striking similarities between these cells.
To further study the mechanisms of vertebrate erythropoiesis, we established an immortalised zebrafish erythroid burst cell line (ZEB). The ZEB cells were initially derived from zebrafish whole kidney marrow cells by spontaneous immortalization and were initially dependent on Epo, Kitlga and Dex. However, their dependence on Kitlga and Dex was lost after 40 days in culture, thus Epo is the only factor critically required for proliferation of these cells.
This cell line currently serves us as a model to understand molecular mechanisms underlying erythroid transformation and differentiation. Specifically, we performed several RNAseq and proteomic experiments that helped us to understand the involvement of various genes in maintenance of these cells. Our results indicate that several proto-oncogenes (*bcl11, mecom, mdk, prox1, tbx*) are upregulated, whereas we identified few tumor suppressors (*prox1, socs1*) to be downregulated. Even though we still do not fully understand the exact mechanisms that led to the origin of the ZEB cell line, we can at least partially explain the mechanisms of maintenance of ZEBs dependent on mis-expression of regulators described above.

### 1.3.2 Ex vivo tools for the clonal analysis of zebrafish hematopoiesis

Zebrafish became a popular model organism to study the genetic underpinnings of hematopoiesis. However, the main disadvantage of this model was unavailability of culture conditions to study hematopoietic cells *ex vivo*. To overcome this, we optimized culture media and generated recombinant zebrafish cytokines that enabled for growth and differentiation of zebrafish hematopoietic cells *ex vivo* for the first time. These results were published in Nature Protocols (Svoboda et al. 2016) and the tools established helped to better understand mechanisms of zebrafish hematopoiesis. Our tools are widely used within the whole zebrafish community and our cytokines are either available through Addgene or directly distributed by us to the scientific community.

### 1.3.3 Study of zebrafish cytokines

As a follow up, and to provide further enrichment of our *ex vivo* toolbox for growth and differentiation of zebrafish hematopoietic cells *ex vivo*, we also focused on investigating and characterizing the role of other zebrafish cytokines that were previously either not studied or the information on them was scattered. Also, since many cytokine orthologues, as well as many other genes, were duplicated in the zebrafish genome to form multiple paralogues, we also tried to understand how the function of some of these paralogs diversified and if all of them still play a role in the same processes as compared to other vertebrates.
Our new findings were summarized in our review (Oltova et al. 2018). In this review we discuss consequences of an extra round of whole genome duplication in teleost fish with the emphasis on hematopoietic cytokine signalling and we provide an overview of recent findings in the zebrafish hematopoietic field regarding activity, role and specificity of these cytokines. In addition, we studied the role of Mcsf and Kitlg signalling in zebrafish. The study of Kitlg signalling was recently submitted to the journal “Blood Advances”.

### 1.3.4 Zebrafish as a model for cancer cell xenotransplantation

In the last decade, human cancer-cell xenotransplantation into zebrafish has gained more attention. Zebrafish embryos can engraft transplanted tumor cells until the adaptive immune system matures, at around two weeks post fertilization. The visualization of cancer cell growth and spreading could be further enhanced by using immune-deficient zebrafish lines in the transparent *casper* background. We perform allograft transplantations as well as xenograft transplantations of cancer cell lines into zebrafish embryos. We have developed a bioluminescence assay which allows for fast, simple and quantitative analysis of cancer cell

growth *in vivo*. This assay is highly sensitive and with less background when compared to conventional fluorophores. We have selected a set of kinase inhibitors for a pilot screen. The activity of inhibitors was tested *in vitro* as well as *in vivo* in zebrafish embryos. In conclusion, our bioluminescent xenotransplantation model seems to be a powerful tool for the critical evaluation of human tumorigenesis inhibition in zebrafish.
Recently, we have published a review article concerning zebrafish as a pre-clinical cancer model with new insights on modeling human cancer in fish (Hason and Bartunek 2019). In this article, we described zebrafish as an important non-mammalian vertebrate model for the study of tumorigenesis. Well-established genetic and transplantation models of cancer are discussed in this article, with a focus on new contributions from zebrafish models to the field of cancer research. With this article we highlighted the importance of zebrafish as a reliable pre-clinical cancer model organism.

## 1. 4. Chemical biology

### 1.4.1 Inhibitors for treatment of purine disorders.

Purines comprise essential molecules in all living organisms. Their cellular pool is maintained by the balance between de novo synthesis (DNPS), recycling and degradation. The entering substrate to DNPS is PRPP and the final product is IMP, which is further converted to AMP and GMP, essential components of many vital biomolecules. Once synthesized, purines are efficiently recycled by enzymes of the salvage pathways and eliminated as uric acid. DNPS requires 10 enzymatic steps, which are catalyzed by 6 enzymes. They are organized in a multienzyme structure: the purinosome, which transiently and reversibly assembles in the cytoplasm in response to increased purine demand. To date, two genetically determined defects of DNPS have been identified: adenylosuccinate lyase (ADSL) deficiency and AICA-ribosiduria, caused by mutations in the ADSL or ATIC genes respectively. Both are accompanied by serious neurological involvement and accumulation of enzymes' substrates in the bodily fluids of patients. Defects in the other four DNPS genes have not been identified, even though the range of the genetic variabilities and allele frequencies available in the ExAC database indicate that there are no evolutionary constraints against loss of function or missense mutations in other DNPS genes except the PPAT gene.

The main pathogenic effect leading to ADSL deficiency has been attributed to the toxic effects of accumulating dephosphorylated ADSL substrates SAdo and SAICAr. The observation of less severe intellectual impairment in patients with similar SAICAr levels but increased SAdo/SAICAr ratios, suggests that SAICAr, an intermediate of the DNPS pathway, is the neurotoxic compound, and that SAdo, an intermediate of the purine nucleotide cycle, may be protective of SAICAr’s effects. In model HeLa cells deficient for the DNPS enzyme GART, no accumulation of substrates was detected. This finding is a result of the instability of the GART substrate phosphoribosylamine, which has a half-life of 5 s under physiological conditions and is hydrolyzed to ribose 5-phosphate, an intermediate of the pentose phosphate pathway. It can be utilized in the treatment of DNPS disorders because the main pathogenic effect leading to the neurological symptoms of known DNPS disorders has been attributed to the toxic effects of accumulated intermediates in the body fluids of patients.

The aim of the project was to develop and characterize selective and potent small molecules for the study of de novo DNPS pathway, treatment of genetically determined DNPS disorders and as a target for anticancer therapy. To achieve this goal, we carried out a high throughput screening of a chemical library consisting of 80 000+ compounds using a GART enzymatic activity assay. The strategy involves compounds profiling in a complex panel of enzymatic and cell-based assays for important genes of purine metabolism.

### 1.4.2 New chemical regulators of juvenile hormone signaling

Two lipophilic hormones govern insect postembryonic development. While a steroid ecdysone promotes metamorphosis and adult differentiation, the sesquiterpenoid juvenile hormone (JH)

counters ecdysone by maintaining the status quo juvenile character of an insect larva until it reaches an appropriate size. When that size is attained, JH secretion ceases and the absence of JH allows metamorphosis to take place. If JH is artificially added at this time, metamorphosis fails as the animal repeats the juvenile stage and dies without reaching reproductive maturity. This is the effect of JH-mimicking insecticides. Conversely, premature depletion of JH triggers precocious metamorphosis.

In addition to the native sesquiterpenoid JH, many similar (methoprene) and disparate compounds, such as the pyridine derivative pyriproxyfen or the carbamate fenoxycarb, exert JH-like effects on insects. It is astonishing that these insecticides have been in use (methoprene since 1975), mainly against mosquitoes, ants, beetles, or fleas in agriculture and households, while their molecular action remained unknown until the characterization of the JH receptor Met in 2011. These compounds are also widely employed as tools in insect endocrinology research. JH mimics are attractive for insect control because they hijack the arthropod-specific endocrine system without affecting vertebrates. Nonetheless, currently available JH-mimicking insecticides have disadvantages such as toxicity to crustaceans and low selectivity to target insect species

JH agonists prevent metamorphosis and extend the larval feeding period, in some cases causing giant larvae, consuming more than they normally would. Another disadvantage is that JH agonists are ineffective until late larval stages, when the natural absence of endogenous JH permits metamorphosis. Thus, JH antagonists that would terminate the larval stage and provoke precocious metamorphosis should be superior to JH mimics in controlling insect growth. They would also be most useful as research tools for studies of JH function in vivo. Strikingly, no genuine JHR antagonists have yet been found although much effort has been devoted to searching natural inhibitors of JH signaling in plants.

In quest for yet unknown JH antagonists, we have developed cellular assays allowing us to screen large compound libraries and to understand the molecular mode of action of active compounds. The HTS assays depend on ligand binding to Drosophila Gce and therefore enable identification of novel JHR antagonists and agonists. Indeed, an HTS campaign yielded hundreds of new agonists and antagonists, with potency exceeding in a few cases, one of the best-known agonists so far. We currently perform triage of these ligands with respect to their chemical structure and ability to modulate different aspects of JH signalling.

### 1.4.3 Carborane-based selective agonists of estrogen receptor β

Estrogen receptor β (ERβ), a member of the nuclear receptor superfamily, is a ligand inducible transcription factor which regulates many physiological processes. ERβ is implicated in several pathologies such as steroid sensitive cancers, inflammatory diseases and Alzheimer's disease. Hence, understanding ERβ mediated biological effects through development of selective ligands can not only serve as excellent molecular probe but might also offer therapeutic opportunity. However, targeting ERβ remains a substantial challenge because of its closely related homology with ERα. In this regard, we have developed novel, selective and potent ligands (agonists) of ERβ based on unique chemistry of carborane-based compounds.

These compounds have been thoroughly characterised in cell-based reporter luciferase assays and biochemical binding assays to generate SAR information and data about selectivity of these ligands with respect to other remaining steroid receptors. We have developed a method to purify ligand-binding domains of ERα and ERβ in order to generate and analyse cocrystals of these receptors with carborane ligands. This will reveal the binding mode of the ligands to respective receptors and the information will be utilised for design of more potent and selective generation of ligands. Further, we have initiated *in vivo* studies of select compounds in transgenic mouse line carrying luciferase reporter construct integrated in the genome, to understand how the selectivity between two receptor isoforms is manifested in different tissues and organs.

### 1.4.4 17β-hydroxysteroid dehydrogenase inhibitors in breast cancer

17β-hydroxysteroid dehydrogenases are a family of enzymes metabolizing not only precursors of steroid hormones, but many other low-molecular-weight biological substances. Specifically, 17βHSDI is an important enzyme converting oestrone (E1) to 17β-estradiol (E2), which is the major estrogenic substance in the body. Oestrogens are important regulators of not only the normal physiological processes, but are important factors in the development of breast cancer. Therefore, therapy of breast cancer with hormone-sensitive forms of carcinomas uses the antihormone treatment including selective oestrogen receptor modulators (SERMs) with specific antihormone activity in the breast tissue, and pure antioestrogens. Both of these types of therapeutics bind to oestrogen receptors in the breast epithelia and block their activity on the promoters of target genes. The enzymes from the family of 17βHSDs are an interesting therapeutic target in the sense that they offer the possibility to block formation of E2 not only in target tissues, but also in other places serving as the source of the hormones.

In collaboration with laboratories at IOCB ASCR and Helmholtz Zentrum Muenchen, we develop steroid derivatives selectively inhibiting 17βHSDI. We use biochemical assays not only with the purified 17βHSDI enzyme, but also with the other related isoenzymes from the family to control the selectivity. Biochemical assays are further supported with data from cell-based reporter assays for the oestrogen and androgen receptors (ERα and AR). The goal is to achieve *in vitro* inhibition of 17βHSDI and, at the same time, to inhibit transcription by ERα and AR. The combined profile would be more complex than the profile of currently used antihormone therapeutics (SERMs, SARMs, antioestrogens and antiandrogens). The inhibitors are further validated in the panel derived from the hormone-sensitive breast and prostate cancers with a wide range of expression of genes controlling cell proliferation (17βHSDI-V, ERα, ERβ, AR, HER2, etc.). These compounds can become a new generation of modern medicaments for breast and/or prostate cancers.

### 1.4.5 Novel approach to identify small molecules maintaining the pluripotency in human embryonic stem cells

Human embryonic stem cells (hESC) and induced pluripotent stem cells (hiPSC) have a potential to differentiate into any cell of the human body tissues. Therefore, they represent an important future source for regenerative medicine. hESC can be cultured *in vitro* in a pluripotent state in the presence of bFGF and TGFβ, however, the existing protocols are very expensive. We performed a high-throughput screen and subsequent high-content analysis to identify small molecules capable of maintaining pluripotency in hESC. We used the H1 Oct4-eGFP hESC line, expressing the marker of pluripotency Oct4 fused with eGFP. In our screening protocol, cells were grown without any factors while standard cultivation medium supplemented with bFGF and TGFβ served as positive control. We have screened in total 4545 compounds from two chemical libraries (Bioactives library and Czech Academic collection). Two potential inducers were detected and further tested in dose-response experiments (EC50 equal to 1nM or 100nM, respectively). Both compounds were confirmed as pluripotency inducers also in a different hESC line, WA22. Exact pathways through which these compounds induce pluripotency are currently being examined. However, neither of these two compounds are able to stimulate cell proliferation at the same time. Therefore, we are currently optimizing a proliferation screening protocol to search for combinations of small molecules, keeping both pluripotency and proliferation of hESC in fully defined chemical medium.

### 1.4.6 Novel method development

To satisfy and support the growing number of our research partners and collaborators we are constantly developing tools, methods and novel approaches that are being used in high-throughput screening campaigns. For example we have established a cell-based reporter assay platform to screen different chemical libraries for their biological activity towards 23 well

characterized nuclear receptors (NRs) and this platform was already used for a number of collaborative projects.
For another project, a non-invasive and label-free method based on a real-time impedance cell assay was established in our facility. We also developed new software for analysis of real time cell analysis (RTCA) data that allows us to handle and normalize data from large scale experiments. We are currently continuing with development of a method of analyzing the data by means of creating RTCA fingerprints that would allow us to cluster chemical compounds by the type of response they elicit in various cell lines.
More recently we have developed a new method for very sensitive detection of hemoglobin production in both primary hematopoietic cells and hematopoietic cell lines. The method is optimized for high-throughput setup using either 1536-well or 384-well plates. The readout is based on automated imaging coupled with automated image analysis and thus allows quick and reliable detection of possible inducers of erythropoiesis.

### 1.4.7 Development of biologically active substances

#### 1.4.7.1 Photodynamic therapy (PDT).

One of the guidelines of our research was devoted to photodynamic therapy (PDT) using photosensitive synthetic compounds (photosensitizers) for the destruction of tumor cells. We discovered the glycolporphyrin derivatives as very effective PDT agents and, to deepen the mechanisms of their action, we analysed the molecular mechanisms of resistance to photodynamic therapy using PDT-resistant clones to various photosensitizers. It was found that the structure of photosensitizers affects the underlying mechanism of resistance. PDT resistance to soluble glycol porphyrins was mediated mainly by increased drug efflux through ABCB1 (P-glycoprotein), while resistance raised to clinically used temoporfin, which is generally more lipophilic than glycol porphyrins, elicited a response based on sequestration of the drug to lysosomes (Kralova et al. 2017).
We also examined the effect of photosensitizer drug delivery on PDT's effectiveness. A new biodegradable nanosystem based on lipid nanoparticles with the non-covalently bound photosensitizer temoporfin, T-SLNP, and two types of temoporfin-loaded silica nanoparticles have been developed and their photodynamic efficacies were compared with a commercial temoporfin formulation both *in vitro* and *in vivo*. The T-SLNP anticancer efficacy was markedly improved showing faster accumulation kinetics in cells and a massive destruction of tumor mass. The T-SLNP nanoparticles thus represent a promising drug delivery system applicable in cancer treatment (Brezaniova et al. 2016). Temoporfin-loaded silica nanoparticles also passed through the blood-brain barrier showing potential for the treatment of brain metastases. Our laboratory contributed to this study by characterizing accumulation kinetics in cells and designing and evaluating *in vivo* experiments (Brezániová et al. 2018). Our long-time research and experiences in photosensitizer-mediated photodynamic therapy in the context of results of others was summarized in a book chapter (Kejík et al. 2017).

#### 1.4.7.2 Compounds with anticancer effect

A new approach combining a biologically active moiety (hydrazone) with a rigid scaffold (Tröger's base) to construct DNA intercalators as novel anticancer drugs was developed. Our team contributed to evaluation of the anticancer activity of these agents using several cancer cell lines (Kaplánek et al. 2015, Kaplánek et al. 2015). A new pentamethinium salt with a conjugated quinoxalin unit was analysed and showed, besides great fluorescent properties, specific mitochondrial localization, high toxicity for tumour cell lines, and, following regular administration, significant suppression of human breast tumor growth in nu/nu mice. Our team contributed to this result by finding mitochondrial localization, determination of cytotoxicity and design and providing materials for *in vivo* experiments (Bříza et al. 2015). Additional pentamethinium salts with side units comprising one or two positive charges were studied. Several important discoveries were made: 1) the affinity of the pentamethinium salts to sulphated polysaccharides correlated with their biological activity, 2) the side heteroaromates displayed a strong effect on the cytotoxicity and selectivity towards cancer cells, 3) doubly charged pentamethinium salts possessing benzothiazolium side units exhibited remarkably

high efficacy against a taxol-resistant cancer cell line. Our laboratory contributed mainly by revealing selective activation of NF-κB signalling, which is directly or indirectly involved in induced cell death (Bříza et al. 2019).

### 1.4.7.3 Development of fluorescent probes

A new pentamethinium salt with a conjugated quinoxalin unit was shown to exhibit bright fluorescence in exclusive mitochondrial localization (Bříza et al. 2015). Dual fluorescent probes were developed with selective localization in mitochondria and lysosomes. They exhibited unusual dichromic behaviour, whereby, in just one step mitochondria and lysosomes could be visualized at fluorescence emission wavelengths distinct from each other. Localization studies were conducted at IMG (Bříza et al. 2017). Four novel fluorescent cores bearing transformable functional group were discovered as lysosomal probes LysosSers showing excellent brightness and low toxicity. Our team contributed with demonstration of co-localization of these probes with commercial LysoTracker (Havlík et al. 2019). This study presents the design and synthesis of fluorescent probes for cholesterol recognition based on heterocyclic sterol derivatives with various attached fluorophores and demonstrates their selectivity by a variety of methods. The most promising probe, a P1-BODIPY conjugate FP-5, exhibited an intensive labelling of cellular membranes followed by intracellular redistribution into various cholesterol rich organelles. Thus, FP-5 has high potential as a new probe for monitoring cholesterol trafficking and its disorders (Králová et al. 2018). A critical evaluation of the recent development of cholesterol probes, including our own results in this field, was summarized in a review (Králová and Král 2019).

### 1.4.8 Cheminformatics

CZ-OPENSCREEN has a very strong background in software development for cheminformatics. One of our main scientific interests is the development of chemical probes. These high-quality chemical tools are small-molecular compounds commonly used in biological experiments to study a gene function, validate molecular targets or dissect complex processes within cells and organisms. Data about chemical probes and their qualities are often scattered over various data sources which makes the search for the right chemical tool a very complex and time-consuming task. To alleviate these problems, we developed *the Probes & Drugs (P&D) portal* (Skuta et al. 2017). *P&D portal* is an up-to-date public resource for chemical biology that should simplify the well-informed selection of high quality chemical tools for biological screening. It allows one to search compounds based on their biological activities, physical-chemical properties, structure-based properties and many others, with literally unlimited number of their combinations. *P&D portal* is currently the most comprehensive source of both well-described non-commercial and pre-picked commercial compound libraries and it is one of the three main resources in this field used by the community.

We also develop tools for data management, analysis and visualization. We created our own laboratory information management system (LIMS), ScreenX, which is used for the management of processes involved in the whole high-throughput screening pipeline (compound management, preparation of experiments, analysis of results and reporting). Apart from our facility, ScreenX is currently used by three other laboratories: The Department of Chemistry at the Masaryk University, Brno, Czech Republic; the Department of Organic Chemistry at the Charles University, Prague, Czech Republic; and the Institute for Molecular and Cell Biology, Porto, Portugal.

Stemming from our need to explore compound sets from different points of view (mainly physical-chemical properties and biological properties), we developed a javascript library for the exploration of chemical space, ChemSpace.js (https://www.openscreen.cz/software/chemspace). This library can be used for the interactive visualization of sets containing up to hundreds thousands of compounds and can be integrated into any web application.

Besides the development of cheminformatics tools, we were also leading the investigation of the possibility to use *in silico* affinities as descriptors of small molecules. Using data from the ChEMBL database, we created a pipeline predicting affinity fingerprints, so called QAFFP,

describing a small molecule, which can be used for common cheminformatics applications (similarity searching, activity prediction, etc.). This approach showed a great promise in retrieving new chemotypes with defined bioactivity. We also investigated the possibilities to use QAFFPs for regression tasks with application to cytotoxicity prediction.

**1.5 Research infrastructure**

Our Open Access facility “CZ-OPENSCREEN: National Infrastructure for Chemical Biology” is on the National Roadmap for Large Research Infrastructures and is the National node of ESFRI initiative EU-OPENSCREEN (www.eu-openscreen.eu). In the period 2015-2019 we participated on a number of projects with partners from both the Czech Republic and abroad and these collaborations resulted in papers and patent applications.

We constantly improve and broaden services that the facility can offer. In the last five years we have acquired over 72,000 new chemical compounds (66,000 commercial compounds, 4,000 donated compounds from academic chemists, and 2,000 private samples) and altogether our library currently contains over 100,000 individual chemical samples. Significant improvements on instrumentation and workflows have been done during the past five years. We improved automated compound storage and liquid handling and implemented a completely novel pipeline to assess the quality of chemical samples by automated plate surveys after each run or more thoroughly by LC-MS. We also implemented a new Liquid Dispensing Unit (Labcyte ECHO 550) capable of transferring water based solutions, which allows us for example to prepare large numbers of qPCR reactions in an automated mode. We also contributed to the scientific community by development of several open software tools, such as Probes & Drugs Portal, a tool for facilitation of large scale sample management RackScanner 2, barcode PrintServer v2.0, EnSpire_control package for automatic run of fluorescence scan assays, RTCA, and set of tools for Echo acoustic dispenser Echo.py.

To expand our capabilities using animal models for disease modelling and validation of bioactive compounds *in vivo*, last year we expanded our zebrafish facility with racks containing 850+ fish tanks. Currently we possess a collection of more than 130 transgenic and mutant zebrafish lines. This makes this unique setting very competitive in the Czech Republic, as well as in Europe. The zebrafish community has been growing rapidly and so is the amount of resources in the form of genetically modified fishlines, further accelerated by the rapid development in the field of zebrafish genome editing. We developed Zebrabase, an interactive web-based animal tracking system tailored specifically to fish species and facility management. It allows advanced fish tracking, data visualization and reporting, experiment planning and facility management via an intuitive user interface (Oltova et al. 2018).

## Research activity and characterisation of the main scientific results

Our research activities during the evaluation period can be summarized as follows:

1. Identification of new phosphorylation events in the DNA damage response
2. Deciphering the role of the truncated PPM1D in cancer development
3. Testing PPM1D as potential pharmacological target
4. Classification of CHEK2 variants identified in cancer patients
5. Description of new genes involved in the cell cycle progression

Detailed description of individual activities is given below.

### Aim 1. Identification of new phosphorylation events in DNA damage response

Here we investigated the role of dynamic protein (de)-phosphorylation during the cell response to DNA damage. We focused on the activity of two major protein kinases ATM and CK2 and identified two new substrates that are involved in the DNA damage response.

**Output 1)** ATM/Wip1 activities at chromatin determine duration of the G2 checkpoint.

Here, we developed a FRET-based biosensor suitable for measuring the activity of ATM kinase in living cells. We found that whereas ATM activity in the nucleoplasm persists long after DNA double strand induction, ATM activity at the chromatin is rapidly counteracted by a chromatin-bound PPM1D/WIP1 phosphatase. One of the ATM/WIP1 target at chromatin is a transcriptional repressor and chromatin remodelling complex TRIM28/KAP1. Following its rapid dephosphorylation, activity of PLK1 starts building up and eventually it counteracts the activity of the G2 checkpoint inducers ATR/CHK1. By controlling the ATM activity at chromatin cells determine the minimal duration of the G2 checkpoint. One of the main conclusions of this study was that decision to re-enter the cell cycle is made very early after the DNA damage and before completion of the DNA repair. This work was published in *EMBO Journal.*

**Output 2)** MRE11 stability is regulated by CK2-dependent interaction with R2TP

Here, we identified MRE11 as a new substrate of casein kinase 2. Constitutive phosphorylation of Ser688/689 by CK2 mediates interaction of MRE11 with PIH1D1 subunit of the chaperone R2TP complex. In the absence of this phosphorylation, folding of the MRN complex is impaired resulting in impaired DNA repair including the removal of DNA adducts. Lack of the interaction with the R2TP complex may explain the low levels of MRE11 in ATLD patients. This work was published in *Oncogene*.

### Aim 2. Deciphering the role of the truncated PPM1D in cancer development

This study builds on our discovery of the truncated PPM1D mutants in cancer patients and represents one of the main research outcomes in the evaluating period. Our results were published in *Cell Death and Disease* journal.

Previously, we identified clinically relevant frameshift or non-sense mutations in the sixth exon of PPM1D resulting in production of the C-terminally truncated PPM1D/WIP1 phosphatase. As PPM1D/WIP1 is a monomeric phosphatase and the catalytic domain resides in the N-terminal part of the protein, truncated PPM1D/WIP1 is enzymatically active. Truncation removes a degron sequence present at the very C-terminus resulting in massive stabilization of PPM1D/WIP1 protein. Here we used CRISP/Cas9 technology and generated truncating mutation in exon 6 of the PPM1D gene in diploid RPE cells. We observed that truncated PPM1D/WIP1 impaired activation of both G1 and G2 checkpoint following a mild DNA damage. As a result, cells with truncated PPM1D/WIP1 continued proliferating despite the presence of the DNA damage likely leading to increased genomic instability. To study the contribution of

truncated PPM1D/WIP1 to cancerogenesis, we developed a new transgenic mouse model that mimics the mutations observed in humans. We observed overall reduced survival of *PPM1D*$^{T/+}$ heterozygotes compared to wild-type animals. As the phenotype occurs in advanced age, we continue with the analysis in a follow up project. Here we investigated contribution of truncated PPM1D/WIP1 to cancer growth induced by other oncogenes. Since we observed high expression of PPM1D in intestinal/colon stem cells we further focused on the tumors in the intestine and colon. We found that the truncated PPM1D/WIP1 increased the number of intestinal polyps induced in APC$^{min}$ mouse model and also reduced mouse survival. In addition, we found that tumors in the colon were more frequent in APC$^{min}$ mice carrying the *PPM1D*$^{T}$ allele. Next we compared normal intestinal stem cells with those carrying truncated PPM1D/WIP1 and found that truncated PPM1D/WIP1 impaired activation of p53 pathway and prevented induction of apoptosis. In addition, we found sustained proliferation at the bottom of intestinal crypts in animals carrying the *PPM1D*$^{T}$ allele that were exposed to whole body irradiation. Based on this in vivo data, we concluded that truncated PPM1D/WIP1 provides proliferation advantage of intestinal stem cells under stress condition and thus can contribute to cancerogenesis.

Next we analyzed a cohort of human patients suffering from colon carcinoma and found that these truncating PPM1D mutations occur predominantly in a subgroup of tumors with microsatellite instability and wild-type p53. Those tumor were frequently driven by oncogenic BRAF-V600E mutation. Currently we use transgenic mouse models to investigate whether truncated PPM1D/WIP1 could promote of BRAF-induced tumors in the intestine.

Finally, we tested the effect of GSK2830371 inhibitor on cancer cell proliferation using 3D organoids derived from the colon tumors induced by APC$^{min}$. Importantly we found that tumors carrying truncated PPM1D/WIP1 were more sensitive to GSK2830371 and also that this compound improved the sensitivity to a commonly used chemotherapeutics 5-FU. We conclude that truncation of PPM1D/WIP1 may modulate therapeutic effect of chemotherapy and may also represent potential pharmacological target.

Importantly, this research activity generated unique tools and preliminary data that we will follow in future research. In particular we will exploit the mouse model of truncated PPM1D and test the impact of truncated PPM1D on development of haematological cancers.

**Aim 3. Testing PPM1D as potential pharmacological target**

Here, we tested the ability of various small-molecule inhibitors of PPM1D to supress proliferation of cancer cells. Using CRISR/Cas9-mediated knock out of PPM1D in U2OS cells we established that GSK2830371 inhibitor specifically inhibits PPM1D/WIP1 (whereas several other commercially available compounds showed non-specific off target effects). GSK2830371 was not sufficient to kill cancer cells but it significantly potentiated cytotoxic effect of chemotherapeutics including doxorubicin and camptothecin. This synergistic response was fully dependent on the presence of the wild-type p53.

While screening the effect of the WIP1 inhibitor we noted that it reduced the efficiency of DNA repair through homologous recombination. The same phenotype was observed after depletion of WIP1 by RNAi confirming that WIP1 is involved in HR. Next we investigated the molecular mechanism and found that inhibition of WIP1 delays recruitment of BRCA1 to the DNA damage foci. In addition, we found that WIP1 interacted with BRCA1 and efficiently dephosphorylated S1453 site that is modified by ATM after DNA damage. Formation of RPA foci (as marker of DNA resection) and RAD51 filaments was unaffected by WIP1 inhibition which led us to conclude that WIP1 may regulate a downstream step in HR. Importantly we noted that WIp1 inhibition of WIP1 potentiated the effect of olaparib, PARP inhibitor currently approved for

treatment of BRCA1/2 mutated ovarian cancers. Our data suggest that inhibition of WIP1 may promote efficiency of PARP inhibitor also in BRCA1 proficient tumors.

Results from this research activity were published in *Oncotarget* and in *Cells*. Motivated by these result we also performed a synthetic lethality screening of commonly used chemotherapeutics and other approved drugs in combination with WIP1 inhibitor. Currently we are verifying several hits from the HTS screen the effect of which was potentiated by treatment with WIP1 inhibitor.

**Aim 4. Classification of CHEK2 variants identified in cancer patients**

Germline mutations in CHEK2 occur in cancer patients and mutants CHEK2 are believed to predispose the mutation carriers to cancer. However, precise evaluation of the relative risk of cancer development is complicated by existence of high number of various CHEK2 mutations that occur with low frequency. Whereas some of the mutations are clearly pathogenic, others are likely benign. This situation makes genetic counselling of CHEK2 variants difficult and in many cases inconclusive. To improve the functional characterization of identified CHEK2 variants we first generated CHEK2 knock-out cells. In the next step, we cloned EGFP-tagged CHEK2 and its 25 variants (identified in Czech population) and these were transfected into CHEK2 knock-out cells. Cells were then probed for the ability to phosphorylate KAP1 at S473 (newly identified CHEK2 phosphorylation site) using high-content microscopy. This allowed us to compare the activity of CHEK2 variants with that of the wild-type CHEK2. Finally, we assayed the occurrence of the tested variants in Czech cancer patients and healthy controls. We found that loss-of function variants were significantly enriched in the cancer patients, whereas distribution of the active CHEK2 variants was equal between both cohorts. This pilot study proved the efficiency of our new assay for distinguishing the benign and pathogenic CHEK2 variants and was published in *International Journal of Cancer*. We now continue with testing of CHEK2 variants within the ENIGMA consortium where we have access to large patient cohorts.

**Aim 5. Description of new genes involved in the cell cycle progression**

Here we performed expression profiling of cells in various phases of the cell cycle aiming to identify new regulators of normal cell cycle progression. To this end we first established a nontransformed RPE cell line expressing a fluorescent cell cycle indicator (FUCCI) that allowed isolation of sufficient amount of G1 and G2 cells from asynchronously growing cell cultures. This is an important difference to previous studies that used mainly cancer cell lines that were synchronized in individual phases of the cell cycle by drugs including thymidine or nocodazole. Both these treatments cause considerable level of cellular stress that could result in significant changes in gene expression. Using our system we identified about 700 transcripts differently expressed in G1 and G2 cells. Our data were in good agreement with previous reports and a large part of differentially expressed genes corresponded to their known function in cell cycle progression. We followed by analysis of a set of gees that were previously not recognized as cell cycle regulators including *FAM110A, FAM72D, PIF1, GAS2L3, MXD3* and *NEURL1b*. So far we obtained most experimental data on the FAM110A and we have described its role in mitotic spindle positioning. Using immune-fluorescence we detected FAM110A at spindle poles, proximal spindle and midbody. Depletion of FAM110A by RNAi slowed down progression through mitosis resulting in slight increase of the mitotic index detected by flow cytometry. Filming of cells expressing GFP-H2B showed slower organization of the chromosomes in the metaphase plate and also higher frequency of segregation errors in anaphase cells with depleted FAM110A. To reveal molecular mechanism of these defects we analyzed proteins interacting with EGFP-FAM110A mass spectrometry and found that mitotic FAM110A forms a complex with casein kinase 1 delta/epsilon. This interaction was further independently confirmed by immunoprecipitation with CSNK1E antibody. During

mitosis, FAM110A is phosphorylated resulting in a mobility shift on SDS-PAGE. Treatment of mitotic cells with CK1 inhibitor or depletion of CSNK1E by siRNA reduced the mobility shift of FAM110A indicating that CK1 phosphorylates FAM110A in mitosis. We identified putative phosphorylation sites on FAM110A and mutated them to alanines which reduced the mobility shift of mitotic FAM110A. Previously, CK1 was implicated in control of spindle positioning in various models. Using life-cell imaging we observed that RPE cells treated with CK1 inhibitor showed similar phenotype as depletion of FAM110A. One of the interacting partners of mitotic EGFP-FAM110A was actin and this interaction was also confirmed by immunoprecipitation. In conclusion, FAM110A might be a linker between the mitotic spindle and cortical actin cytoskeleton and thus can contribute to the positioning of the spindle during mitosis. Manuscript describing these data described is currently ready for submission to *Cellular and Molecular Life Sciences*.

In addition, we developed a sensitive kinase assay for detection of CDK activity in cells and we used this tool to study the involvement of CDKs in G2 checkpoint recovery. In collaboration with Arne Lindqvist (Karolinska Institute, Stockholm) we found that residual CDK1/2 activity after DNA damage promotes permanent cell cycle exit. These findings were published in *Aging Cell*.

## Research activity and characterisation of the main scientific results

In the period of 2015-2019 our laboratory was actively engaged in the fields of research described above. It was well funded and generated interesting and exciting data within our own projects, as well as within various collaborations. On the following pages we will discuss the projects where our contribution was critical.

Src-family kinases (SFKs) are key protein tyrosine kinases involved in signal transduction through numerous receptors within as well as outside the immune system. In leukocytes, these include TCR, BCR, and other ITAM-bearing immunoreceptors, G-protein coupled receptors, integrins, growth factor and cytokine receptors, MHC glycoproteins and others. SFKs are targeted to the cellular membranes via myristoylation and palmitoylation of their N-termini. However their major negative regulator Csk lacks these N-terminal membrane targeting sequences and instead relies on binding to membrane-associated adaptor proteins to reach the SFKs at the plasma membrane. Before 2015 we were identifying novel membrane adaptor proteins that would bind Csk and, thus, would have the ability to regulate SFKs and SFK-dependent signaling. We named one of these new membrane adaptors SCIMP. We found it by searching protein sequence databases for membrane adaptors containing Csk consensus binding site. Our initial characterization in cell lines showed that SCIMP not only binds Csk, but also SFK LYN and very important adaptor proteins from SLP76/SLP65 family, known to be critical for antigen receptor signaling in lymphocytes. Our analysis also revealed that SCIMP supports signaling downstream of MHCII glycoproteins in B cells. Interestingly, it did not regulate SFKs globally, but rather used Csk to regulate SCIMP's own activity (Draber et al. Mol Cell Biol. 2011;31(22):4550-62). In order to determine physiologically most relevant functions of SCIMP we obtained SCIMP deficient mice. Their analysis was carried out within the evaluated period. It revealed that in steady state these mice are healthy and do not show any unusual phenotype. We also did not detect any alterations to the signaling downstream of MHCII and in B cell responses. On the other hand, our analysis of dendritic cells and macrophages revealed that SCIMP is strongly phosphorylated after stimulation of pattern recognition receptor Dectin-1 and that SCIMP is important for maintaining sustained MAP kinase signaling and sustained proinflamatory cytokine TNFα and IL-6 production downstream of Dectin-1. Since Dectin-1 is a major receptor involved in detection of fungal infections, we concluded that SCIMP contributes to macrophage and dendritic cell responses to these pathogens. We published these data in The Journal of Biological Chemistry (Kralova et al J Biol Chem. 2016;291(32):16530-40). More than 90% of the work was performed in our group. We also had a contribution from Bernard Malissen from Immunophenomics Centre, Marseilles, France, who helped us with designing and executing part of the flow cytometry analysis. This project was funded by Czech Science Foundation grant P302/12/1712 "Function of Csk-anchoring proteins SCIMP and PSTPIP2 in leukocyte signaling and inflammation" (2012-2015).

Another membrane adaptor that we identified within our effort to find proteins recruiting Csk to the cellular membranes was PSTPIP2. We co-purified it from WEHI231 cells with a construct containing SH2 and SH3 domains of Csk. In contrast to SCIMP this was not a completely new unknown protein and its membrane binding was mediated by an F-bar domain rather than the transmembrane segment. However, what was known about this protein was very interesting. Its deficiency in mouse models resulted in autoinflammatory disease chronic multifocal osteomyelitis, characterized by sterile inflammation of hind paw and tail bones and surrounding soft tissues. It was known that this disease is dependent on a potent pro-inflammatory cytokine IL-1β and that it is most likely caused by IL-1β hyperproduction by neutrophils. However, the molecular mechanism of how PSTPIP2 suppresses IL-1β production and inflammation was unclear. Uncovering this mechanism is important not only for this particular disease model, but also for better understanding of related human diseases and the regulation of inflammation in general.

As an adaptor protein, PSTPIP2 was expected to act by recruiting inhibitory molecules to the receptors or signaling complexes located at the cellular membranes. Of these inhibitory molecules only PEST-family phosphatases were known to interact with PSTPIP2. Binding of these enzymes to the region of PSTPIP2 containing W232 was important for suppression of differentiation and functional responses of osteoclasts and megakaryocytes in vitro, but no such information was available for neutrophils, cells most critical for chronic multifocal osteomyelitis development in the animal model. Studies of osteoclasts also revealed that three tyrosine phosphorylation sites at PSTPIP2 C-terminus are critical for its suppressive function. However, the mechanism of their action or identity of their binding partners was unknown. Our findings suggested that Csk is an inhibitory effector that PSTPIP2 recruits to suppress neutrophil functions. We found that its binding is partially dependent on PEST family phosphatases (PEST-PTPs). However, the critical C-terminal tyrosines of PSTPIP2 did not have any role in Csk binding. Instead, our large-scale PSTPIP2 immunoprcipitations followed by identification of co-purifying proteins by mass spectrometry revealed that these tyrosines bind phosphoinositide phosphatase SHIP1. SHIP1 was known negative regulator of leukocyte signaling. By its identification we increased the portfolio of known inhibitory enzymes interacting with PSTPIP2 by two enzymes, Csk and SHIP1 and offered an explanation for the role of C-terminal tyrosines. We demonstrated that both, W232, which binds the complex of Csk and PEST-PTPs, and C-terminal tyrosines binding SHIP1 are critical for suppression of IL-1β processing in neutrophils differentiated from immortalized progenitors. In the same cells we showed that chemical inhibition of SHIP1 results in increased IL-1β processing in wild-type neutrophils, while in PSTPIP2-deficient cells this effect was relatively mild. These data supported the conclusion that both PEST-PTP-Csk complex and SHIP1 are involved in the suppression of IL-1β production by PSTPIP2 in neutrophils. Another interesting outcome of this study was finding that neutrophils from PSTPIP2-deficient mice show rather generalized hyper-responsiveness to multiple stimuli acting through different receptors, including silica particles, LPS or Fc-receptor activators. These results suggested that PSTPIP2 has rather central position in the signaling network of neutrophils affecting multiple pathways at the same time. This study was published in The Journal of Immunology (Drobek et al. 2015;195(7):3416-26.). Our team generated majority of the data. Other contributions included generation of some of the PSTPIP2 cDNA constructs and generation of PSTPIP2 monoclonal antibodies by P.Angelisova and P.Otahal from the Laboratory of Molecular Immunology at our institute, which later became part of our team. They also included assistance with generation of immortalized neutrophil progenitors by M.Alberich-Jorda from the Laboratory of Hematooncology at our institute and mass spectrometry analysis performed by P.Novak from the Institute of Microbiology. The rest of the experimental work was performed by our team. The project was funded by Czech Science Foundation grant P302/12/1712 “Function of Csk-anchoring proteins SCIMP and PSTPIP2 in leukocyte signaling and inflammation” (2012-2015) already mentioned above.

This work also resulted in another interesting observation that treatment with silica particles results in much higher reactive oxygen species (ROS) production in PSTPIP2-deficient neutrophils than in wild-type cells. We found that this dysregulation is rather universal and can be observed downstream of many different stimuli, including silica,TNF, fMLP and number of others. Moreover we also demonstrated that in PSTPIP2-deficient animals increased ROS production in the areas later affected by inflammation can be observed in vivo, weeks before visible disease onset. The reason for this ROS hyperproduction was dysregulation of Nox2 NADPH oxidase which showed hyperphosphorylation of its key regulatory subunit p47phox resulting in increased activity and superoxide generation. To study the effects of this increased ROS production on disease development in Pstpip2-deficient mice we bred these mice with the strain lacking Nox2 to generate PSTPIP2-deficient mice where neutrophils are unable to produce large amounts of ROS. Indeed ROS production by neutrophils was undetectable by our methods in these mice. Very interestingly, these mice still developed inflammation of hind paws and tail with only slight delay and slightly lower severity when compared to the regular PSTPIP2-deficient animals. However, the most interesting observation was that their bones were almost completely intact and did not show virtually any

signs of inflammatory destruction. These data demonstrated that ROS production is critical factor contributing to bone damage in PSTPIP2-deficient animals. Importantly in Nox2 x PSTPIP2 double-deficient mice and neutrophils, IL-1β production remained intact, suggesting that ROS dysregulation works in parallel to IL-1β deregulation specifically enhancing bone damage in these animals. This work has been published in The Journal of Immunology early in 2020 (Kralova et al J Immunol. 2020 Mar 15;204(6):1607-1620, accepted on Jan 13th) and so it does not fall within the evaluated period. Nevertheless, all the data for this study were generated during the evaluated period and working on this project represented substantial part of our research activity. There are 3 first authors on this article. J.Kralova and A.Drobek were from our team, J.Prochazka from the Laboratory of Transgenic Models of Diseases and Czech Centre for Phenogenomics at our Institute. Their team contributed all the mouse imaging data and histology analysis. M.Gregor and P.Kovarik assisted in generation of neutrophil-deficient mice for this project. The remaining data were generated by our team, though it should be clarified that one of the first authors, also to a small extent contributed to this work after moving to the Laboratory of Adaptive Immunity in our institute. This work was funded by Czech Science Foundation grant 17-07155S “Molecular mechanisms of the regulation of inflammatory responses by adaptor protein PSTPIP2” (2017-2019).

It appears that signaling by multiple receptors is dysregulated in PSTPIP2-deficient mice. It is likely that activation of one or more of these receptors in vivo leads to exaggerated response and sterile inflammation. However, it is not clear which of these receptors and what ligands are triggering the disease in vivo. Article published by others (Lukens et al. Nature. 2014;516(7530):246-9) demonstrated that gut microbiota play a critical role, however the inflammatory lesions in the bones appear sterile and it is unclear how the reaction of the immune system to gut microbiota eventually affects bone inflammation. To gain some information about the types of receptors required for disease development we have generated PSTPIP2-deficient mice with defective TLR signaling (lacking key adaptors MyD88 in leukocytes or TRIF in the whole body). Interestingly, we found that disease develops independently of these adaptors and so TLR-mediated leukocyte response to gut microbiota or any other stimulus is not involved. We have also generated PSTPIP2 x CD45 double-deficient mice. CD45 is a major positive regulator of SFKs, and so this way we indirectly probed the requirement for these enzymes. In these mice the disease severity is significantly milder, suggesting the role for SFK-dependent (or CD45-regulated) receptors. Most of this work was completed before the end of 2019 and currently it is in the stage of manuscript preparation. It has been funded by Czech Science Foundation grants 17-07155S “Molecular mechanisms of the regulation of inflammatory responses by adaptor protein PSTPIP2” (2017-2019) and 19-05076S “The role of interactions of adaptor protein PSTPIP2 in the development of autoinflammatory disease” (2019-2021).

The latest member of transmembrane adaptor protein family we have been working on is known as WBP1L or OPAL1. Our interest in this protein resulted from a collaboration with the team at 2$^{nd}$ Faculty of Medicine and Motol University Hospital in Prague. Together, we were generating and testing antibodies to novel markers of childhood leukemia for potential diagnostic use. WBP1L was one of the proteins they were very much interested in due to the correlation between its expression at mRNA level and favorable prognosis of childhood leukemia. A potential explanation for this correlation was later provided by a study showing increased WBP1L mRNA in a subset of leukemia characterized by ETV6-RUNX1 (TEL-AML1) gene fusion, which by itself is associated with good prognosis. Interestingly, within this group WBP1L expression level still correlated with favorable outcome, when the patients were treated using low risk (i.e. less aggressive) protocol suggesting that its expression and prognosis may be functionally linked. We have generated antibodies to this protein. They performed especially well when used for new diagnostic method of Size Exclusion Chromatography-Microsphere-based Affinity Proteomics developed by our collaborators, which revealed that WBP1L/OPAL1 has increased expression in ETV6-RUNX1$^{+}$ leukemia not only at mRNA, but also at the protein level (Kanderova et al Mol Cell Proteomics. 2016 Apr;15(4):1246-61). To this work we have contributed just these antibodies. However, the amino acid sequence of WBP1L/OPAL1 also suggested that it is a transmembrane adaptor

protein. Its function or anything else except for its expression profile was unknown. We expected that if we can reveal its physiological function we could also obtain some idea about any potential impact on the leukemia treatment outcome. This effort resulted in finding that WBP1L binds and activates multiple E3 ubiquitin ligases from NEDD4 family. Some of these ligases negatively regulate key chemokine receptor CXCR4, critical for retention and function of hematopoietic stem and progenitor cells. We found that downregulation or genetic ablation of WBP1L/OPAL1 in multiple leukocyte and progenitor subsets in mice or in human ETV6-RUNX1$^{+}$ leukemic cell line REH resulted in increased activity of CXCR4. In primary murine KIT$^{+}$ progenitors we also detected significant increase in CXCR4 surface expression, enhanced migration towards CXCR4 ligand CXCL12 and increased bone marrow homing. Collectively these data suggested that WBP1L negatively regulates CXCR4 via the recruitment of NEDD4 family ubiquitin ligases. We also obtained WBP1L-deficient mice. Their analysis showed alterations in B cell development. However, the most interesting finding was that during bone marrow or early progenitor transplantation in competitive setting WBP1L$^{-/-}$ cells engrafted 2-3 times better than WT cells and this ratio was maintained for at least 4 months after transplantation, demonstrating that WPB1L reduces the ability of HSPCs to engraft efficiently during the bone marrow transplantation. The major conclusions about WBP1L function in this article, thus, are that WBP1L negatively regulates CXCR4 signaling likely via the recruitment of NEDD4-family ubiquitin ligases, it is also involved in the regulation of B cell development and it diminishes the ability of hematopoietic stem and progenitor cells to reconstitute murine hematopoietic system after transplantation. Returning back to leukemia, we can now hypothesize that there may be a similar negative effect of WBP1L on leukemic (stem) cell retention or “engraftment” in the bone marrow. Since bone marrow niches have protective effects on leukemic cells, higher WBP1L expression can make them more vulnerable to the toxicity of the drugs used in the treatment. This work was published in the Journal of Cellular and Molecular Medicine in December 2019 (Borna et al, J Cell Mol Med. 2020;24(2):1980-1992. Epub 2019 Dec 17). Our team generated majority of the data, including analysis of the mice and experiments on cell lines and primary cells. I. Splichlova from Laboratory of Immunobiology in our institute performed analysis of embryonic hematopoiesis in WBP1L-deficient mice. Team from 2$^{nd}$ Faculty of Medicine provided us with REH leukemic cell line with deletion of ETV6-RUNX1 and with data on WBP1L expression in leukemic cell lines. Our collaborators from Nencki Institute for Experimental Biology, Warsaw, performed analysis of WBP1L palmitoylation and our collaborators from University of Birmingham generated one of the WBP1L antibodies. All the remaining work was performed by our team. The project has been funded by the Czech Science Foundation grant 16-07425S “Analysis of the role of transmembrane adaptor protein OPAL1 in the regulation of leukocyte receptor signaling with focus on chemokine receptor CXCR4” (2016-2018).

During the evaluated period we have also been working on another transmembrane adaptor LST1/A encoded by MHCIII locus. This is again a relatively new protein which we described in our previous work. In that work we showed that it is mainly expressed in leukocytes of myeloid lineage and that it contains ITIM phosphorylation motifs, which recruit inhibitory phosphatases SHP1 and SHP2. We demonstrated that it is capable of inhibiting ITAM-dependent signaling. However it remained unclear, what is its function in vivo. This we published in 2012 (Draber et al J Biol Chem. 2012;287(27):22812-21). After several years break we restarted the work on this adaptor by obtaining and analyzing LST1-deficient mice. In these animals we found alterations in the composition of NK and myeloid cell subsets, but they otherwise appeared normal and healthy. Our analysis of a large number of signaling pathways did not show any differences between wild-type and LST1-deficient animals. It was published previously that LST1 expression is increased in patients with inflammatory bowel disease. Czech Centre for Phenogenomics at our institute had established the mouse model of inflammatory bowel disease, DSS-induced colitis. Therefore we set up a collaboration to analyze DSS colitis in LST1-deficent mice. The data clearly showed that the disease is significantly less severe in the absence of LST1 demonstrating that LST1 is involved in the regulation of inflammation. Even though we do not know the precise molecular mechanism, we have decided to publish this study at its current stage, mainly due to the time and financial

constrains. The manuscript is currently in final stages of preparation. Almost all data for this manuscript were collected during the evaluated period. Majority of this project was funded by large collaborative grant for support of excellence in basic research from Czech Science Foundation P302/12/G101 “Molecular mechanisms of signaling through leukocyte receptors – their role in health and disease” (2012-2018).

Transmembrane adaptor proteins also were a major topic in our long-lasting collaboration with M. Cebecauer group at J Heyrovsky Institute of Physical Chemistry. We have analyzed the role of palmitoylation and transmembrane domain structure in plasma membrane sorting of these leaderless proteins. Our study showed that there are no general rules how their plasma membrane sorting is determined, but rather that it is dependent on the unique combinations of palmitoylation, transmembrane domain length and composition and characteristics of its flanking sequences. The results were published in The Journal of Cell Science (Chum et al. J Cell Sci. 2016;129(1):95-107). Our group generated majority of cDNA constructs for this study and contributed to microscopy analysis. In addition the first author was a visiting student in our group splitting his time between our group and group in Heyrovsky institute as project development required.

This collaboration was later extended to analysis of the relationship between structural features of T cell correceptors CD4 and CD8 and their localization at nanoscale, i.e. as detected by superresolution microscopy. So far this resulted in one additional publication describing novel method of clustering analysis of molecules observed with super-resolution microscopy. We have generated CD4 constructs and transfectants used in the validation of this method (Lukes et al. Nat Commun. 2017 Nov 23;8(1):1731). It was funded by a joint grant project from Czech Science Foundation 15-06989S „Organisation and function of CD4 co-receptor on the surface of T cells at nanoscale“ (2015-2017).

In the evaluated period we have also been involved in the research activities more specifically targeting Src-family kinases. One of these projects was a highly collaborative effort including our team and teams at 2nd Faculty of Medicine and University Hospital in Motol, Prague. They have identified a patient with activating mutation in Src-family kinase Hck. The patient suffers from exaggerated inflammatory response with inflammatory infiltrates and fibrosis in lungs, severe vasculopathy in the skin and other symptoms. This is the first patient with this type of mutation described so far and so we believe this is an important work. Our team has carried out experiments showing that the mutation in Hck is a gain of function mutation increasing kinase activity. We also prepared constructs with the same mutation in Hck and generated cell lines expressing this construct. We contributed to functional analysis of these cell lines. We also set up humanized mouse model to test the behavior of patient’s leukocytes in vivo. Overall we contributed ca 30% of the workload of this project. The results of this research directly contributed to the improvement in patient’s treatment strategy. The manuscript describing this study is currently in final stages of preparation. There will be three shared first authors and three shared senior authors, one of each will be from our team.

Another project on Src-family kinases, which was executed exclusively by our team, was inspired by our previous work as well as by the work of other researchers. It tried to explain multiple observations that it is not possible to inhibit B cell antigen receptor signaling in B cells by SFK inhibitors, while it is very easy to achieve complete inhibition of T cell antigen receptor signaling in T cells with the same inhibitors. There were multiple possible explanations. The most widely accepted stated that SYK kinase expressed in B cells is able to phosphorylate ITAM motifs in BCR and this way substitute for the lack of SFK activity. It was thought that ZAP-70, a SYK counterpart expressed in T cells is not capable of phosphorylating these motifs. We have generated SYK and ZAP-70 deficient B and T cell lines and reconstituted each with SYK or ZAP-70 and also with TCR or BCR in various combinations. Our analysis of these cell lines revealed that the threshold for SFK activity required for initiation of antigen receptor signaling is in T cells much higher than in B cells and this makes T cells more sensitive to SFK inhibition. We also found that any potential unique ability of SYK to phosphorylate ITAM motifs does not contribute to lowering this threshold. Setting the threshold is a multilevel process, dependent on other features of SYK and ZAP-70 (possibly differences in kinase activity), structure of antigen receptors, and separation of transmembrane adaptor protein LAT from

TCR (LAT is a major organizer of TCR signalosome and is not expressed by B cells). The experimental part of the study was finished in 2019 and the resulting manuscript was submitted to The Journal of Biological Chemistry. Currently we are preparing a revised version for resubmission.

Many transmembrane adaptors and Src-family kinases were in the past shown to partition to so called membrane rafts, dynamic plasma membrane structures of specific lipid and protein composition. In the past prevailing method of their biochemical analysis was based on detergent-mediated solubilization, which was prone to multiple artefacts, resulting in confusion and even doubts about membrane rafts existence. The main research interest of Václav Hořejší (and Pavla Angelisova) has been focused on disintegration of immunocyte membranes without the use of detergents, under the conditions preserving native lipid environment of membrane proteins, especially those residing in T cell membrane rafts. To this aim, amphiphilic copolymers of styrene and maleic acid (SMA) have been used instead of mild detergents to cut out small „nanodiscs“ from plasma membrane, while raft-associated proteins were present in much larger SMA-resistant membrane fragments. Initial results were published (Angelisova et al. BBA Biomembranes 1861 (2019) 130–141). The proteomic and lipidomic composition of these SMA-resistant raft-derived membrane fragments was remarkably similar to that described earlier for detergent-resistant raft-derived membranes. This was collaborative work, where our group members performed biochemical experiments, while remaining co-authors provided SMA co-polymers and performed limited number of specialized analyses. Contribution of our team was ca 60-70%.This research topic is currently supported by a 3-year (2019 – 2021) grant of the Czech Science Foundation (“Biochemical studies of membrane rafts and immunoreceptors based on cell membrane-disintegrating copolymers”). It is based on collaboration with a team from the Institute of Macromolecular Chemistry CAS. Major aims of this project are: (1) Extension of the previous results on T cells on receptor complexes and membrane microdomains of various other cell types; (2) Preparation of novel amphiphilic copolymers, with potentially better properties compared to the presently existing ones; (3) Proteomic and lipidomic analysis of membrane nanodiscs (produced by means of various amphiphilic copolymers) containing immunologically important membrane proteins, and of similar analysis of copolymer-resistant, presumably raft-derived membrane fragments; (4) Elucidate the biological nature of the disintegration-resistant raft-derived membrane fragments and factors and molecular components essential for these membrane entities; (5) Effects of low, sublytic concentrations of amphiphilic copolymers on cells. The results of some of these experiments are now being prepared for publication. During these studies we made an exciting discovery - a class of synthetic peptides disintegrating cell membranes apparently very similarly as the above amphiphilic copolymers. This topic is currently under intense investigation.

## Research activity and characterisation of the main scientific results

The laboratory of Haematooncology contributed to the publication of 17 manuscripts during the last five years. Part of the research activity was performed mostly by the team at IMG, resulting in 3 manuscripts published in international peer-review journals. In addition, some of our main scientific results were published in collaboration with other groups. Since our contribution to those manuscripts was crucial and substantial, the results will be discussed here (3 manuscripts published in international peer-review journals). Below is a summary of the main research activity carried out by the laboratory of Haematooncology and main scientific results. In all instances the contribution of the team is clarified. At the end of the section a list of the publications related to these results is provided.

One of the main of objectives of our laboratory is to identify novel C/EBPa target genes and define their function in granulopoiesis. To this aim we have performed gene expression profile analysis of cell lines with and without C/EBPa activation. Further, by performing chromatin immunoprecipitation followed by sequencing we determined whether the identified C/EBPa targets where regulated in a direct or indirect manner by C/EBPa. We selected several interesting candidates, and evaluated their role in granulocytic differentiation, and their possible contribution to the development of human acute myeloid leukemia. Part of our work has focused on deciphering the role of the transmembrane protein EVI2B (Ecotropic virus integration site 2) in hematopoiesis. We demonstrated that EVI2B is a direct C/EBPa target gene, we identified its regulatory regions, and showed that C/EBPa controls its expression in a direct manner. During granulocytic differentiation, EVI2B levels are upregulated, and prevention of this upregulation results in a block of neutrophilic production in mouse and human cells. Next, we demonstrated that AML patient samples with mutated C/EBPa presented low EVI2B levels, possibly contributing to the block of granulocytic development characteristic of this disorder. The results of our work were published in the journal Cell Death and Differentiation (Zjablovskaja et al, 2017) and the Journal of Visual Experiments (Zjablovskaja et al, 2018). In the first manuscript, most authors were from the IMG, and we performed most of the experiments presented in the manuscript. The second publication was archived by solely the laboratory of Haematoloncology. Another C/EBPa target gene we have been investigating deeply is C/EBPg. C/EBPg was originally identified as a negatively regulated C/EBPa target gene, however the role of C/EBPg during granulopoiesis was expected but unknown. To address this open question we generated a conditional C/EBPg knockout mouse model and determined the phenotype in hematopoiesis. To our surprise, ablation of C/EBPg in hematopoietic cells did not affect myeloid commitment and/or neutrophilic differentiation. Since we observed that C/EBPg forms heterodimers with C/EBPb, and this transcription factor is crucial for emergency granulopoiesis, we next investigated whether deletion of C/EBPg would perturb granulopoiesis in the context of infection. Several inducers of stress granulopoieisis were employed, but C/EBPg knockout mice responded to the stress similarly to wildtype mice. Altogether, these results were compiled and published in the journal Haematologica (Kardosova et al, 2019). In this publication, most of the work was performed at IMG. In addition to identifying coding genes regulated by C/EBPa, we expanded our interest to the identification and characterization of small non-coding RNAs, in particular miRNAs. Several studies have shown a critical role of miRNA in regulating cell differentiation, and miRNA disruption has been related to the development of cancer. Interestingly, we observed that miRNA-182 and C/EBPa participate in a negative regulatory loop. When C/EBPa or miRNA-182 expression was altered, the loop was deregulated and granulocytic differentiation was impaired. Next, we observed that miRNA-182 expression was highly elevated in AML patients with C-terminal C/EBPa mutations, thereby depicting a mechanism by which C/EBPa blocks miRNA-182 expression. Altogether, our data demonstrated the importance of a controlled balance between C/EBPa and miRNA-182 for the maintenance of healthy granulopoiesis. These results were published in Nature Communications (Wurm et al, 2017), and the contribution from the researchers at

the laboratory of Haematooncology was critical for the high-impact publication. In particular, the co-authors from IMG intellectually contributed to the development of the project, designed, performed, and analyzed all murine in vivo experiments. Another miRNA we have been investigating in the context of granulocytic differentiation is miRNA-143. We revealed that hematopoietic cells undergoing granulocytic differentiation exhibited increased miRNA-143 expression. We manipulated miRNA-143 levels in hematopoietic cells, and observed that overexpression or ablation of miRNA-143 expression resulted in accelerated granulocytic differentiation or block of differentiation, respectively. Argonaute2-RNA-Immunoprecipitation assay revealed ERK5, a member of the MAPK-family, as a target of miRNA-143 in myeloid cells. Altogether, our data demonstrated that miRNA-143 is a relevant factor in granulocyte differentiation, whose expression may be useful as a prognostic and therapeutic factor in AML therapy. These results were published in the Journal of Cell Death and Disease (Hartmann et al, 2018), and the contribution from the researchers at the laboratory of Haematooncology was critical for the high-impact publication. In particular, the co-authors from IMG intellectually contributed to the development of the project, designed, performed, and analyzed all murine in vivo experiments. In addition to the identification and characterization of C/EBPa target genes, our laboratory has been interested in understanding the factors that modulate C/EBPa expression, and identified the transcription factor ZNF143 as a potential C/EBPa regulator. In particular, part of our efforts have focussed in understanding the mechanisms that control C/EBPa transcriptional activation during myeloid differentiation. Importantly, we identified an evolutionarily conserved octameric sequence, CCCAGCAG, ~100 bases upstream of the C/EBPa transcription start site, and demonstrated through mutational analysis that this sequence is crucial for C/EBPa expression. Next, we identified that ZNF143 specifically binds to this 8-bp sequence to activate C/EBPa expression in myeloid cells through a mechanism that is distinct from that observed in liver cells and adipocytes. Altogether, our data suggest that ZNF143 plays an important role in the expression of C/EBPa in myeloid cells. Our laboratory contributed to the successful publication of these results in the Journal of Biological Chemistry (Gonzalez, 2017). The last author position in this manuscript is shared by a member of the laboratory of Haematooncology at IMG, since her contribution (intellectually and experimentally) was critical for the completion of the project.

As mentioned above, the main results of our research activity gave rise to 6 publications listed here. Authors from the laboratory of Haematooncology are in bold, and co-authorships are indicated by asterisk (*).


- **Zjablovskaja P, Kardosova M, Danek P**, Angelisova P, Benoukraf T, Wurm AA, Kalina T, Sian S, Balastik M, Delwel R, Brdicka T, Tenen DG, Behre G, Fiore F, Malissen B, Horejsi V, **Alberich-Jorda M**. EVI2B is a C/EBPα target gene required for granulocytic differentiation and functionality of hematopoietic progenitors. *Cell Death Differ*. 2017
- Wurm AA**, Zjablovskaja P, Kardosova M,** Gerloff D, Bräuer-Hartmann D, Katzerke C, Hartmann JU, Benoukraf T, Fricke S, Hilger N, Müller AM, Bill M, Schwind S, Tenen DG, Niederwieser D, **Alberich-Jorda** M, Behre G. Disruption of the C/EBPα-miR-182 balance impairs granulocytic differentiation. Nat Commun. 2017
- Gonzalez D, Luyten A, Bartholdy B, Zhou Q, **Kardosova M**, Ebralidze A, Swanson KD, Radomska HS, Zhang P, Kobayashi SS, Welner RS, Levantini E, Steidl U, Chong G, Collombet S, Choi MH, Friedman AD, Scott LM, **Alberich-Jorda M***, Tenen DG*. ZNF143 protein is an important regulator of the myeloid transcription factor C/EBPα. (* last two authors equal contribution). J Biol Chem. 2017
- **Zjablovskaja P*, Danek P*, Kardosova M, Alberich-Jorda M**. Proliferation and Differentiation of Murine Myeloid Precursor 32D/G-CSF-R Cells. (*first two authors equal contribution). J Vis Exp. 2018
- Hartmann JU, Bräuer-Hartmann D, **Kardosova M**, Wurm AA, Wilke F, Schödel C, Gerloff D, Katzerke C, Krakowsky R, Namasu CY, Bill M, Schwind S, Müller-Tidow C, Niederwieser D,

**Alberich-Jorda M**, Behre G. MicroRNA-143 targets ERK5 in granulopoiesis and predicts outcome of patients with acute myeloid leukemia. Cell Death Dis. 2018

- **Kardosova M, Zjablovskaja P, Danek P**, Angelisova P, Lobo de Figueiredo-Pontes L, Welner RS, Brdicka T, Lee S, Tenen DG*, **Alberich-Jorda M***. C/EBPγ is dispensable for steady-state and emergency granulopoiesis. (* last two authors equal contribution). Haematologica. 2018

## Research activity and characterisation of the main scientific results

Our research team was established in 2016 with first members arriving in mid-2016 to an empty laboratory space. Hence, we were able to perform experimental work approximately since beginning of 2017. The laboratory has been since then steadily developing, both in terms of instrumentation and personnel. Crucially, the team currently consists of several highly motivated and capable scientists, each with a unique expertise, and each pursuing his/her own project, as well as collaborating together.

Since 2016 we published the following publications with affiliation to IMG-
The Dean et al. 2016, publication utilized a biochemical approaches to identify constituents of discrete cytoskeletal regions, which I mainly developed during my postdoctoral stay at the University of Oxford. I contributed to composing the manuscript and revisions after re-locating to the IMG.

While a part of the work reported in the Varga et al., 2017, publication was done during my postdoctoral stay at the University of Oxford, the experimental work continued and was finalised at the IMG. Preparation of the manuscript plus experimental work associated with revisions were done solely at the IMG. The publication is significant for our laboratory and for the field of flagellum research as it is so far the most comprehensive piece of work focusing on the structures at the flagellum tip. The work identifies a number of novel constituents of the structures at the tip of the trypanosome flagellum, sub-localizes them and characterizes their functions and how these contribute to the overall function of structures.

The Abeywickrema et al., 2019 publication stemmed from a collaboration between our laboratory and the laboratory of Dr. Jack Sunter at the Oxford Brookes University. In our laboratory antibody validation by immunofluorescence, morphometrics measurements and manuscript preparation were performed. The publication introduced the first molecular markers, which allow to distinguish between two daughter cells of a trypanosome cell division. This facilitated description of their morphological differences and changes associated with their maturation. The work also introduces the concept of non-equivalence, i. e. daughter cells with a different history and morphology, which maturate to become morphologically and functionally identical.

In addition to the already published work we have been pursuing several other projects. For some of these the major body of experimental work was performed in the relevant period, and are currently at various stages of the publication process. These include-
The Vachova et al., to be shortly re-submitted to Molecular and Biochemical Parasitology after addressing referees' comments, which introduces and characterizes in a detail a novel approach for creating in locus over-expression mutants in *Trypanosoma brucei*. The approach does not require molecular cloning and is therefore particularly useful to study large proteins, such as many cytoskeletal and flagellar proteins. The work reported in the manuscript was done by members of our laboratory with the exception of RT-qPCR analysis performed by members of bioinformatics facility at the IMG.

The Kiesel et al., manuscript, currently re-submitted after addressing referees comments to Nature Structural and Molecular Biology, is a highly significant study mainly by Pigino laboratory using cryo-electron microscopy to describe ultrastructure of mammalian primary cilia. Our laboratory contributed by confirming localizing of EB1 protein to the primary cilia by live cell imaging.

The manuscript by Mgr. Peter Gorilak, our PhD student, and other member of our laboratory, is currently in preparation, with all experimental work finished. It establishes a robust protocol

for preparation of parasitic kinetoplastids, *T. brucei* and Leishmania spp., for expansion microscopy. This super-resolution method, which is based on a physical 4-fold expansion of a sample in all directions, is particularly suited for imaging of kinetoplastid cells; their cytoskeleton is well defined, highly elaborated, with spacing of individual elements such that it precludes their resolution by a classic light microscopy. In addition to establishing the protocol we validated a number of classic antibody markers to various structures of kinetoplastid cytoskeletons in the protocol. Furthermore, we identified antibodies suitable to localize genetically-tagged proteins. This, together with the high genetic tractability of kinetoplastids gives the expansion microscopy-based localization approach great flexibility. This has been proven by the fact we were already approached by several laboratories in order to start collaborative projects on localization of various proteins in kinetoplastids by this approach. The results reported in the Gorilak et al manuscript were obtained exclusively by members of our laboratory with help in image analysis by a member of imaging facility.

In addition, several important projects were started and significantly progressed since establishing the laboratory.
In the first of these projects we have re-visited some 40 years old observations in Chlamydomonas of the flagellar cytoskeleton being constructed by addition of material exclusively to its distal end. We now confirmed that for a protein of every large axonemal complex using an appropriate experimental set up, whereby the proteins were tagged at their endogenous loci with a self-labelling tag. The experimental work, mainly by our former master student Martina Pruzincova, will be finished by the end of 2020.

A significant effort went towards our major project, focused on identification and characterization of the flagellum/cilium tip-localizing proteins, as these are the prime candidates for regulators of the tip-specific processes. By developing novel biochemical approaches (such as those described in Varga et al., 2017) and via collaboration with the TrypTag project, which aims to localize in a cell all proteins encoded in the trypanosome genome, we have identified over 60 flagellum tip-localizing proteins. These likely represent an almost complete set of flagellum tip-localizing proteins in *T. brucei*. Hence, *T. brucei* is the first organisms with such a deep level of knowledge on this region of the flagellum and put as in a excellent position to achieve its holistic understanding. We re-created fluorescently-tagged cell lines for all the proteins, studied in detail their localization pattern, and whether they are structural or detergent-soluble. Moreover, we created a cell line capable of inducible depletion of each of the protein by RNAi and characterized the resulting cells for their ability to proliferate, form flagella and swim. The analysis of these cell lines is ongoing. However, it already identified tip proteins necessary for construction of the axoneme, proteins that negatively regulate the flagellar length in a cell cycle-dependent manner, and proteins which modulate, both positively and negatively, the swimming velocity. We have studied ultrastructure of the axoneme in these mutants using transmission electron microscopy and for some identified structural abnormalities.

Crucially, in collaboration with Dr. Bill Wickstead from the University of Nottingham, we performed an evolutionary analysis of all the proteins and identified mammalian orthologues for a number of them. We have been systematically screening those by localizing their tagged variants in mammalian tissue culture cells forming primary cilia. This led so far to identification of a previously unrecognized Zn-finger domain containing family of cilia-associated proteins. Intriguingly, one member of the family localizes to the tip of the primary cilium and in addition in a dotted pattern also along the cilium. We have been testing the hypothesis that the protein localizes to the distal end of every microtubule in the primary cilium; it was recently demonstrated that many microtubules of the primary cilium terminate sub-distally. We do see a correlation between the localization pattern of the tagged Zn-finger protein in the cilium and the distribution of axonemal microtubule ends for individual cell lines. Moreover, the positions

of the Zn-finger protein localization correspond to sites where the intraflagellar transport particles, which transport material along the axonemal microtubules, frequently pause or change direction of their movement. This behaviour of the intraflagellar transport particles has not been so far described, but will be critical for understanding the processes of the primary cilium axoneme assembly and turnover. We are convinced that having a reliable marker of the ends of axonemal microtubules is a crucial step towards understanding how the microtubule pattern is formed and what is its significance.

**Publications-**

Dean S., Moreira-Leite F., Varga V. and Gull K. (2016). Cilium transition zone proteome reveals compartmentalization and differential dynamics of ciliopathy complexes. PNAS 113(35):E5135-43

Varga V.*, Moreira-Leite F., Portman N. and Gull K.* (2017). Protein diversity in discrete structures at the distal tip of the trypanosome flagellum. PNAS 114(32):E6546-E6555

Abeywickrema M., Vachova H., Farr H., Mohr T., Wheeler R., Lai D., Vaughan S., Gull K., Sunter J.* and Varga V.* (2019). Non-equivalence in old- and new-flagellum daughter cells of a proliferative division in Trypanosoma brucei. Mol. Microbiol. 112(3):1024-1040

**Manuscripts under review-**

Vachova H., Alquicer G., Sedinova M., Sachova J., Hradilova M. and Varga V.* A rapid approach for in locus overexpression of Trypanosoma brucei proteins and its use to study flagellum biology – addressing referees comments to Mol. Biochem. Parasitol.

Petra Kiesel, Gonzalo Alvarez Viar, Nikolai Tsoy, Riccardo Maraspini, Peter Gorilak, Vladimir Varga, Alf Honigmann, Gaia Pigino. The molecular structure of primary cilia revealed by cryo-electron tomography – resubmitted to Nature Structural and Molecular Biology

underlined – members of our laboratory
* - corresponding author

## Research activity and characterisation of the main scientific results

We are listing all the accepted publications from our lab in the period of 2016-2019 below. The authors affiliated to our lab are underlined. We indicate the contribution of our lab to the manuscript. The publications are arbitrarily divided into four research directions to manifest the focus of our research in a comprehensive manner.

Lots of the activities of our group after its establishment in mid-2016 were finished/published in 2020, i.e., in the year following the evaluated period. The respective publications and manuscripts are listed in the section “Activity plan of the team for the period of 2020-2024”.

1. Research direction “TCR signaling”:

Courtney AH, Shvets AA, Lu W, Griffante G, Mollenauer M, Horkova V, Lo WL, Yu S, Stepanek O, Chakraborty AK, Weiss A: CD45 functions as a signaling gatekeeper in T cells. **Sci Signal** 2019 12(604).

T cells require the protein tyrosine phosphatase CD45 to detect and respond to antigen because it activates the Src family kinase Lck, which phosphorylates the T cell antigen receptor (TCR) complex. CD45 activates Lck by opposing the negative regulatory kinase Csk. Paradoxically, CD45 has also been implicated in suppressing TCR signaling by dephosphorylating the same signaling motifs within the TCR complex upon which Lck acts. We sought to reconcile these observations using chemical and genetic perturbations of the Csk/CD45 regulatory axis incorporated with computational analyses. Specifically, we titrated the activities of Csk and CD45 and assessed their influence on Lck activation, TCR-associated ζ-chain phosphorylation, and more downstream signaling events. Acute inhibition of Csk revealed that CD45 suppressed ζ-chain phosphorylation and was necessary for a regulatable pool of active Lck, thereby interconnecting the activating and suppressive roles of CD45 that tune antigen discrimination. CD45 suppressed signaling events that were antigen independent or induced by low-affinity antigen but not those initiated by high-affinity antigen. Together, our findings reveal that CD45 acts as a signaling "gatekeeper," enabling graded signaling outputs while filtering weak or spurious signaling events.

*Contribution of our group: generation of cell line models for antigen-specific TCR signalling ex vivo*

Lo WL, Shah NH, Rubin SA, Zhang W, Horkova V, Fallahee IR, Stepanek O, Zon LI, Kuriyan J, Weiss A: Slow phosphorylation of a tyrosine residue in LAT optimizes T cell ligand discrimination. **Nat Immunol** 2019 20(11): 1481-1493.

Self–non-self discrimination is central to T cell-mediated immunity. The kinetic proofreading model can explain T cell antigen receptor (TCR) ligand discrimination; however, the rate-limiting steps have not been identified. Here, we show that tyrosine phosphorylation of the T cell adapter protein LAT at position Y132 is a critical kinetic bottleneck for ligand discrimination. LAT phosphorylation at Y132, mediated by the kinase ZAP-70, leads to the recruitment and activation of phospholipase C-γ1 (PLC-γ1), an important effector molecule for T cell activation.

The slow phosphorylation of Y132, relative to other phosphosites on LAT, is governed by a preceding glycine residue (G131) but can be accelerated by substituting this glycine with aspartate or glutamate. Acceleration of Y132 phosphorylation increases the speed and magnitude of PLC-γ1 activation and enhances T cell sensitivity to weaker stimuli, including weak agonists and self-peptides. These observations suggest that the slow phosphorylation of Y132 acts as a proofreading step to facilitate T cell ligand discrimination.

*Contribution of our group: generation of cell line models for antigen-specific TCR signalling ex vivo*

Lo WL, Shah NH, Ahsan N, Horkova V, Stepanek O, Salomon AR, Kuriyan J, Weiss A: Lck promotes Zap70-dependent LAT phosphorylation by bridging Zap70 to LAT. **Nat Immunol** 2018 19(7): 733-741.

T cell-antigen receptor (TCR) signaling requires the sequential activities of the kinases Lck and Zap70. Upon TCR stimulation, Lck phosphorylates the TCR, thus leading to the recruitment, phosphorylation, and activation of Zap70. Lck binds and stabilizes phosho-Zap70 by using its SH2 domain, and Zap70 phosphorylates the critical adaptors LAT and SLP76, which coordinate downstream signaling. It is unclear whether phosphorylation of these adaptors occurs through passive diffusion or active recruitment. We report the discovery of a conserved proline-rich motif in LAT that mediates efficient LAT phosphorylation. Lck associates with this motif via its SH3 domain, and with phospho-Zap70 via its SH2 domain, thereby acting as a molecular bridge that facilitates the colocalization of Zap70 and LAT. Elimination of this proline-rich motif compromises TCR signaling and T cell development. These results demonstrate the remarkable multifunctionality of Lck, wherein each of its domains has evolved to orchestrate a distinct step in TCR signaling.

*Contribution of our group: generation of cell line models for antigen-specific TCR signalling ex vivo*

Bosch AJT, Bolinger B, Keck S, Stepanek O, Ozga AJ, Galati-Fournier V, Stein JV, Palmer E: A minimum number of autoimmune T cells to induce autoimmunity? **Cell Immunol** 2017 316: 21-31.

While autoimmune T cells are present in most individuals, only a minority of the population suffers from an autoimmune disease. To better appreciate the limits of T cell tolerance, we carried out experiments to determine how many autoimmune T cells are required to initiate an experimental autoimmune disease. Variable numbers of autoimmune OT-I T cells were transferred into RIP-OVA mice, which were injected with antigen-loaded DCs in a single footpad; this restricted T cell priming to a few OT-I T cells that are present in the draining popliteal lymph node. Using selective plane illumination microscopy (SPIM) we counted the number of OT-I T cells present in the popliteal lymph node at the time of priming. Analysis of our data suggests that a single autoimmune T cell cannot induce an experimental autoimmune disease, but a "quorum" of 2-5 autoimmune T cells clearly has this capacity.

*Contribution of our group: mathematical analysis of biological data, data interpretation*

Palmer E, Drobek A, Stepanek O: Opposing effects of actin signaling and LFA-1 on establishing the affinity threshold for inducing effector T-cell responses in mice. **Eur J Immunol**, 2016 46(8): 1887-901.

Mature CD8(+) T cells use a narrow antigen affinity threshold to generate tissue-infiltrating cytotoxic effector T cells and induce autoimmune pathology, but the mechanisms that establish this antigen affinity threshold are poorly understood. Only antigens with affinities above the threshold induce stable contacts with APCs, polarization of a T cell, and asymmetric T-cell division. Previously published data indicate that LFA-1 inside-out signaling might be involved in establishing the antigen affinity threshold. Here, we show that subthreshold antigens weakly activate all major distal TCR signaling pathways. Low-affinity antigens are more dependent on LFA-1 than suprathreshold antigens. Moreover, augmenting the inside-out signaling by hyperactive Rap1 does not increase responses to the subthreshold antigens. Thus, LFA-1 signaling does not contribute to the affinity-based antigen discrimination. However, we found that subthreshold antigens do not induce actin rearrangement toward an APC, mediated by Rho-family GTPases, Cdc42, and Rac. Our data suggest that Rac and Cdc42 contribute to the establishment of the antigen affinity threshold in CD8(+) T cells by enhancing responses to high-affinity antigens, or by reducing the responses to low-affinity antigens.

*Contribution of our group: ex vivo experiments such as GTPase activity measurement, interpretation of the data.*

Hrdinka M, Sudan K, Just S, Drobek A, Stepanek O, Schlüter D, Reinhold D, Jordan BA, Gintschel P, Schraven B, Kreutz MR: Normal Development and Function of T Cells in Proline Rich 7 (Prr7) Deficient Mice. **PLoS One** 2016 11(9): e0162863.

Transmembrane adaptor proteins (TRAPs) are important organisers for the transduction of immunoreceptor-mediated signals. Prr7 is a TRAP that regulates T cell receptor (TCR) signalling and potently induces cell death when overexpressed in human Jurkat T cells. Whether endogenous Prr7 has a similar functional role is currently unknown. To address this issue, we analysed the development and function of the immune system in Prr7 knockout mice. We found that loss of Prr7 partially impairs development of single positive CD4+ T cells in the thymus but has no effect on the development of other T cell subpopulations, B cells, NK cells, or NKT cells. Moreover, Prr7 does not affect the TCR signalling pathway as T cells derived from Prr7 knockout and wild-type animals and stimulated in vitro express the same levels of the activation marker CD69, and retain their ability to proliferate and activate induced cell death programs. Importantly, Prr7 knockout mice retained the capacity to mount a protective immune response when challenged with Listeria monocytogenes infection in vivo. In addition, T cell effector functions (activation, migration, and reactivation) were normal following induction of experimental autoimmune encephalomyelitis (EAE) in Prr7 knockout mice. Collectively, our work shows that loss of Prr7 does not result in a major immune system phenotype and suggests that Prr7 has a dispensable function for TCR signalling.

*Contribution of our group: analysis of PRR7 expression in tissues*

2. Research direction “T-cell diversity”:

Drobek A, Moudra A, Mueller D, Huranova M, Horkova V, Pribikova M, Ivanek R, Oberle S, Zehn D, McCoy KD, Draber P, Stepanek O: Strong homeostatic TCR signals induce formation of self-tolerant virtual memory CD8 T cells. **EMBO J** 2018 37(14).

Virtual memory T cells are foreign antigen-inexperienced T cells that have acquired memory-like phenotype and constitute 10-20% of all peripheral CD8+ T cells in mice. Their origin, biological roles, and relationship to naïve and foreign antigen-experienced memory T cells are incompletely understood. By analyzing T-cell receptor repertoires and using retrogenic monoclonal T-cell populations, we demonstrate that the virtual memory T-cell formation is a so far unappreciated cell fate decision checkpoint. We describe two molecular mechanisms driving the formation of virtual memory T cells. First, virtual memory T cells originate exclusively from strongly self-reactive T cells. Second, the stoichiometry of the CD8 interaction with Lck regulates the size of the virtual memory T-cell compartment via modulating the self-reactivity of individual T cells. Although virtual memory T cells descend from the highly self-reactive clones and acquire a partial memory program, they are not more potent in inducing experimental autoimmune diabetes than naïve T cells. These data underline the importance of the variable level of self-reactivity in polyclonal T cells for the generation of functional T-cell diversity.

*Contribution of our group: vast majority of experiments and managing of the project*

3. Research direction “Inflammatory signaling”:

Lafont E, Draber P, Rieser E, Reichert M, Kupka S, de Miguel D, Draberova H, von Mässenhausen A, Bhamra A, Henderson S, Wojdyla K, Chalk A, Surinova S, Linkermann A, Walczak H: TBK1 and IKKε prevent TNF-induced cell death by RIPK1 phosphorylation. **Nat Cell Biol** 2018 20(12): 1389-1399.

The linear-ubiquitin chain assembly complex (LUBAC) modulates signalling via various immune receptors. In tumour necrosis factor (TNF) signalling, linear (also known as M1) ubiquitin enables full gene activation and prevents cell death. However, the mechanisms underlying cell death prevention remain ill-defined. Here, we show that LUBAC activity enables TBK1 and IKKε recruitment to and activation at the TNF receptor 1 signalling complex (TNFR1-SC). While exerting only limited effects on TNF-induced gene activation, TBK1 and IKKε are essential to prevent TNF-induced cell death. Mechanistically, TBK1 and IKKε phosphorylate the kinase RIPK1 in the TNFR1-SC, thereby preventing RIPK1-dependent cell death. This activity is essential in vivo, as it prevents TNF-induced lethal shock. Strikingly, NEMO (also known as IKKγ), which mostly, but not exclusively, binds the TNFR1-SC via M1 ubiquitin, mediates the recruitment of the adaptors TANK and NAP1 (also known as AZI2). TANK is constitutively associated with both TBK1 and IKKε, while NAP1 is associated with TBK1. We discovered a previously unrecognized cell death checkpoint that is mediated by TBK1 and IKKε, and uncovered an essential survival function for NEMO, whereby it enables the recruitment and activation of these non-canonical IKKs to prevent TNF-induced cell death.

*Contribution of our group: planning and analysis of experiments*

Peltzer N, Darding M, Montinaro A, Draber P, Draberova H, Kupka S, Rieser E, Fisher A, Hutchinson C, Taraborrelli L, Hartwig T, Lafont E, Haas TL, Shimizu Y, Böiers C, Sarr A, Rickard J, Alvarez-Diaz S, Ashworth MT, Beal A, Enver T, Bertin J, Kaiser W, Strasser A, Silke J, Bouillet P, Walczak H: LUBAC is essential for embryogenesis by preventing cell death and enabling haematopoiesis. **Nature** 2018 557(7703): 112-117.

The linear ubiquitin chain assembly complex (LUBAC) is required for optimal gene activation and prevention of cell death upon activation of immune receptors, including TNFR1. Deficiency in the LUBAC components SHARPIN or HOIP in mice results in severe inflammation in adulthood or embryonic lethality, respectively, owing to deregulation of TNFR1-mediated cell death. In humans, deficiency in the third LUBAC component HOIL-1 causes autoimmunity and inflammatory disease, similar to HOIP deficiency, whereas HOIL-1 deficiency in mice was reported to cause no overt phenotype. Here we show, by creating HOIL-1-deficient mice, that HOIL-1 is as essential for LUBAC function as HOIP, albeit for different reasons: whereas HOIP is the catalytically active component of LUBAC, HOIL-1 is required for LUBAC assembly, stability and optimal retention in the TNFR1 signalling complex, thereby preventing aberrant cell death. Both HOIL-1 and HOIP prevent embryonic lethality at mid-gestation by interfering with aberrant TNFR1-mediated endothelial cell death, which only partially depends on RIPK1 kinase activity. Co-deletion of caspase-8 with RIPK3 or MLKL prevents cell death in Hoil-1−/− (also known as Rbck1−/−) embryos, yet only the combined loss of caspase-8 with MLKL results in viable HOIL-1-deficient mice. Notably, triple-knockout Ripk3−/−Casp8−/−Hoil-1−/− embryos die at late gestation owing to haematopoietic defects that are rescued by co-deletion of RIPK1 but not MLKL. Collectively, these results demonstrate that both HOIP and HOIL-1 are essential LUBAC components and are required for embryogenesis by preventing aberrant cell death. Furthermore, they reveal that when LUBAC and caspase-8 are absent, RIPK3 prevents RIPK1 from inducing embryonic lethality by causing defects in fetal haematopoiesis.

*Contribution of our group: planning and analysis of experiments*

4. Research direction “Ciliogenesis/Polarized transport”:

Arakel EC, Huranova M, Estrada AF, Rau EM, Spang A, Schwappach B: Dissection of GTPase-activating proteins reveals functional asymmetry in the COPI coat of budding yeast. **J Cell Sci** 2019 132(16).

The Arf GTPase controls formation of the COPI vesicle coat. Recent structural models of COPI revealed the positioning of two Arf1 molecules in contrasting molecular environments. Each of these pockets for Arf1 is expected to also accommodate an Arf GTPase-activating protein (ArfGAP). Structural evidence and protein interactions observed between isolated domains indirectly suggest that each niche preferentially recruits one of the two ArfGAPs known to affect COPI, i.e. Gcs1/ArfGAP1 and Glo3/ArfGAP2/3, although only partial structures are available. The functional role of the unique non-catalytic domain of either ArfGAP has not been integrated into the current COPI structural model. Here, we delineate key differences in the consequences of triggering GTP hydrolysis through the activity of one versus the other ArfGAP. We

demonstrate that Glo3/ArfGAP2/3 specifically triggers Arf1 GTP hydrolysis impinging on the stability of the COPI coat. We show that the Snf1 kinase complex, the yeast homologue of AMP-activated protein kinase (AMPK), phosphorylates the region of Glo3 that is crucial for this effect and, thereby, regulates its function in the COPI-vesicle cycle. Our results revise the model of ArfGAP function in the molecular context of COPI.

*Contribution of our group: Microscopy experiments (FRAP)*

Niederlova V, Modrak M, Tsyklauri O, Huranova M, Stepanek O: Meta-analysis of genotype-phenotype associations in Bardet-Biedl syndrome uncovers differences among causative genes. **Hum Mutat** 2019 40(11): 2068-2087.

Bardet-Biedl syndrome (BBS) is a recessive genetic disease causing multiple organ anomalies. Most patients carry mutations in genes encoding for the subunits of the BBSome, an octameric ciliary transport complex, or accessory proteins involved in the BBSome assembly or function. BBS proteins have been extensively studied using in vitro, cellular, and animal models. However, the molecular functions of particular BBS proteins and the etiology of the BBS symptoms are still largely elusive. In this study, we applied a meta-analysis approach to study the genotype-phenotype association in humans using our database of all reported BBS patients. The analysis revealed that the identity of the causative gene and the character of the mutation partially predict the clinical outcome of the disease. Besides their potential use for clinical prognosis, our analysis revealed functional differences of particular BBS genes in humans. Core BBSome subunits BBS2, BBS7, and BBS9 manifest as more critical for the function and development of kidneys than peripheral subunits BBS1, BBS4, and BBS8/TTC8, suggesting that incomplete BBSome retains residual function at least in the kidney.

*Contribution of our group: vast majority of analyses and management of the project*

## Research activity and characterisation of the main scientific results

Dr Hana Hanzlikova (laboratory 49 deputy head) started to work in the field of poly-ADP-ribose polymerase (PARP) biology in 2014 when she joined my laboratory at the Genome Damage and Stability Centre in Sussex. Already in 2017, we have published in *Nature* our conceptually ground-breaking discovery that the neurological disease that is triggered by unrepaired DNA single-strand breaks is caused in part by excessive activation of the DNA damage sensor protein, PARP1. This discovery identifies PARP1 as a plausible new therapeutic target in the treatment of neurological disease; a truly exciting concept in which novel and/or existing PARP1 inhibitors might be repurposed beyond their current use in the cancer clinic. Excitingly, excessive PARP activity has now also been implicated in several common neurological diseases including Parkinson's disease and Huntington's disease, expanding the possible translational significance of our discovery.

Following this period, Hana relocated back to the Institute of Molecular Genetics in Prague, to set up a new Laboratory of Genome Dynamics (guest group), where she continued to work in the field of PARP biology. Here, she extended her studies on PARP metabolism beyond the molecular mechanisms of human disease in postmitotic cells to include disease mechanisms in proliferating cells. We have discovered and published in ***Molecular Cell*** (2018) that the primary source of endogenous DNA single-strand breaks that are detected by PARP1 during normal cell division are not stochastic DNA lesions, but instead are normal Okazaki fragment intermediates of DNA replication. This unexpected and striking discovery implicates PARP-dependent DNA repair as an alternative pathway for the processing of canonical DNA replication intermediates; a paradigm-shifting discovery that challenges the "text book" view of how DNA is replicated. This work has major implications for cancer research, because it identifies these obligatory DNA replication intermediates as the likely source of synthetic lethality that is triggered in certain cancer cells by PARP1 inhibition.

In summary, we have identified the primary source of the genome damage that is detected by the PARP1 sensor protein; a paradigm-shifting discovery that has changed the direction in which the field of genome instability is thinking. Our discoveries have established new avenues of therapeutic intervention not only in neurodegeneration, but also in the diagnosis and treatment of cancer (Summarised in ***Trends in Genetics*** in 2019).

Publications:

1. **Hanzlikova H***, **Kalasova I**, Demin AA, Pennicott LE, **Cihlarova Z**, **Caldecott KW***. The Importance of Poly(ADP-Ribose) Polymerase as a Sensor of Unligated Okazaki Fragments during DNA Replication. ***Mol. Cell.*** 2018 Jul 19;71(2):319-331. *corresponding authors

We have described Okazaki fragment intermediates of DNA replication as the primary source of endogenous DNA single-strand breaks in cells and implicated PARP-dependent DNA repair as an alternative pathway for their processing. Our work has major implications for cancer research, because it identifies these obligatory DNA replication intermediates as the likely source of synthetic lethality that is triggered in certain cancer cells by PARP1 inhibition. This work was conducted at IMG. Four authors, the first as well as the corresponding authors are IMG employees.

2. **Hanzlikova H*** & **Caldecott KW***. Perspectives on PARPs in S Phase. ***Trends Genet.*** 2019 Jun 35(6): 412-422. *corresponding authors

Trends in Genetics is a review journal of broad and general interst. In this review, we highlight and discuss the broader implications of our *Molecular Cell* paper, in which we identified Okazaki fragments as a primary source of PARP1 activity in proliferating cells. This

review extends our primary publication and presents, for the first time, our groundbreaking concepts concerning the impact of canonical DNA replication intermediates in cancer biology and cancer therapeutics. Both authors are IMG employees.

Other research:

3. **Kalasova I[#]**, **Hanzlikova H[#,*]**, Gupta N*, Li Y, Altmüller J, Reynolds JJ, Stewart GS, Wollnik B, Yigit G, **Caldecott KW***. Novel PNKP mutations causing defective DNA strand break repair and PARP1 hyperactivity in MCSZ. ***Neurol Genet.*** 2019 Mar 25;5(2):e320. [#]equal contribution, *corresponding authors

Mutations in the single-strand break repair enzyme polynucleotide 5′-kinase 3′-phosphatase (PNKP) are associated with three apparently distinct neurologic diseases: *microcephaly with early onset seizures* (MCSZ), *ataxia with oculomotor apraxia, and Charcot-Marie-Tooth disease.* Here, we identify two novel mutations in PNKP which expand the spectrum of PNKP mutations associated with MCSZ and show that PARP1 hyperactivation at unrepaired topoisomerase-induced DNA breaks is a molecular feature of this disease. This work was conducted at IMG. The first as well as the corresponding authors are IMG employees. Other co-authors are clinicians and researchers who provided us with research materials and services.

4. Mahjoub A[#], **Cihlarova Z[#]**, Tétreault M, MacNeil L, Sondheimer N, **Caldecott KW**, **Hanzlikova H***, Yoon G*. Homozygous pathogenic variant in BRAT1 associated with nonprogressive cerebellar ataxia. ***Neurol Genet.*** 2019 Sep 4;5(5):e359. [#]equal contribution, *corresponding author

We have identified a novel homozygous BRAT1 pathogenic variant in two siblings with nonprogressive cerebellar ataxia, a phenotype considerably milder than previously reported. Surprisingly, in contrast to the molecular role currently proposed for BRAT1 in ataxia-telangiectasia mutated (ATM) regulation, we show that this disorder is unlikely to result from defective ATM kinase signalling or mitochondrial dysfunction. We are now exploring the molecular role of BRAT1 to identify the mechanism of the pathogenicity. This work was conducted at IMG. The first as well as the corresponding authors are IMG employees. Other co-authors are clinicians and researchers who provided us with research materials and services.

5. **Caldecott KW**. XRCC1 protein; Form and Function. ***DNA Repair***. 2019 Sep;81:102664.

This review summarises and discusses our current structural and functional understanding of XRCC1; a critical scaffold protein that coordinates the activity of multiple DNA strand break repair proteins involved in neuroprotection in humans.

## Research activity and characterisation of the main scientific results

### 1. Research tools and techniques

Since 2015, we have generated and partially characterized mice with plectin ablation in the intestinal epithelium, using constitutive and inducible intestine-specific cre-mice under control of the villin promoter: Plecfl/fl;Villin-cre and Plecfl/fl;Villin-creERT2 mice. To grasp the complexity of interactions of immune cells with plectin-deficient mucosa, we also generated mice with tissue-specific plectin ablation in myeloid cell lineage, using Cre-expressing mice under control of either lysozyme 2 (Plecfl/fl;Lyz2-cre) or MRP8 (Plecfl/fl;S100A8-cre). To study the function of plectin in livers, we generated hepatocyte- and cholagiocyte-specific conditional Plecfl/fl;Alb-cre mice and hepatic stellate cell (HSC)-specific Plecfl/fl;Lrat-cre mice. In collaboration with the Laboratory of Cell Differentiation (IMG CAS, Prague), we have also generated two plectin isoform-specific knock-in lines using CRISPR/Cas9 targeting and *i*-GONAD approach. Both mouse lines carry mutations which were identified in human patients (premature stop codons in Plec1f and Plec1a first exons) and recapitulate patient conditions (limb-girdle muscle dystrophy and *Epidermolysis bullosa*).

To complement our *in vivo* studies, we also developed plectin-targeting constructs based on CRISPR/Cas9 technology, enabling either complete gene inactivation (also in isoform-specific manner) or gene editing of functional domains (actin and intermediate filament binding domains). All targeting constructs were successfully validated in both murine and human cell lines. To date, we have generated plectin-deficient intestinal epithelial cell lines (both non-transformed and cancer: hCC, Caco-2, HT-29, SW480), cholangiocytes, hepatocellular carcinoma cell lines (Huh-7, Hep3B, PCL/PRF, SNU-423, SNU-475, and SNU-398), HSC lines (Lx-1 and Lx-2), mouse lung fibroblasts (CCL206), and MDCK cells.

From the very beginning, our team has strived to establish several methods which would enable us to stimulate living systems mechanically (by well-controlled external forces) and then determine their mechanical and functional responses. In collaboration with the team of Prof. B. Fabry (Biophysics Group, Center for Medical Physics and Technology, Erlangen), we developed a uniaxial cell stretching system for parallel application of a fixed strain to single cells or cell sheets by deforming an elastic PDMS substrate. The experimental setup with six PDMS membrane holders allows a combined analysis of cell architecture (immunofluorescence microscopy) and mechanosignalling pathways (immunoblot and transcript profiling). The cell stretching system is currently fully functional and available to the local scientific community. A collaborative manuscript with the Fabry group is in preparation. In addition, in collaboration with the team of Dr. Kubinova (Laboratory of Biomathematics, Institute of Physiology, CAS, Prague) we are also developing a cell stretching system which will be combined with spinning disc confocal microscopy. Currently, we are testing a functional prototype. In order to determine traction forces exerted by adherent cell or cell clusters quantitatively, we successfully established 2D traction force microscopy (TFM) on PAAm hydrogels. In collaboration with the team of Prof. B. Fabry, we developed a computational approach to calculate deformation, traction forces, shear stress, contractile energy, and line tensions at cell-cell borders (a collaborative manuscript in preparation). In addition, we established approaches to visualize tensile forces at cell-ECM or cell-cell junctions, using molecular tension FRET sensors (talin-, E-cadherin-, and desmoplakin-based) and time-correlated single-photon counting (TCSPC) FLIM-FRET microscopy. This work was funded by the Czech-BioImaging Open Access Project “Measurement of actomyosin tension in plectin deficient hepatocellular carcinoma cells using talin-based FRET tension sensor”, 2019. The unique combination of 2D TFM with molecular force sensors provides an efficient tool to decipher the mechanisms of mechanosensing and mechanotransduction at cell junctions.

**2. Completed Research Projects (2015-2019)**

**2.1. The role of plectin in biliary tree architecture and stability in cholestasis** (funded by GACR 15-23858S "The impact of liver-specific plectin deficiency on the pathogenesis of liver diseases", 2015-2017 and GAUK 896916 "The role of plectin in the architecture and function of the liver epithelium", 2016-2018). Using liver-specific plectin knockout mouse model (Plecfl/fl;Alb-cre), we were first to demonstrate that genes associated with *Epidermolysis bullosa* (plectin, keratins) play an essential role in the adaptation of liver tissues to cholestasis. Plectin deficiency in hepatocytes and biliary epithelial cells (BECs) led to aberrant keratin filament network organization, biliary tree malformations, and an age-related collapse of bile ducts (BDs) and ductules. Using bile duct ligation (BDL) and 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) feeding of mice as cholestatic liver injury models, we found that plectin ablation aggravated biliary damage, particularly in BDL-induced cholestasis. Coincidently, we observed a significant expansion of A6-positive progenitor cells in plectin-deficient livers. After BDL, plectin-deficient BDs were prominently dilated with more frequent ruptures corresponding to an increased number of bile infarcts. A transmission electron microscopy analysis revealed a compromised tight junction formation in plectin-deficient BECs. In addition, protein profiling showed increased expression of the adherens junction protein E-cadherin and inefficient upregulation of the desmosomal protein desmoplakin in response to BDL.

In order to further characterize the response to cholestatic conditions in Plecfl/fl;Alb-cre mice, we fed Plefl/fl and Plecfl/fl;Alb-cre mice with the 0.5% cholic acid (CA)-supplemented diet for 14 days. Mice of both genotypes had comparable relative liver weights and similar levels of alkaline phosphatase. In line with the results obtained from the BDL and DDC models, CA-feeding resulted in slightly higher bilirubin levels in Plecfl/fl;Alb-cre when compared to the levels in Plefl/fl mice. In addition, we observed significantly higher levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), and also increased total serum bile acids (tBA) in Plecfl/fl;Alb-cre mice. The inspection of H&E stained liver sections from mice subjected to CA-feeding revealed a typical increase in cell size and disseminated hepatocyte necroses, which were more frequent in Plecfl/fl;Alb-cre livers. Immunofluorescence microscopy for canalicular marker ZO1 showed significantly dilated, more meandering, and prominently misshaped bile canaliculi in Plecfl/fl;Alb-cre livers when compared to Plefl/fl livers. Although abnormalities in a canalicular network were also seen in untreated Plecfl/fl;Alb-cre livers, the difference between Plefl/fl and Plecfl/fl;Alb-cre phenotypes was more pronounced upon CA insult. Higher serum levels of liver injury markers and histopathological changes after CA-diet suggest that Plecfl/fl;Alb-cre mice also fail to prevent the hepatocellular accumulation of toxic bile acids, likely due to the inability to activate a cholestasis-induced adaptive response.

**Publications:**

- **Jirouskova M.**, **Nepomucka K.**, **Oyman-Eyrilmez G.**, **Kalendova A.**, **Havelkova H.**, **Sarnova L.**, Chalupsky K., Schuster B., Benada O., Miksatkova P., Kuchar M., Fabian O., Sedlacek R., Wiche G., **Gregor M.**: Plectin Controls Biliary Tree Architecture and Stability in Cholestasis. *J Hepatol.*, 68:1006-1017, 2018.

**Publication description:** Using a mouse model, we were first to demonstrate that genes associated with *Epidermolysis bullosa* (plectin, keratins) play an essential role in the adaptation of liver tissues to cholestasis. Plectin deficiency led to biliary epithelial instability, and mutated mice failed to activate a cholestasis-induced adaptive response in models of experimental cholestasis. Subsequently, Wu et al. (*Hepatology* 2019) identified plectin mutations in patients with progressive familial intrahepatic cholestasis.

**Group contribution:** Seven co-authors (out of 15), including two co-first authors and the corresponding author, are group members (in **bold**, corresponding author). Group contribution entails the study concept and design, all experiments, analysis and interpretation of data, drafting of the manuscript, and obtained funding. Contribution of external co-authors

entails bile MS analysis (Chalupsky K., Miksatkova P., Kuchar M.), CRISPR/Cas9 targeting constructs (Schuster B.), transmission electron microscopy (Benada O.), histopathological analysis (Fabian O.), funding and infrastructural support (Sedlacek R.), and mouse model (Wiche G.).

- **Korelova K.**, **Jirouskova M.**, **Sarnova L.**, **Gregor M.**: Isolation and 3D Collagen Sandwich Culture of Primary Mouse Hepatocytes to Study the Role of Cytoskeleton in Bile Canalicular Formation In Vitro. *J Vis Exp.* 2019 Dec 20;(154). doi: 10.3791/60507.

**Publication description:** The methodological publication describes a protocol that we developed for the isolation of mouse hepatocytes from the adult mouse liver, using a modified collagenase perfusion technique. It also describes the production of culture in a 3D collagen sandwich setting, which is used for immunolabeling of cytoskeletal components to study bile canalicular formation and its response to treatments *in vitro*. We show that hepatocyte 3D collagen sandwich cultures respond to treatments with toxins (ethanol) or actin cytoskeleton altering drugs (e.g., blebbistatin) and serve as a valuable tool for *in vitro* studies of the bile canaliculi formation and function.

**Group contribution:** All co-authors, including the corresponding author, are group members (in **bold**, corresponding author).

- Müller M., Wetzel S., Köhn-Gaone J., Chalupsky K., Lüllmann-Rauch R., Barikbin R., Bergmann J., Wöhner B., Zbodakova O., Leuschner I., **Gregor M.**, Tiegs G., Rose-John S., Sedlacek R., Tirnitz-Parker J.E.E., Saftig P., Schmidt-Arras, D.: A disintegrin and metalloprotease 10 (ADAM10) is a central regulator of murine liver tissue homeostasis. *Oncotarget*, 7:17431-41, 2016.

**Publication description:** The loss of ADAM10 in the murine liver results in hepatocyte necrosis and concomitant liver fibrosis. ADAM10 directly regulates expression of bile acid transporters, but it is dispensable for Notch2-dependent formation of the biliary system. In the absence of ADAM10, the activation of liver progenitor cells is enhanced through increased c-Met signalling, and the differentiation of liver progenitor cells to hepatocytes is augmented.

**Group contribution:** Only one co-author (out of 17) is a group member (in **bold**). His contribution entails planning and supervising experiments and writing the manuscript.

**2.2. The impact of plectin deficiency on the architecture and barrier function of intestinal epithelia** (funded by MEYS/COST Action CA15214, EuroCellNet "Plectinopathies: Disease Aspects in Simple Epithelia.", 2015-2017; CAS postdoctoral fellowship to A. Krausova "Dysregulation of the digestive epithelia cytoskeleton in models of *Epidermolysis bullosa*", 2016–2017, and GAUK 192119 "Experimental colitis in a mouse model of plectinopathy", 2019-2021).
Previous studies demonstrated the importance of KF-associated cell junctions (hemidesmosomes and desmosomes for the maintenance of the intestinal epithelial barrier and protection against intestinal inflammation). Furthermore, it was shown that hemidesmosomal integrity protects the colon against colorectal cancer; some authors suggested classifying hemidesmosomal integrin □6 as a tumour suppressor. It is generally accepted that functional junctions are required to interlink intestinal epithelial cells (IECs) with the basement membrane (BM) to form a robust physical barrier, which is exposed to substantial mechanical stress. Indeed, genetic studies identified multiple links between junction/BM-associated genes and the development of IBD. Surprisingly, mechanobiological aspects of the intestinal epithelium remain largely elusive. No previous studies have

addressed how intestinal KFs and KF-associated junctions are integrated into the structural and functional continuum that provides the epithelial barrier with mechanical resilience.
In this study, we investigated this question using two newly generated mouse lines with induced IEC-specific ablation of plectin either during development (Plecfl/fl;Villin-cre mice) or in adults (Plecfl/fl;Villin-creERT2 mice). Strikingly, all plectin-deficient mice spontaneously developed a colitic phenotype characterized by extensive detachment of IECs from the BM, increased intestinal permeability, and formation of inflammatory lesions. We also found that plectin expression was reduced in a cohort of more than 50 UC patients and that plectin expression levels negatively correlated with the severity of colitis. Mechanistically, plectin ablation in IECs led to aberrant keratin filament network organization and formation of dysfunctional hemidesmosomes and intercellular junctions. In addition, the hemidesmosomal □6□4 integrin receptor showed attenuated association with KFs, and protein profiling revealed prominent downregulation of junctional constituents. Consistent with the effects of plectin loss in the intestinal epithelium, plectin-deficient IECs exhibited remarkably reduced mechanical stability and limited adhesion capacity *in vitro*. Feeding mice with a low-residue liquid diet reduced mechanical stress and successfully mitigated epithelial damage in the plectin-deficient colon. Antibiotic treatment also decreased epithelial injury and mucosal inflammation. Besides colitis, plectin-deficient mice displayed progressive hepatic injury and enhanced hepatocarcinogenesis.

**Publications:**

- **Krausova A.**, **Buresova P.**, **Sarnova L.**, **Oyman-Eyrilmez G.**, Skarda J., Wohl P., Bajer L., Sticova E., Bartonova L., Pacha J., Koubkova G., Prochazka J., Spörrer M., Dürrbeck Ch., Stehlikova Z., Vit M., Ziolkowska N., Sedlacek R., Jirak D., Kverka M., Wiche G., Fabry B., Korinek V., **Gregor M.**: Plectin Ensures Intestinal Epithelial Integrity and Protects Colon Against Colitis. *Gut*. Under review.

**Publication description:** In this study, we found that plectin expression was reduced in patients with ulcerative colitis (UC) and that plectin expression levels negatively correlated with the severity of colitis. To study the underlying molecular mechanisms, we generated two new mouse lines: one constitutive and the other with tamoxifen-inducible plectin ablation in IECs. The phenotypic characterization of these mice demonstrated that loss of plectin leads to spontaneous development of a colitic phenotype characterized by extensive detachment of IECs from the BM, increased intestinal permeability, and formation of inflammatory lesions. In addition, defects in the intestinal epithelium were associated with progressive hepatocellular damage and a higher propensity for experimental HCC. These results demonstrate absolute indispensability of plectin for the maintenance of intestinal epithelium integrity. Moreover both mouse lines provide a useful model system for investigating disease aetiology and testing
palliative therapies.

**Group contribution:** Five co-authors (out of 24), including two co-first authors and the corresponding author, are group members (in **bold**, corresponding author). Group contribution entails the study concept and design, all experiments, analysis and interpretation of data, drafting of the manuscript, and obtained funding. Contribution of external co-authors entails the collection and histopathological analysis of human biopsies (Skarda J., Wohl P., Bajer L., Sticova E. and Bartonova L.), TEER measurements (Pacha J.), pilot experiments (Koubkova G.), whole-body imaging (Prochazka J.), magnetic tweezer rheology and spinning disc assays (Spörrer M., Dürrbeck Ch., Fabry B.), MR imaging (Vit M., Ziolkowska N., Jirak D.), microbiota analysis (Stehlikova Z., Kverka M.), funding and infrastructural support (Sedlacek R.), and mouse model (Korinek V., Wiche G.).

- Brauer R., Tureckova J., Kanchev I., Khoylou M., Skarda J., Prochazka J., Spoutil F., Beck I.M., Zbodakova O., Kasparek P., Korinek V., Chalupsky K., Karhu T., Herzig K.H., Hajduch M., **Gregor M.**, Sedlacek R.: MMP-19 deficiency causes aggravation of

colitis due to defects in innate immune cell function. *Mucosal Immunol.*, 9:974-85, 2015.

**Publication description:** In this report, we demonstrated that MMP-19 was critically involved in increased susceptibility and exacerbation of colitis, maintaining the epithelial barrier function, and regulation of the innate immune response, especially the influx of neutrophils. The dysregulated immune response in Mmp-19 knockout mice was rescued by bone marrow transplantation from WT mice. Moreover, we found that MMP-19 directly generated a soluble chemokine domain of CX3CL1 and that Mmp-19 KO mice showed diminished processing of CX3CL1. We conclude that MMP-19 mediates beneficial effects in intestinal inflammation; it is an essential factor in healing and homeostasis of the mucosa.

**Group contribution:** Only one co-author (out of 17) is a group member (in **bold**). His contribution entails study supervision, data acquisition, analysis and interpretation, statistical analysis, manuscript draft and revision.

**3. Current Research Projects (2015-2019)**

**3.1. The role of plectin in cholestasis-induced adaptive response in the liver** (pending grant application). Cholestatic liver diseases are highly prevalent causes of progressive liver disease with significant morbidity and mortality. A prominent group of genes that become upregulated in response to cholestatic insult encode keratins and plakin cytolinkers. Using liver-specific knock-out mice (Plecfl/fl;Alb-cre), we have recently shown that deficiency for plectin, a highly versatile cytolinker, has a detrimental effect in cholestatic liver injury. To elucidate the mechanisms by which plectin governs the cholestasis-induced adaptive response, we will analyze the role of plectin in: 1) adaptation of hepatobiliary transport systems; 2) activation and expansion of hepatic progenitor cells (HPCs); and 3) hepatocyte-driven liver regeneration. To fulfil these aims, we will combine analysis of mouse models of cholestasis, HPC induction, and hepatectomy injury with *ex vivo*/*in vitro* analyses of primary and CRISPR/Cas9-targeted cells grown in 2D and 3D cultures. This project should expand our knowledge of the development and progression of liver diseases.

**3.2. Analysis of extracellular niche in mouse models of liver fibrosis** (funded by GACR 18-02699S “Analysis of extracellular niche in mouse models of liver fibrosis”, 2018-2020). Liver fibrosis is an excessive scarring process resulting from chronic insults of heterogeneous aetiology. Together with primary liver cancer, it represents the end-stage liver pathology with high mortality. The major hallmark of liver fibrosis is the deposition of the fibrous extracellular matrix (ECM) synthesized mainly by hepatic stellate cells (HSCs). Using mouse models, we propose to analyze changes in the composition of ECM during the development and resolution of two different types of liver fibrosis by differential mass spectroscopy analysis. Potential new targets for fibrosis treatment/resolution will be confirmed in samples from human patients. Further, we will study the interplay between HSCs and altering ECM niche, using a newly developed mouse model harbouring HSCs with compromised ECM-receptor signalling (Plecfl/fl;Lrat-cre). These phenomena will be studied *in vitro*, using a human HSC line and mouse primary HSCs to understand better the processes regulating the deposition and composition of ECM in liver fibrosis.
**Current results:** Liver samples obtained from mice subjected to fibrogenesis ($CCl_4$- and DDC-induced fibrosis models) and recovery were analysed by mass spectrometry. We analysed six mice at each time point and treatment condition. We identified 5,184 of proteins in total: out of these, 4,431 were quantified (defined by protein identification in at least three samples in at least one condition). Principal component analysis and unsupervised hierarchical cluster analysis showed clear separation of each time point and each group of samples in each treatment condition ($CCl_4$ vs. DDC-treatment). Cluster analysis in both treatment groups showed enrichment of matrisome proteins (core matrisome, matrisome-associated, ECM glycoproteins) in clusters that were upregulated with treatment progression.

Experimental fibrosis was also induced in Plecfl/fl;Lrat-cre mice and their littermate controls Plecfl/fl by $CCl_4$ injection or DDC-feeding for two or four weeks. At the end of treatment, we obtained plasma to measure plasma liver damage markers, and we harvested the liver for immunoblot, histochemistry, and immunohistochemistry analyses. $CCl_4$-treated Plecfl/fl;Lrat-cre livers showed unexpectedly higher activation of SMAD3 after two weeks of treatment, but significantly lower parenchymal damage and fibrosis deposits after two weeks treatment. Similarly, DDC feeding resulted in lower parenchymal damage and lower cholestasis, as judged by lower ALT, AST, and ALP levels in Plecfl/fl;Lrat-cre than in Plecfl/fl mice after two, but not four weeks. Immunoblot analysis showed decreased levels of □SMA, fibronectin, and type I collagen in treated Plecfl/fl;Lrat-cre livers, accompanied by lower activation of SMAD3 and lower expression of integrin □4 after two weeks of DDC treatment. Interestingly, initial collagen deposition and □SMA expression were higher in treated Plecfl/fl;Lrat-cre livers, as judged by immunofluorescence microscopy, but with prolonged treatment, plectin Plecfl/fl;Lrat-cre livers contained less type I collagen and □SMA. We verified plectin deletion in primary HSC isolated from two-week DDC-fed animals by immunoblotting, and we analyzed protein expressions in these activated HSCs.

**3.3. Inflammatory bowel disease in primary sclerosing cholangitis: Prognostic markers and models** (funded by MH/AZV 17-31538A "Inflammatory bowel disease in primary sclerosing cholangitis: Prognostic markers and models", 2017-2020). Primary sclerosing cholangitis (PSC) frequently associates with inflammatory bowel diseases (IBD), in particular ulcerative colitis (UC) and colorectal cancer. Although different clinical courses and features (higher carcinogenic potential) suggest that IBD in PSC represents a disease entity distinct from IBD without the hepatobiliary component, underlying molecular mechanisms remain unknown. In this project, we will analyze the transcriptome of colon biopsy samples collected in a prospective study from patients diagnosed with PSC without UC, UC without PSC, PSC with concurrent UC, and from a control group. Using whole-genome transcriptome profiling, we will identify expression signatures of different diagnoses and corroborate these in expanded patient cohorts. In parallel, we will characterize available experimental PSC mouse models with respect to alterations in colonic tissues and genes identified in the transcriptome analysis.
**Current results:** All patients were divided into cohorts according to their diagnoses: PSC without UC (PSC), UC without PSC (UC), PSC with concurrent UC (PSC-UC), and a healthy control group (Control). Subjects were included as healthy controls only after all clinical examinations had concluded no signs of autoimmune inflammatory disease and colon cancer. Since our prospective study was launched (September 2015), over 150 patients have been examined and colon biopsy samples collected. For the UC, PSC, PSC-UC groups, standard endoscopic biopsies were extracted from the inflamed non-dysplastic mucosa of the right-side colon (caecum), the left-side colon (rectum), and the small bowel (ileum). For the control group, three biopsies were taken from the same locations. Transcriptome profiles of the extracted biopsies were obtained using Illumina RNA-seq technology. Resulting data were preprocessed by a standard pipeline, which includes technical and biological quality controls, rRNA removal, and read trimming. The preprocessed reads were mapped to the human reference genome (GRCh38/hg38), and transcriptome profiles were quantified at the gene level with gene annotation provided by the ensEMBL genome browser. To find specific transcriptional signatures of the studied cohorts, we determined genes differentially expressed among the cohorts using the DESeq2 package of Bioconductor. To assist in the biological interpretation of the data, we also performed gene set enrichment (GSEA) and network analyses of the differentially expressed genes.

**3.4. The role of plectin in nuclear mechanosensing and proliferation** (funded by GACR 20-16520Y "The role of plectin in nuclear mechanosensing and proliferation", 2020-2022). Mechanosignalling regulates many fundamental cellular processes such as migration, proliferation, and cell fate commitment. However, the link between physical stimuli and biological responses remains strangely elusive. Using intestine-specific knock-out

(Plecfl/fl;Villin-cre) mice, we have recently shown that plectin, a highly versatile cytolinker, controls cytoarchitecture and nuclear envelope (NE) organization of colonic epithelial cells (CECs). In addition, plectin deficiency has a detrimental effect on colonic crypt architecture and abrogates the tightly regulated balance between proliferation and differentiation of CECs. The primary objective of this project is a detailed analysis of underlying molecular mechanisms, consisting of the governing role of plectin for cytoskeletal networks, junctional complexes, and the NE. Using conditional Plecfl/fl;Villin-cre mice and CRISPR-generated human cell lines, we aim to unravel: 1) how plectin controls NE composition; 2) how changes in NE composition translate into gene expression; and 3) how altered NE composition and gene expression are reflected in proliferation, differentiation, and carcinogenesis.

**Current results:** Histochemical staining of Ki-67 on colonic sections revealed a striking increase in the number of proliferating CECs in Plecfl/fl;Villin-cre crypts. This observation was further confirmed by increased levels of proliferating cell nuclear antigen (PCNA) found in KO colonic lysates by immunoblotting. Since proliferating cells eventually differentiate into specialized epithelial cells, we tested if the percentage of differentiated CECs was also altered in the Plecfl/fl;Villin-cre crypts. Indeed, the number of goblet cells, secretory cells producing mucus, was increased in Plecfl/fl;Villin-cre colonic crypt, as indicated by periodic acid Schiff's base staining. Plectin deficiency thus results in hyperproliferation and aberrant differentiation of CECs, affecting the spatiotemporal organization of intestinal epithelium in Plecfl/fl;Villin-cre mice.

Given that plectin mediates mechanical signalling from the plasma membrane to the nucleus and that lamins within the lamina are responsible for the signal propagation, we quantified lamin A and lamin C levels in the Plecfl/fl;Villin-cre colonic lysates. Both lamin A and C were decreased, suggesting changes in nuclear envelope composition and mechanical properties upon plectin deletion. Strikingly, these changes were associated with severe DNA damage, as evidenced by significantly increased levels of the marker of DNA double-strand breaks, □H2AX. To determine gene sets that are deregulated upon plectin deletion, we compared transcriptomes of Plecfl/fl and Plecfl/fl;Villin-cre scraped colonic mucosa. Consistently with Plecfl/fl;Villin-cre CECs hyperproliferation, our preliminary Ingenuity Pathway Analysis revealed major changes in genes related to proliferation, cell cycle regulation, and DNA damage response. Taken together, these results prompted us to hypothesize that plectin is essential for converging mechanical stimuli, which are sensed by nuclear lamina and are then translated into proliferation and gene expression. We expected that the increased proliferation of plectin-deficient CECs in our mouse model might precede neoplastic changes and cancer progression. This was confirmed by histological scoring, revealing prominent dysplasia and dramatic incidence of spontaneously developed carcinoma in colons of 40- and 53-week-old Plecfl/fl;Villin-cre mice. Similarly, Plecfl/fl;Villin-cre mice displayed earlier cancer onset and higher cancer incidence in the model of azoxymethane (AOM)-induced carcinogenesis.

In order to verify our observations in the mouse model in a human system, we generated plectin-deficient non-cancerous human colonic cell lines (hCC) using the CRISPR/Cas9 system as described elsewhere. Successful plectin ablation was confirmed by sequencing/immunoblot analysis. KO hCC cells displayed reduced lamin A/C levels, recapitulating the phenotype of primary plectin deficient CECs in the mouse colon. In addition, we generated two plectin-deficient human colon cancer cell lines: well-differentiated/colon-like HT-29 and undifferentiated Caco-2. To further analyze the mechanical properties of nuclear lamina of WT and KO cells, we established a micro-channel based assay in collaboration with Prof. B. Fabry (Friedrich-Alexander University Erlangen, Germany). A pilot experiment with hCC cells revealed more frequent envelope ruptures and nuclear bleb formation in KO cells, confirming the plectin effect on nuclear mechanics.

Altogether, these data suggest 1) a critical role of plectin in the maintenance of spatiotemporal proliferation/differentiation pattern of colonic crypts; 2) direct involvement of plectin in nuclear mechanotransduction and biomechanics of nuclear lamina; and 3) a detrimental effect of plectin deficiency on colorectal carcinogenesis. We believe that our

preliminary results represent a sound basis for further research and experiments proposed within this project.

**3.5. Targeting mechanical properties and mechanotransduction in hepatocellular carcinoma** (pending grant application). Hepatocellular carcinoma (HCC) is primary malignancy of the liver. HCC is a heterogeneous tumour with high metastatic potential and complex pathophysiology. There is increasing evidence that tissue mechanics play a critical role in tumour onset and progression. Therefore new approaches targeting tumour mechanical properties constitute promising therapeutic strategies. The main goal of this project is to determine the role of the tumour marker and potential therapeutic target protein plectin in biomechanics and mechanotransduction of HCC. To achieve this goal, we will analyze plectin's role in migration, mechanosignalling, and tensional homeostasis of HCC cells. Using autochthonous and orthotopic mouse models, we will address the impact of tensional homeostasis on HCC carcinogenesis. Finally, using proteomics-based approaches, we will define response signatures of HCC cells associated with pharmacological targeting of plectin by the anticancer compound plecstatin. Understanding plectin's role in mechanobiology of HCC is of critical importance for screening strategies and future targeted therapy.

**Current results:** To examine the expression of plectin in HCC, we analyzed TCGA public datasets and found that plectin was significantly elevated in HCC tumours compared to their corresponding healthy tissues. This finding was confirmed by immunohistochemistry of tissue microarrays containing paired HCC and non-tumour liver tissues from 17 HCC patients. In order to verify our results in a reliable mouse model, we established HCC induction with diethylnitrosamine (DEN) in C57BL/6J mice. Consistent with human patient samples, our immunoblot analysis revealed upregulation of plectin in murine HCC when compared to healthy tissue.

To further characterize the role of plectin in HCC, we obtained nine HCC cell lines covering the whole HCC differentiation spectrum, corresponding to individual stages of tumour progression. These HCC cell lines were classified into two subsets of "well-differentiated" cells that retained expression of epithelial markers (e.g., E-cadherin) and of highly motile and invasive "poorly differentiated" cells characterized by higher expression of mesenchymal markers (e.g., vimentin. Using immunoblot analysis, scratch-wound assays, and Matrigel transwell invasion assays, we showed that plectin expression was markedly elevated in poorly differentiated HCC cells (SNU-423, SNU-475, SK-Hep1) and correlated with cell migratory and invasion potentials. To achieve plectin deletion in different types of liver cell lines, we had already developed and successfully validated targeting constructs based on CRISPR/Cas9 technology in both murine and human cells. Using the same strategy, we prepared a wide array of plectin-deficient (KO) HCC cell lines (Huh-7, Hep3B, PCL/PRF, SNU-423, SNU-475, and SNU-398) and characterized them in the scratch-wound assay, the spheroid invasion assay, Matrigel transwell invasion, and colony formation assays. We have shown that plectin ablation resulted in a dramatic drop in migratory, invasive and tumorigenic potentials in all tested HCC cell lines when compared to their wild-type (WT) counterparts. Taken together, these data suggest that plectin plays an important role in HCC carcinogenesis and metastasis.

Moreover, we have shown that in Huh7 ("well-differentiated") and SNU-475 ("poorly differentiated") cell lines, plectin deficiency was accompanied by decreased expression of integrin □1 and upregulation of the well-known tumour suppressor E-cadherin, corresponding with the results previously obtained *in vitro* (using siRNA) and *in vivo* (using Cre-loxP system). As prior studies linked changes in integrin □1 and E-cadherin expression to cytoskeletal tension, we also compared F-/G-actin ratio in WT and KO Huh-7 cells, using fluorescently labelled DNAse I and Phalloidin. A significantly reduced F-/G-actin ratio in KO Huh-7 (indicative of lower tension) was consistent with previously found compromised cellular tension in plectin-deficient fibroblasts and myoblasts. For future studies, we established measurements with a talin-based tension FRET sensor containing a donor fluorophore connected to an acceptor fluorophore via a nanospring. This approach was

validated in Huh-7 and SNU-475 cells. As expected, the FRET index (inversely related to the tension) was lower in more motile (and contractile) SNU-475 than in Huh-7 cells.
To complement our studies in CRISPR/Cas-9 targeted plectin-deficient HCC cell lines with pharmacological targeting of plectin, we obtained the organorutenium compound plecstatin, a recently described high-affinity plectin ligand with anticancer activity. Consistent with effects of plectin ablation, plecstatin (in a dose-dependent manner) suppressed migration of WT Huh-7 cell in the scratch-wound assay. In contrast, KO Huh-7 cells showed only a negligible decrease in migratory potential, showing that plecstatin effectively inhibits HCC migration in a plectin-dependent manner. Based on our preliminary results, we hypothesize that plectin deletion in hepatocytes/HCC cells both *in vitro* and *in vivo* leads to changes in biomechanical properties of cells and impaired mechanotransduction associated with increased expression of E-cadherin and a decreased amount of □1 integrin on their surface. This might affect the time-course and extent of plectin-deficient tumour stiffening and the ability of plectin-deficient cells to perceive mechanical cues from ECM. That makes plectin-deficient cells excellent tools to further our understanding of biomechanics and mechanotransduction in HCC progression and metastasis.

**3.6. The impact of plectin deficiency on keratin filament and desmosome interdependence in simple epithelia** (funded by CAS postdoctoral fellowship to M. Prechova "Mechanobiological principles of epithelial pathologies", 2019–2021). Desmosomes (Ds) are intercellular junctions essential for mediating strong intercellular cohesion. Desmosomal structure, dynamics and function are tightly regulated by desmoplakin-mediated recruitment of keratin filaments. Ablation of plectin, a putative binding partner of desmoplakin, leads to destabilization of the barrier formed by plectin-deficient biliary epithelial cells (BECs) both *in vivo* and *in vitro*. We hypothesize that the underlying molecular mechanism resides in effect, either direct through desmoplakin or via keratin filament organization, in Ds which further translates into AJ and TJ aberrations in BEC continuum. As a consequence, the plectin-deficient BEC barrier fails to reorganize cytoarchitecture and intercellular junctions and is less resilient to mechanical stress exerted by increased bile pressure during cholestatic insult. To test this hypothesis, we will analyze the role of plectin in structural-functional interdependence of keratin filaments and Ds in plectin-deficient BEC and MDCK monolayers.
**Current results:** In order to study the molecular details of plectin involvement in the mechanical stability of the simple epithelium, we generated plectin-deficient (KO) epithelial cell lines (cholangiocytes and MDCKs), using the CRISPR/Cas9 technology. Plectin deletion caused a collapse of keratin filaments, associated with irregularly shaped desmosomes. Interestingly, this phenotype was rescued by ectopic expression of several plectin isoforms in the KO cells. In collaboration with the University of Vienna (Dr. S. M. Meier-Menches), we also demonstrated that the treatment of wild-type (WT) cells with the plectin inhibitor plecstatin mimics the KO phenotype.
Using super-resolution microscopy imaging, we showed that, in WT cells, keratin filaments form a delicate network composed of a circumferential keratin rim and a finely organised system of radial keratin spokes. However, in plectin KO cells, the circumferential keratin rim was missing, and the radial keratin spokes were thicker, associated with distorted desmosomes. Altered desmosomal morphology suggests that, in KO cells, desmosomes might be under tensile stress exerted by collapsed keratin networks. To test this hypothesis, we have established a FLIM-FRET based tension measurement using a desmoplakin tension sensor; experiments to address this question are currently underway.
Interestingly, the aberrant network of keratin filaments led to compensation by the actin cytoskeleton. We observed an increased amount of F-actin in KO cells when compared to their WT counterparts. In collaboration with Prof. Fabry's laboratory in Erlangen, we established a traction force microscopy-based method to measure line tension on cell-cell borders. Using this approach, we showed that KO cells were more contractile and that their intercellular junctions were under higher tension.

To test the mechanical stability of the epithelium, we performed experiments where monolayers of cells, seeded on elastic membranes, were uniaxially stretched using a newly-designed cell stretching system. Mechanical stretch led to larger dilation of desmosomes resulting in extensive ruptures in KO monolayers.
Based on our results, we propose that aberrantly-formed desmosomes, combined with increased actomyosin tension on cell-cell borders, bundled keratin networks, and the absence of a circumferential keratin rim, lead to decreased mechanical stability of simple epithelia consisting of KO cells.

## Research activity and characterisation of the main scientific results

Introduction

To ensure genetic diversity as well as fertility, mammalian meiotic division induces programmed DNA breaks and their repair by homologous recombination. This process needs to be tightly controlled, as both too low and too high numbers of meiotic DNA breaks result in decreased fertility. The distribution of recombination-inducing DNA breaks across genome is non-random and occurs at some sites (termed hotspots) with high frequency. Mouse and human meiotic DNA break hotspots depend on the *Prdm9* gene product (e.g., doi.org/10.1126/science.1183439). PRDM9 protein binds DNA and trimethylates histone 3 on lysines K4 and K36, which opens the chromatin and specifies the hotspot sites for meiotic recombination. While PRDM9 is dispensable in the dog, it is important for fertility in some mice. Moreover, one fertile human lacking PRDM9 function was found. On the other side, *PRDM9* polymorphisms were revealed in sterile human patients. Therefore, the causal relationship between sterility and PRDM9 variability remains unclear.

The causes and consequences of the tight control of the levels of a meiotic epigenetic protein

Histone methyltransferase PRDM9 directs meiotic DNA breaks via site-specific chromatin modifications. The site specificity is determined by its quickly evolving DNA-binding domain. However, PRDM9 alleles with different DNA domains do not induce meiotic breaks with the same efficiency in heterozygous human and mouse males (allelic dominance). To explain this observation and thus decipher the mechanism of PRDM9 action, we asked whether PRDM9 alleles can bind each other and thus influence DNA binding at recombination hotspots. In collaboration with The Jackson Laboratory, our paper (Baker et al 2015) manifested that the PRDM9 protein binds DNA as a multimer and the resulting isoforms compete for hotspot activation. We then reasoned that multimerization together with limited protein expression could cause not both skewed hotspot distribution and gene dosage sensitivity (haploinsufficiency) of *Prdm9* for germ cells. Indeed, our team found that halved *Prdm9* copy number resulted in lower fertility (offspring production) via reduced meiotic synapsis and thereby lower number of normal pachytene spermatocytes. The results of our team were thus crucial to show that PRDM9 levels are tightly controlled and to manifest the functional importance of the finding of PRDM9 multimerization. Because both human and mouse populations carry high frequencies of *Prdm9* heterozygotes, the effects of multimerization coupled with limited expression can seriously influence patterns of inheritance. Our results are also important for reproductive medicine, as they support the view that the heterozygous *PRDM9* mutations previously disclosed in human patients could cause their infertility.

The separation of the effects of crossover frequency and localization on fertility

DNA breaks and their repair by homologous recombination resulting in non- and crossovers must be tightly controlled to assure fertility. One way of control is directing the positions of meiotic DNA breaks using the *Prdm9* gene encoding histone modifier. This gene affects sterility of intersubspecific mouse male hybrids and thus bridges mammalian speciation genetics and epigenetics. The head of this team and his PhD student contributed to this discovery before the establishment of the team under evaluation (2009). In collaboration with the IMG team of Jiri Forejt, our team studied the genomic architecture of crossover production in the intersubspecific male hybrids (Balcova et al 2016), because a correlation between the degree of fertility and crossover rate frequency has been previously suggested. The task of our team was to assess whether *Prdm9* controls just the positions or also the crossover rate frequency, defined as the number of DNA breaks repaired to crossovers per meiotic cell. Some studies in goat and human suggested that PRDM9 could control both. We found that that the mouse *Prdm9* dosage does not play a role in the male crossover frequency, thus separating the effects of frequency and position on meiosis progression. Our results were vital to interpret the findings of the paper: recombination rate frequency affects hybrid sterility via gene(s) located on chromosome X. We thus contributed towards understanding the role of meiotic recombination in reproductive isolation, which is an important step in speciation.

Tackling the function of evolutionary young exons in a speciation gene

The activity of transposable elements often leads to transcription of new genomic regions, but how these new genes or exons acquire a new function remains unclear. We asked whether new exons, which arose in the speciation gene *Prdm9*, contributed to its function. Two alternative *Prdm9* mRNA isoforms occur naturally in the germ cells of mouse testes, one formed via splicing to a 3'-terminal exon consisting of short interspersed retrotransposomal repeat element B2 and one isoform including an alternative internal exon of 28 base pairs. Both isoforms result in premature translation termination. These alternatives probably emerged recently, as they are not present in the rat. We characterized a transgenic mouse, where these two transcripts (but not the constitutive one) were overexpressed (Mihola and Trachtulec 2017). However, their expression did not differ in sterile versus fertile hybrids. Moreover, both intersubspecific hybrid males and *Prdm9*-deficient laboratory mice of both sexes carrying this transgene remained sterile, indicating that these alternative transcripts are not important for hybrid sterility or fertility of the laboratory mice. These results are in agreement with other studies suggesting that the number of young exons and genes with discernible phenotype effect might be low.

Buffering male fertility reduction caused by a loss of meiotic epigenetic factor and its possible mechanism

Until our publication, *Prdm9* deficiency was shown to result in complete meiotic arrest of both sexes of all tested classical mouse strains, including C57BL/6J (B6) of *Mus musculus domesticus* origin. This sterility was ascribed to recombination hotspot relocation from nonfunctional sites to promoters and enhancers. However, dogs or birds do not require PRDM9 for fertility and carry many recombination hotspots in promoters. To learn whether other mice need PRDM9 to accomplish meiosis, we prepared *Prdm9*-deficient PWD/Ph strain of *M. m. musculus* origin (Mihola et al 2019). In contrast to B6, these males were able to produce a low amount of sperm (although not offspring) despite recombination hotspots in promoters. The *Prdm9* deficiency induced variable fertility decrease in additional mice and crosses, some combinations even retained the ability to bear offspring. We predicted that one of the possible mechanisms of infertility suppression could be more efficient DNA repair of relocated recombination sites to crossovers. Indeed, we manifested a correlation between the number of crossovers per meiotic cell and sperm presence in *Prdm9*-deficient males. This work was a cooperation, but most results came from our team - except the DMC1 chromatin immunoprecipitation coupled with single-stranded DNA sequencing and bioinformatics. We argue that our mice are a superior tool to study human male fertility compared to previous ones, because the only known human case with homozygous mutation in *PRDM9* is fertile. Our results have an implication for reproductive medicine, as we can predict that drugs or conditions inducing mild increase in DNA breaks targeted into testes of human infertility patients with low recombination rate could improve fertility.

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