Characteristics of main research directions investigated at the institute and the achievements 2010−2014

Institute	Institute of Molecular Genetics of the CAS, v. v. i.

The research directions pursued at the Institute combine continuation of the topics from the previous period and innovations brought by young researchers. The Institute has a policy strongly supporting establishing new independent junior groups, which bring new research topics and experimental approaches (see section 3). The Institute sees the diversity as its strength because it creates an inspiring environment in which original solutions to the studied phenomena can arise. The main research directions and their achievements in the evaluated period have been as follows:

(1) **Cancer cell biology, including DNA damage**

This research direction has a long tradition at the IMG; in recent years it became rather prominent after several of our new groups interested in this field had been established. The most important achievements in this area in the last five years are as follows:

Mechanisms of DNA damage response (the teams of J. Bartek and L. Macurek):

Nucleoporin NOP153 was identified as a novel factor specifically required for nuclear import of 53BP1 (an essential mediator of DNA damage response (DDR) and a tumour suppressor whose accumulation on damaged chromatin promotes DNA repair and enhances DDR signalling via importin and as such is involved in the maintenance of genome integrity. (**Moudry, P., C. Lukas, L. Macurek, B. Neumann, J.K. Heriche, R. Pepperkok, J. Ellenberg, Z. Hodny, J. Lukas, and J. Bartek. 2012. Nucleoporin NUP153 guards genome integrity by promoting nuclear import of 53BP1. *Cell Death Differ.* 19:798-807).**

The activity of the ATR kinase (which controls chromosome integrity and chromatin dynamics) at the nuclear envelope was discovered to respond to mechanical stress. It was proposed that mechanical forces derived from chromosome dynamics and torsional stress on nuclear membranes activate ATR to modulate nuclear envelope plasticity and chromatin association to the nuclear envelope. (**Kumar, A., M. Mazzanti, M. Mistrik, M. Kosar, G.V. Beznoussenko, A.A. Mironov, M. Garre, D. Parazzoli, G.V. Shivashankar, G. Scita, J. Bartek, and M. Foiani. 2014. ATR mediates a checkpoint at the nuclear envelope in response to mechanical stress. *Cell.* 158:633-646.)**

Drug-induced DNA damage response has been shown to be an activating complex network comprising more than two dozens of various cytokine species. These cytokines produced both in normal and tumour senescent cells activate corresponding signalling pathways (JAK/STAT) and gene expression in autocrine and paracrine ways and they can modulate the tissue microenvironment. These findings have implications for understanding several patho/physiological processes including wound healing, inflammation and tumour immunosurveillance. (**Novakova, Z., S. Hubackova, M. Kosar, L. Janderova-Rossmeislova, J. Dobrovolna, P. Vasicova, M. Vancurova, Z. Horejsi, P. Hozak, J. Bartek, and Z. Hodny. 2010. Cytokine expression and signaling in drug-induced cellular senescence. *Oncogene.* 29:273-284).**

Novel gain-of-function mutations of Wip1 phosphatase were identified that result in expression of truncated Wip1 affecting the DNA damage response pathway. The mutations are present both in the tumours and in other tissues of breast and colorectal cancer patients, indicating that they arise early in development and may predispose to cancer. (**Kleiblova, P., I.A. Shaltiel, J. Benada, J. Sevcik, S. Pechackova, P. Pohlreich, E.E. Voest, P. Dundr, J. Bartek, Z. Kleibl, R.H. Medema, and L. Macurek. 2013. Gain-of-function mutations of PPM1D/Wip1 impair the p53-dependent G1 checkpoint. *J Cell Biol.* 201:511-521.).**

Furthermore, Wip1 phosphatase was shown to be regulated in context of the cell cycle. Low levels of Wip1 allow cells to activate ATM kinase during unperturbed mitosis without any external source of DNA damage. Removal of Wip1 increases the sensitivity of cells to a low level of DNA damage present during normal mitosis. (**Macurek L, Benada J, Müllers E, Halim VA, Krejčíková K, Burdová K, Pecháčková S, Hodný Z, Lindqvist A, Medema RH, Bartek J. (2013) Downregulation of Wip1 phosphatase modulates the cellular threshold of DNA damage signaling in mitosis. Cell Cycle; 12:251-62).**

The role of Wnt pathway in enterocyte malignant transformation (the team of V. Korinek, collaborations with additional teams):

Investigations of the roles of Wnt pathway in homeostasis of the intestinal epithelium and of the Troy gene as a novel target of the canonical Wnt signalling pathway. Importantly, Troy was identified as a novel marker of adult stem cells in the intestine. **(Fafilek, B., Krausova, M., Vojtechova, M., Pospichalova, V., Tumova, L., Sloncova, E., Huranova, M., Stancikova, J., Hlavata, A., Svec, J., Sedlacek, R., Luksan, O., Oliverius, M., Voska, L., Jirsa, M., Paces, J., Kolar, M., Krivjanska, M., Klimesova, K., Tlaskalova-Hogenova, H., Korinek, V. 2013. Troy, a Tumor Necrosis Factor Receptor Family Member, Interacts with Lgr5 to Inhibit Wnt Signaling in Intestinal Stem Cells. Gastroeneterology, 144: 381–391).**

Monensin, a natural antibiotic, was identified as a potent inhibitor of Wnt signalling and a prospective anticancer drug. The inhibitory effect of monensin on Wnt/beta-catenin signalling was tested in mammalian cells, in zebrafish, and in Xenopus embryos. In the mouse model of intestinal cancer, monensin suppressed progression of the intestinal tumours. **(Tumova, L., Pombinho, A.R., Vojtechova, M., Stancikova, J., Gradl, D., Krausova, M., Sloncova, E., Horazna, M., Kriz, V., Machonova, O., Jindrich, J., Zdrahal, Z., Bartunek, P., Korinek, V. 2014. Monensin Inhibits Canonical Wnt Signaling in Human Colorectal Cancer Cells and Suppresses Tumor Growth in Multiple Intestinal Neoplasia Mice. Molecular Cancer Therapeutics 13: 812-822.)**

The pathologic role of DNA release by leukemic cells (the team of M. Dvorak in the lab of J. Kralova):

Using the animal model of acute leukaemia it was demonstrated that leukemic cells release fragmented chromatin which enters the nuclei of surrounding cells in the bone marrow microenvironment, where it incites the DNA damage response and subsequently also the cell death. The leukemic cell-liberated DNA also integrates in the genome of bone marrow cells. Thus, the chromatin produced by tumour cells can damage the surrounding tissue by cell death and mutation. (**Dvořáková, M., Karafiát, V., Pajer, P., Kluzáková, E., Jarkovská, K., Peková, S., Krutílková, L. and Dvořák, M. 2013. DNA released by leukemic cells contributes to the disruption of the bone marrow microenvironment. *Oncogene*. 32: 5201-5209).**

(2) **Developmental biology**

This research area has been successfully pioneered in IMG mainly by Z. Kozmik and his lab, which became a world leader in the field of eye development and its evolutionary aspects. More recently, the lab of P. Bartunek achieved important results on development of the hematopoietic system using the zebra fish model and published them repeatedly in a top haematological journal, and the team of P. Svoboda achieved truly excellent results in the field of the earliest phases of embryo development.

Mechanisms of eye development (the team of Z. Kozmik):

Cell types of the amphioxus (our most primitive chordate relative) frontal eye were characterized molecularly and their possible relatedness to the cell types of vertebrate eyes was tested. The results support homology of the amphioxus frontal eye and the vertebrate eyes and yield insights into their evolutionary origin. **(Vopalensky P, Pergner J, Liegertova M, Benito-Gutierrez E, Arendt D, Kozmik Z. (2012) Molecular analysis of the amphioxus frontal eye unravels the evolutionary origin of the retina and pigment cells of the vertebrate eye. Proc Natl Acad Sci U S A. 109:15383-8).**

Using conditional inactivation of Pax6 at distinct time points of mouse neuroretina development, the impact of this gene on early eye morphogenesis has been elucidated. (**Klimova L, Kozmik Z. (2014) Stage-dependent requirement of neuroretinal Pax6 for lens and retina development. Development. 2014 Mar;141(6):1292-302.**)

Development of hematopoietic system using the zebra fish model (the team of P. Bartunek)

Identification of hematopoietic progenitor cells in the zebrafish (*Danio rerio*) has been hindered by a lack of functional assays to gauge the proliferative potential and differentiation capacity. To investigate the nature of myeloerythroid progenitor cells, clonal methylcellulose assays were developed by using recombinant zebrafish erythropoietin and granulocyte colony-stimulating factor. (**Stachura, D.L., Svoboda, O., Lau, R.P., Ball, K.M., Zon L.I., Bartunek, P., and Traver D. (2011) Clonal analysis of hematopoietic progenitor cells in the zebrafish, *Blood*, 118: 1274-82.).**
Zebrafish granulocyte colony-stimulating factor (Gcsf) was found to be required for the specification and proliferation of hematopoietic stem cells, suggesting that Gcsf is an ancestral cytokine responsible for the broad support of HSPCs. These findings show how hematopoietic cytokines had evolved following the diversification of teleosts and mammals from a common ancestor. (**Stachura DL, Svoboda O, Campbell CA, Espín-Palazón R, Lau RP, Zon LI, Bartunek P, Traver D. (2013) The zebrafish granulocyte colony-stimulating factors (Gcsfs): 2 paralogous cytokines and their roles in hematopoietic development and maintenance. *Blood* 122: 3918-28.).**

Results obtained in studies on the zebrafish model of haematopoiesis strongly suggest that mammalian megakaryocytes are homologues of non-mammalian thrombocyte progenitors and evolved from them during the course of vertebrate evolution. (**Svoboda O, Stachura DL, Machoňová O, Pajer P, Brynda J, Zon LI, Traver D, Bartůněk P. (2014) Dissection of vertebrate hematopoiesis using zebrafish thrombopoietin. *Blood* 124: 220-8.)**

<u>Oocyte-to-embryo transition</u> (the team of P. Svoboda)

The endogenous RNA interference pathway was found to have a larger impact on the transcriptome than the miRNA pathway. The results suggests that suppression of endogenous maternal miRNAs could be the first step in maternal-to-zygote reprogramming. **(Ma J., Flemr M., Stein P., Berninger P., Malik R., Zavolan M., Svoboda P.*, and Schultz R.M.* (2010) MicroRNA Activity Is Suppressed in Mouse Oocytes, Current Biology 20 (3), 1-6).** In contrast to the general belief about dsRNA toxicity in somatic cells, tolerance to expressed dsRNA in a transgenic mouse model was demonstrated. The results also show that RNAi is highly effective only in the oocyte. **(Nejepinska, J., Malik, R., Filkowski, J., Flemr, M., Filipowicz, W., and Svoboda, P. (2012) dsRNA expression in the mouse elicits RNAi in oocytes and low adenosine deamination in somatic cells. Nucleic Acids Res 40(1): 399-413.)** Finally, a mouse oocyte-specific Dicer isoform (Dicer(O)) was discovered, which is responsible for highly active endogenous RNAi in oocytes as compared to somatic cells. Expression of the Dicer(O) isoform is driven by an intronic MT-C retrotransposon promoter, deletion of which causes loss of Dicer(O) and female sterility. The alternative Dicer isoform demonstrates evolutionary plasticity of RNA-silencing pathways. (**Flemr M., Malik R., Franke V., Nejepinska J., Sedlacek R., Vlahovicek K., Svoboda P. (2013) A retrotransposon-driven Dicer isoform directs endogenous siRNA production in mouse oocytes. Cell 155(4):807-16).**

(3) **Molecular genetics and (functional) genomics**

This strong research direction with a long tradition at the IMG has been particularly successful in recent years. The main topics include: genomic analysis of various organisms (in collaboration with extramural partners), mapping of genes for parasite resistance and other immunologic traits using the model of mouse recombinant congenic strains, elucidation of the phenomenon of mouse subspecies male hybrid sterility, mechanisms of RNA splicing and spliceosome function, and mechanisms of gene expression in mammalian oocytes and zygotes (this last topic was especially successful most recently).

<u>Genomics</u> (the team of C. Vlcek):

The complete genomic sequence of *Rhodobacter capsulatus* SB 1003 was determined. The bacterium belongs to the group of purple nonsulphur bacteria. The genome of this purple nonsulphur bacterium encodes genes for photosynthesis, nitrogen fixation, utilization of xenobiotic organic substrates, and synthesis of polyhydroxyalkanoates. These features made it a favourite research tool for studying these processes. (**Strnad H, Lapidus A, Pačes J, Ulbrich P, Vlček Č, Pačes V, Haselkorn R. Complete genome sequence of the photosynthetic purple nonsulfur bacterium Rhodobacter capsulatus SB 1003. J**

Bacteriol 2010 192(13): 3545-3546.). Also, the complete genomic sequence of *Achromobacter xylosoxidans* strain A8 was determined. The bacterium isolated from the soil contaminated with polychlorinated biphenyls can use these pollutants as a sole sources of carbon and energy, making it a good starting microorganism for derivation of a bioremediation system. Besides genes for the utilization of xenobiotic organic substrates, the genome was found to contain genes associated with pathogenesis, toxin production, and resistance. (**Strnad H, Rídl J, Pačes J, Kolář M, Vlček Č, Pačes V. Complete genome sequence of the haloaromatic acid-degrading bacterium Achromobacter xylosoxidans A8. J Bacteriol 2011 193(3): 791-792.)**

Mechanisms of hybrid sterility (the team of J. Forejt)

The mechanistic details of the role of the Prdm9 gene (discovered earlier by J. Forejt team and published in 2009 in Science) in mouse subspecies hybrid sterility were partially elucidated; the results indicate an important role of interallelic and intergenic incompatibilities of the gene. Additions and subtractions of Prdm9 copies, as well as allelic replacements, improved meiotic progression and fecundity. (**Flachs, P., O. Mihola, P. Šimeček, S. Gregorová, J.C. Schimenti, Y. Matsui, F. Baudat, B. de Massy, J. Piálek, J. Forejt and Z. Trachtulec 2012. Interallelic and intergenic incompatibilities of the *Prdm9* (*Hst1*) gene in mouse hybrid sterility. *PLoS Genetics* 8(11):e1003044).**

Further results indicate that asynapsis of heterospecific chromosomes in meiotic prophase provides a recurrently evolving trigger for the meiotic arrest of the interspecific F1 hybrids. Asynapsis was not preceded by a failure of double-strand break induction, and the rate of meiotic crossing over was not affected in synapsed chromosomes. (**Bhattacharyya, T., S. Gregorova, O. Mihola, M. Anger, J. Sebestova, P. Denny, P. Simecek and J. Forejt 2013. Mechanistic Basis of Male Infertility in Mouse Intersubspecific Hybrids. *Proc Natl Acad Sci U S A*. Feb 5;110(6):E468-77)**.
Furthermore, it was shown that hybrid sterility is a consequence of a failure of mutual recognition of chromosomes originating from different subspecies and that one of the two major hybrid sterility genes, which control synapsis of meiotic chromosomes and male sterility, is localized to a 4.7Mb interval on the Chromosome X. (**Bhattacharyya, T., R. Reifova, S. Gregorova, P. Simecek, V. Gergelits, M. Mistrik, I. Martincova, J. Pialek and J. Forejt 2014. X Chromosome Control of Meiotic Chromosome Synapsis in Mouse Inter-Subspecific Hybrids. *PloS Genetics* 10(2):e1004088.)**

RNA splicing and spliceosome function (the team of D. Stanek)

Fluorescence correlation spectroscopy and FRAP approaches were used to monitor the dynamic interaction of splicing factors with pre-mRNA to get insight into spliceosome assembly in living cells. The results support a step-wise assembly model of the spliceosome and allowed estimation of pre-mRNA splicing kinetics in living cells. *(***Huranová, M., Ivani, I., Benda, A., Poser, I., Brody, Y., Hof, M., Shav-Tal, Y., Neugebauer K.M. and Staněk D. 2010. The differential interaction of snRNPs with pre-mRNA reveals splicing kinetics in living cells. *J. Cell Biol.* 191:75-86).**

Furthermore, chromatin modifications and specifically histone H4 acetylation were shown to modulate alternative splicing. **(Hnilicová J, Hozeifi S, Dušková E, Icha J, Tománková T, Staněk D. 2011. Histone deacetylase activity modulates alternative splicing. *PLoS One*. 6:e16727)**

<u>Mapping of genes for parasite resistance</u> (the team of M. Lipoldova)

Highly sensitive PCR-ELISA for detection and quantification of *Leishmania* parasites in host tissues was designed. The sensitivity of this technique is 0.3 fg of parasite DNA/reaction. This technique was subsequently adopted by other laboratories to detect other pathogens. (**Kobets, T., J. Badalová, I. Grekov, H. Havelková, M. Svobodová, and M. Lipoldová. 2010. *Leishmania* parasite detection and quantification using PCR-ELISA. *Nat. Protoc.* 5: 1074- 1080.)** Genes for mouse susceptibility to T*rypanosoma brucei brucei* infection were mapped using unique recombinant congenic strains. Definition of susceptibility genes will improve the understanding of the pathways and genetic diversity underlying the disease and may lead to new strategies to overcome the active subversion of the immune system by *T. b. brucei*. (**Šíma, M., H. (Havelková, L. Quan, M. Svobodová, T. Jarošíková, J. Vojtíšková, A.P.M. Stassen, P. Demant, and M. Lipoldová. 2011.Genetic control of resistance to *Trypanosoma brucei brucei* infection in mice. PLoS Negl. Trop. Dis. 5: e1173.)**
The first animal (mouse) model suitable for genetic, pathological and therapeutic studies in *Leishmania tropica* infection has been established. Comparison of *L. tropica* and *L. major* infections indicates that the strain patterns of response are species-specific, with different sex effects and largely different host susceptibility genes. **(Kobets, T., H. Havelková, I. Grekov, V. Volkova, J. Vojtíšková, M. Slapničková, I. Kurey, Y. Sohrabi, M. Svobodová, P. Demant, and M. Lipoldová. 2012. Genetics of host response to *Leishmania tropica* in mice – Different control of skin pathology, chemokine reaction, and invasion into spleen and liver. PLoS Negl. Trop. Dis. 6 (6): e1667.)**
Genetic loci controlling susceptibility to parasite *Leishmania tropica* in the mouse have been identified using unique recombinant congenic strains. One of the detected loci, *Ltr3*, exhibits the recently discovered phenomenon of trans-generational parental effect. This research not only describes the regulatory mechanisms controlling susceptibility to *L. tropica*, but also elucidates the complex mechanisms of genetic regulation. **(Sohrabi, Y., H. Havelková, T. Kobets, M. Šíma, V. Volkova, I. Grekov, T. Jarošíková, I. Kurey, J. Vojtíšková, M. Svobodová, P. Demant, and M. Lipoldová. 2013. Mapping the genes for susceptibility and response to *Leishmania tropica* in mouse. PLoS Negl. Trop. Dis. 7(7): e2282**

(4) **Molecular and cellular immunology**

Immunology (especially its transplantation aspects) used to be one of the most important IMG research areas in the distant past (co-discovery of immunological tolerance over 50 years ago). In recent years it has shifted from the classical (transplantation) immunology mainly to molecular immunology, with emphasis on the mechanisms of immunoreceptor signalling, and it remains one of the productive areas of research at IMG:

<u>Mechanisms of mast cell degranulation and IgE receptor signalling</u> (the team of Pe. Draber)

In contrast to previous claims, aggregation of FcεRI in mast cells and its association with detergent-resistant membranes is not necessary for tyrosine phosphorylation of the receptor. The results suggested a new model in which the cross-talk between protein tyrosine kinases and phosphatases plays a key role in initial stages of the FcεRI signalling. (**Heneberg, P., L. Dráberová, M. Bambousková, P. Pompach, and P. Dráber. 2010. Down-regulation of protein tyrosine phosphatases activates an immune receptor in the absence of its translocation into lipid rafts. *J. Biol. Chem.* 285: 12787-12802.)**
Using mast cells derived from mice deficient in transmembrane adaptor protein NTAL, a functional link between NTAL and the small GTPase RhoA was discovered, and its importance for regulation of mast cell spreading and migration via actin polymerization was pointed out. **(Tůmová, M., A. Koffer, M. Šimíček, L. Dráberová, and P. Dráber. 2010. The transmembrane adaptor protein NTAL signals to mast cell cytoskeleton via the small GTPase Rho. *Eur. J. Immunol.* 40: 3235-3245.)**
The store-operated Ca^{2+} entry (SOCE) regulator, STIM-1, was identified as a positive regulator of major changes in cell morphology, including formation of protrusions containing microtubules, in antigen-activated mast cells. (**Hájková, Z., V. Bugajev, E. Dráberová, S. Vinopal, L. Dráberová, J. Janáček, P. Dráber, and P. Dráber. 2011. STIM1-directed reorganization of microtubules in activated mast cells. *J. Immunol.* 186: 913-923.)** Using mast cells deficient in the expression of adaptor proteins NTAL, LAT, and tetraspanin CD9, it was demonstrated that chemotaxis toward antigen is controlled by a complex cross-talk among the high-affinity IgE receptor, CD9, NTAL, LAT, and cytoskeleton-regulatory proteins of the ezrin/radixin/moesin family. (**Hálová, I., L. Dráberová, M. Bambousková, M. Machyňa, L. Stegurová, D. Smrž, and P. Dráber. 2013. Crosstalk between tetraspanin CD9 and transmembrane adaptor protein non-T cell activation linker (NTAL) in mast cell activation and chemotaxis. *J. Biol. Chem.* 288: 9801-9814.)**
Transmembrane adaptor protein PAG was shown to function both as a positive or negative regulator of mast cell signalling, depending upon the signalling pathway involved. Mice with PAG knockout have a clear phenotype *in vivo*, namely decreased passive systemic anaphylaxis. **(Draberova, L., V. Bugajev, L. Potuckova, I. Halova, M. Bambouskova, I. Polakovicova, R. J. Xavier, B. Seed, and P. Draber. 2014. Transmembrane adaptor protein PAG/CBP is involved in both positive and negative regulation of mast cell signaling. *Mol. Cell Biol.* 34: 4285-4300.)**

<u>Molecular components of immunoreceptor signalling pathways, the roles of membrane microdomains</u> (the team of V. Horejsi)

Three new transmembrane adaptor proteins (PRR7, SCIMP, LST1/A) expressed in leukocytes were discovered and functionally characterized, which may be involved in regulation of immunoreceptor signalling. **(Hrdinka, M., Dráber, P., Stepánek, O., Ormsby, T., Otáhal, P., Angelisová, P., Brdicka, T., Paces, J., Horejsí, V., Drbal K. 2011. PRR7 Is a transmembrane adaptor protein expressed in activated T cells involved in regulation of T cell receptor signaling and apoptosis. *J. Biol. Chem.* 2011; 286: 19617-19629; Draber, P., Vonkova, I., Stepanek, O., Hrdinka, M., Kucova, M., Skopcova, T., Otahal, P., Angelisova, P., Horejsi, V., Yeung, M., Weiss, A., Brdicka, T. 2011. SCIMP, a transmembrane adaptor protein involved in major histocompatibility complex class II signaling. *Mol Cell Biol.* 31:4550-4562; Draber, P., Stepanek, O., Hrdinka, M., Drobek, A., Chmatal, L., Mala, L., Ormsby, T., Angelisova, P., Horejsi, V., Brdicka, T. 2012. LST1/A Is a myeloid leukocyte-specific transmembrane adaptor protein recruiting protein tyrosine phosphatases SHP-1 and SHP-2 to the plasma membrane. *J. Biol. Chem.* 287:22812-21.).**

A new type of membrane rafts ("heavy rafts") containing a number of biologically important leukocyte membrane signalling proteins was described, the existence of which may explain paradoxical results of a previous paper that casted doubt on the existence and importance of membrane rafts. **(Otáhal, P., Angelisová, P., Hrdinka, M., Brdicka, T., Novák, P., Drbal, K., and Horejsi, V. 2010. A new type of membrane raft-like microdomains and their possible involvement in TCR signaling. *J. Immunol.* 184:3689-3696.)** The functional importance of targeting a key Src-family regulator, cytoplasmic tyrosine kinase Csk, into "classical" rafts as opposed to "heavy" rafts and non-raft membrane was demonstrated. The results also have important implications for a current model of Lck involvement in TCR signalling. **(Otáhal, P., Pata, S., Angelisová, P., Horejsi, V., Brdicka, T. 2011. The effects of membrane compartmentalization of Csk on TCR signaling. *Biochim. Biophys. Acta – Mol. Cell. Res.* 1813:367-376.)**

New insights into the controversial question of mutual importance of Src-family kinases vs. Syk kinase in initiation of BCR signalling were obtained, using experiments based on controlled BCR triggering *ex vivo* on primary murine B cells and on murine and chicken B cell lines. **(Stepanek, O., Draber, P., Drobek, A., Horejsi, V., Brdicka, T. 2013. Nonredundant roles of Src-family kinases and Syk in the initiation of B-cell antigen receptor signaling. *J. Immunol.* 190:1807-1818.)**

The roles of embryonic macrophages and intestinal defensins (the team of D. Filipp)

Toll-like receptors (TLR) expressed on embryonic macrophages were demonstrated to couple inflammatory signals to iron metabolism during early ontogenesis. TLRs expressed on embryonic macrophages thus vitally contribute to homeostasis of the developing early embryo, playing a regulatory role in the integration of immune-related signals with the essential metabolic needs of developing embryos. **(Balounová, J., Vavrochová, T., Benešová, M., Ballek, O., Kolář, M. and Filipp, D. *2014.* Toll-like receptors expressed on embryonic macrophages couple inflammatory signals to iron metabolism during early ontogenesis. *Eur J Immunol.* May;44(5):1491-502).**

This team has been also working for three years on the link between gastrointestinal autoimmunity and enteric anti-microbial peptides and very recently published the resulting important study in the top gastroenterology journal (**Dobes, J., Neuwirth, A., Dobesova, M., Voboril. M., Balounova, J., Ballek. O., Lebl, J., Meloni, A., Krohn, K., Kluger, N., Ranki, A., Filipp. D. 2015. Gastroenterology, in press**).

Mechanisms involved in tumour - immune system interactions (the team of M. Reinis)

The most important results of this group were published in respectable journals as follows:

Vlková, V., I. Štěpánek, V. Hrušková, F. Šenigl, V. Mayerová, M. Šrámek, J. Šímová, J. Bieblová, M. Indrová, T. Hejhal, N. Dérian, D. Klatzmann, A. Six, M. Reiniš. 2014. Epigenetic regulations in the IFNγ signalling pathway: IFNγ-mediated MHC class I upregulation on tumour cells is associated with DNA demethylation of antigen-presenting machinery genes. *Oncotarge*t 5: 6923-6935.

Rosalia, R.A., I. Štěpánek, V. Polláková, J. Šímová, J. Bieblová, M. Indrová, S. Moravcová, H. Přibylová, H.J. Bontkes, J. Bubeník, T. Sparwasser, M. Reiniš. 2013. Administration of anti-CD25 mAb leads to impaired α-galactosylceramide-mediated induction of IFN-γ production in a murine model. *Immunobiology*. 218: 851-859.

(5) **Cell biology of nucleus and cytoskeleton**

Cell biology of the cytoskeleton (mainly microtubules) has been one of rather traditional topics systematically dealt with by the group of Pa. Draber.

The topic of the nucleus cell biology based mainly on advanced microscopic approaches has been brought to IMG by P. Hozak when his team joined IMG in 2007. In recent years they pioneered a novel and original concept of involvement of phosphatidylinositol 4,5-bisphosphate (PIP2) in gene transcription and nucleolar organization:

<u>Cytoskeleton cell biology</u> (the team of Pa. Draber)

γ-tubulin was for the first time shown to be present in nuclei/nucleoli in mammalian interphase cells, functionally associated with tumour suppressor protein C53. (**Hořejší B., S. Vinopal, V. Sládková, E. Dráberová, V. Sulimenko, T. Sulimenko, V. Vosecká, A. Philimonenko, P. Hozák, C.D. Katsetos, P. Dráber. 2012. Nuclear gamma-tubulin associates with nucleoli and interacts with tumor suppressor protein C53. *J. Cell Physiol.* 227: 367-382)**

<u>Nucleus cell biology</u> (the teams of D. Staněk and P. Hozak)

New data about the structure and function of nuclear compartments were obtained. The combined approach of live-cell measurements of snRNP dynamics by FRAP and mathematical modelling demonstrated that snRNP assembly rate in Cajal bodies is ten times faster than in the nucleoplasm. **(Novotný, I.[§], Blažíková, M.[§], Staněk, D.*, Heřman, P*. and Malínský, J. 2011. In vivo kinetics of U4/U6•U5 tri-snRNP formation in Cajal Bodies. *Mol Biol Cell.* 22: 513-523)**

Furthermore, nuclear structures called gems were shown to contain components of the splicing factor U1 snRNP. This is the first example of snRNP components found in gems, which points to a role of gems

in U1 snRNP metabolism. **(Stejskalová, E. and Staněk, D. 2014. Splicing factor U1-70K interacts with the SMN complex and is essential for nuclear gem integrity. *J. Cell Sci.* 127:3909-15)**

Phosphatidylinositol 4,5-bisphosphate (PIP2) was identified as a part of the protein complex on the active ribosomal promoter during transcription. The results indicate that PIP2 interacts with a subset of Pol I transcription machinery, and promotes Pol I transcription. This is the very first description of a direct PIP2 involvement in the regulation of transcription. **(Yildirim S, Castano E, Sobol M, Philimonenko VV, Dzijak R, Venit T, Hozák P., 2013. Involvement of phosphatidylinositol 4,5-bisphosphate in RNA polymerase I transcription. J Cell Sci., 126:2730-9.)**

Additional results indicate that PIP2 is required not only during rRNA production and biogenesis, but also plays a structural role as an anchor for the Pol I pre-initiation complex during the cell cycle. Thus, PIP2 appears to be a novel component of the NOR complex, which is further engaged in the renewed rRNA synthesis upon exit from mitosis. (**Sobol M, Yildirim S, Philimonenko VV, Marášek P, Castaño E, Hozák P.,2013. UBF complexes with phosphatidylinositol 4,5-bisphosphate in nucleolar organizer regions regardless of ongoing RNA polymerase I activity. Nucleus., 4:478-86.)**

(6) **Molecular virology**

(Retro)virology has been (together with immunology) the most traditional research area at IMG. J. Svoboda is one of the father founders of the retrovirology field. This research direction remains (in ***the team led by J. Hejnar***) very strong and highly scientifically productive, as documented by the following collection of topically related papers in excellent international journals:

High oncogene expression is needed for tumour growth; it was demonstrated that avian sarcoma virus proviruses with high and stable expression of transduced oncogene accumulate in genomic regions transcribed in multiple tissues. This requirement is an important concern for retroviral vectors in gene therapy trials. **(Plachý, J., Kotáb, J., Divina, P., Reinišová, M., Šenigl, F., Hejnar, J. 2010. Proviruses selected for high and stable expression of transduced genes accumulate in broadly transcribed genome areas. *J. Virol*. 84: 4204-4211.)**

Syncytin-1, human fusogenic glycoprotein encoded by the *env* genes of the endogenous retrovirus, drives differentiation of syncytiotrophoblast. In non-trophoblastic cells, however, the expression of syncytin-1 is suppressed by DNA methylation and chromatin modifications. The results demonstrate retroviral splicing as an additional level controlling the endogenous retroviruses. **(Trejbalová, K., Blažková, J., Matoušková, M., Kučerová, D., Pecnová, L., Vernerová, Z., Heráček, J., Hirsch, I., Hejnar, J. 2011. Epigenetic regulation of transcription and splicing of syncytins, fusogenic glycoproteins of retroviral origin. *Nucleic Acids Res*. 39: 8728-8739.)** Efficient retrovirus silencing in human cells dependent on the *de novo* DNA methyltransferase activity has been demonstrated. Proviruses integrated close to the transcription start sites of active genes into the regions enriched in H3K4 trimethylation are resistant to the transcriptional silencing. These findings are important for HIV-1

latency and retroviral vectors. (**Šenigl, F., Auxt, M., Hejnar, J. 2012. Transcriptional provirus silencing as a crosstalk of de novo DNA methylation and epigenomic features at the integration site. Nucleic Acids Res. 40: 5298-5312.)** Defective splicing (intronic deletions that disrupt the receptor mRNA splicing**)** has been identified as a mechanism of resistance to avian sarcoma virus; intronic polymorphisms thus play an important role in virus-host coevolution. **(Reinišová, M., Plachý, J., Trejbalová, K., Šenigl, F., Kučerová, D., Geryk, J., Svoboda, J., Hejnar, J. 2012. Intronic deletions that disrupt mRNA splicing of the tva receptor gene result in decreased susceptibility to infection by avian sarcoma and leukosis virus subgroup A. J. Virol. 86: 2021-2030.)** Rous sarcoma virus (RSV) is able to infect and transform mammalian cells but it does not produce infectious virus particles. Using a model of RSV virus-transformed rodent cells, it was established that the lack of virus replication is due to the absence of chicken factor(s), which can be supplemented by cell fusion with chicken cells. The main block is at the level of RNA splicing and nuclear export of specific viral mRNAs. (**Lounková, A., Dráberová, E., Šenigl, F., Trejbalová, K., Geryk, J., Hejnar, J., Svoboda, J. 2014. Molecular events accompanying rous sarcoma virus rescue from rodent cells and the role of viral gene complementation. J. Virol. 88: 3505-3515.)** Analysis of transcription of porcine endogenous retroviruses (PERVs) demonstrated heavy methylation in the majority of PERV 5' long terminal repeats (LTR) in porcine tissues. Only a minor fraction of proviruses are responsible for the PERV expression and transmission. These results may have implications for pig-to-human xenotransplantation. (**Matoušková, M., Veselý, P., Daniel, P., Mattiuzzo, G., Hector, R.D., Scobie, L., Takeuchi, Y., Hejnar, J. 2013. Role of DNA methylation in expression and transmission of porcine endogenous retroviruses. J. Virol. 87: 12110-12120)**

(7) **Molecular pharmacology**

This topic was brought to the IMG in 2007, when ***the team led by J. Blahos*** joined the IMG. This topic is technically quite close to several other groups dealing with receptor signalling. The following paper is the most significant result of their long-term collaboration with a French lab:

Sequential inter- and intrasubunit rearrangements during activation of dimeric metabotropic glutamate receptor 1 by agonist binding were demonstrated using the dynamic FRET approach. The resulting active state of these receptors was found to be asymmetrical. The results clarify the mechanisms involved in activation of these family C GPCRs.

(**Hlavackova V., Zabel U., Frankova D., Bätz J., Hoffmann C., Prezeau L., Pin JP, Blahos J., Lohse MJ.; Sequential inter- and intrasubunit rearrangements during activation of dimeric metabotropic glutamate receptor 1. *Science Signaling 2012* 5(237):ra59.)**

(8) **Structural biology**

This area is covered by ***the team led by P. Rezacova*** (it is the only group located outside of the main building of IMG, as it is a joint lab with the Institute of Organic Chemistry and Biochemistry). This team routinely publishes in very good structural biology journals:

Crystal structures of cancer-specific carbonic anhydrase isoenzyme IX demonstrated that various carborane clusters act as active-site-directed inhibitors of this enzyme. The results provide information that can be applied to the structure-based design of specific inhibitors. (**Brynda,J; Mader, P; Šícha, V; Fábry, M; Poncová, K; Bakardiev, M; Grüner, B; Cígler, P; Řezáčová, P. Carborane-based carbonic anhydrase inhibitors. Angew. Chem. Int. Ed. 2013, 52, 13760-13763).**
The crystal structure of human carbonic anhydrase CA II in a complex with 6,7-dimethoxy-1-methyl-1,2,3,4-tetrahydroisoquinolin-2-ylsulfonamide revealed unusual inhibitor binding. The results provide clues for the future design of even more selective inhibitors for druggable isoforms such as the cancer-associated hCA IX and neuronal hCA VII. (**Mader, P; Brynda J; Gitto, R; Agnello, S; Pachl, P; Supuran, CT; Chimirri, A; Rezacova, P.: Structural Basis for the Interaction Between Carbonic Anhydrase and 1;2;3;4-tetrahydroisoquinolin-2-ylsulfonamides. J. Med. Chem. 2011, 54, 2522-2526.**

Other achievements

In addition to the scientific (publication) achievements listed above, we consider as a major success the very good functioning of the Transgenic Unit, Animal Facility and Light Microscopy and Flow Cytometry Facility. The transgenic facility is able to produce tens of genetically modified mouse strains per year. It is a core of the crucial part of the BIOCEV project for the near future. In addition, the recently built National Centre for Chemical Biology – OPENSCREEN – established a robotic platform for extensive screening of chemical libraries. All these facilities are surely the best ones of this kind in this country; more and more of our best scientific results would not be possible without them.

More generally, all of our service facilities function very well, on a level directly comparable to that of similar institutions in Western Europe.

IMG also supports public outreach. A significant achievement in this area is the public laboratory project Bioskop (www.bioskop.cz) developed by a team of students and postdocs lead by Petr Svoboda and Petr Bartunek. Bioskop was subsequently implemented at the Masaryk University in Brno, where it enjoys a great success. Petr Svoboda is a member of its scientific board; IMG remains the owner of the trademark Bioskop and the web domain bioskop.cz.

Research Report of the team in the period 2010–2014

Institute	Institute of Molecular Genetics of the CAS, v. v. i.

Scientific team	Laboratory of Tumour Immunology

2.1. Introduction

As a long-term research programme, we have been investigating the interactions between tumour cells and the immune system, especially their modulation with chemo- and immunotherapy. Special attention has been paid to the (epigenetic) mechanisms by which tumour cells are capable to escape from immune responses and also to the immunological impacts of anti-tumour therapy using epigenetic agents. Within the last five years, the most important findings at our Laboratory were related to the description of epigenetic mechanisms underlying regulation of antigen-presenting machinery genes and interferon (IFN) γ signalling pathway in tumour cells. We have documented connection of increased expression of genes encoding antigen-presenting machinery (APM) components, which are regulated by IFNγ, with their DNA demethylation after the treatment with DNA methyltransferase inhibitor 5-azacytidine (5AC). On the other hand, we have shown that activation of the APM genes by IFNγ was associated with DNA demethylation, demonstrating that IFNγ is an epigenetic agent (*Simova et al., Brit J Cancer 2011; Vlkova et al., Oncotarget 2014*). Secondly, we have obtained priority data in the field of immune suppression regulation with chemotherapy. We have studied the impacts of chemotherapy on myeloid-derived suppressor cells (MDSCs), a cell population playing a critical role in mediating suppression of the anti-tumour immunity. We characterized the MDSC population raised upon the treatment with cyclophosphamide and, further, we demonstrated that 5AC treatment could display both cytotoxic and differentiation effects on these cells, which may be of therapeutic interest (*Mikyskova et al., J Immunother 2012*; *J Leukoc Biol 2014).*

Recently, our interest has been turned to the topic of cellular senescence induction in tumour cells upon the treatment with genotoxic agents and to the role of cytokines in the senescence induction and maintenance. We are convinced that better understanding of the mechanisms by which senescent tumour cell can influence the tumour microenvironment and, on the other hand, whether and how the immune response can induce cellular stress and senescence in tumour cells, can bring clues important for our better insight into the tumour development mechanisms, as well as for finding new therapeutic schemes against recurrent tumours. Since in this topic immunology meets oncology, we have established a fruitful collaboration with the Department of Genome Integrity at our Institute (*Hubackova et al, Oncogene, in print; Kyjacova et al. Cell Death Differ 2014*).

Besides these projects, we have paid attention to our long-term field of interest, experimental immunotherapy combined with chemotherapy, especially against tumours that escape from anti-tumour immunity, namely MHC class I-deficient tumours. Notably, we have also been involved in the contractual research focused on the development of a dendritic cell-based therapeutic vaccine against prostate cancer.

Most of the published experiments were performed at our Laboratory. However, during the last five years we took part in the Clinigene Network of Excellence for the Advancement of Gene Transfer and Therapy (EU-FP6 Project) until 2011, we collaborated with several laboratories both in Europe and the Czech Republic, and we also set up strong intramural collaboration, mainly with the Department of Genome Integrity.

2.2. Epigenetic regulations of antigen-presenting machinery genes in the MHC class I-deficient tumour cells; IFNγ is an epigenetic agent

Since both genetic and epigenetic regulations play crucial roles in tumorigenesis, it is evident that epigenetic mechanisms are also important in the tumour cell escape from immune responses, such as in MHC class I down-regulation or altered expression of other components important for antigen presentation. Therefore, chemotherapy with DNA methyltransferase inhibitors can influence the tumour cell sensitivity to immunotherapy and augment its therapeutic effect. In our Laboratory, the research in this field dates back to 2008, when we demonstrated that epigenetic agents, including 5AC, could induce expression of genes involved in the antigen-processing machinery and surface expression of MHC class I molecules on MHC class I-deficient tumour cells, which was associated with demethylation of the regulatory sequences of the corresponding APM genes (*Manning et al., Immunology 2008*). The aim of the following study, published in 2011 (*Simova et al., Brit J Cancer 2011*), was to determine the *in vivo* effects of 5AC on expression of the MHC class I molecules and co-stimulatory molecules on tumour cells. The second goal was to elucidate how 5AC as epigenetic agent can influence the anti-tumour immune responses *in vivo* and whether the treatment with epigenetic agents can be successfully combined with immunotherapeutic protocols. In this study, we demonstrated 5AC adjuvant/additive effects against MHC class I-deficient tumours when combined with unmethylated CpG oligodeoxynucleotides or with IL-12-producing cellular vaccine. The efficacy of the chemoimmunotherapy was partially dependent on the $CD8^+$-mediated immune responses. Increased expression levels of the antigen-presenting machinery-related genes, their demethylation, as well as induction of genes encoding selected components of the IFNγ signalling pathway (IRF-1 and IRF-8) in tumours explanted from 5AC-treated animals were observed. These data suggested that chemotherapy of MHC class I-deficient tumours with 5AC combined with immunotherapeutic procedures could be an attractive setting in the treatment of MHC class I-deficient tumours.

Based on our findings that silenced antigen-presenting machinery genes were activated and demethylated upon the treatment with epigenetic agents, we have hypothesised that IFNγ can act as an DNA demethylating agent when activating a set of silenced genes within the MHC chromosomal locus. Indeed, we documented strong association of DNA demethylation of selected antigen-presenting machinery genes located in the MHC genomic locus (*TAP-1, TAP-2, LMP-2, LMP-7*) upon IFNγ treatment with MHC class I up-regulation on tumour cells in several MHC class I-deficient murine tumour cell lines (TC-1/A9, TRAMP-C2, MK16 and MC15) (*Vlkova et al., Oncotarget 2014*). Our data also documented higher methylation levels in these genes in TC-1/A9 cells, as compared to their parental MHC class I-positive TC-1 cells. IFNγ-mediated DNA demethylation was relatively fast in comparison with demethylation induced by 5AC, and associated with increased histone H3 acetylation in the promoter regions of APM genes. Comparative transcriptome analysis in distinct MHC class I-deficient cell lines upon their treatment with either IFNγ or epigenetic agents revealed that a set of genes, significantly

enriched for the antigen presentation pathway, was regulated in the same manner. Our data demonstrate that IFNγ acts as an epigenetic modifier when upregulating the expression of antigen-presenting machinery genes. These priority data contribute to our knowledge of the mechanisms underlying tumour cell escape from specific immunity and its possible reversal, as well as of the role of DNA methylation changes in the IFNγ-mediated up-regulation of selected genes in tumour cells. These findings are also important for better understanding of the immunological impacts of the cancer therapy with epigenetic agents. The principal task now is to elucidate the mechanisms of the IFNγ-induced gene demethylation. We are addressing this point of particular interest in an ongoing project. This work was performed in our Laboratory with the help of our French colleagues with the transcriptome data analysis. The design of the DNA demethylation analysis protocols was established in collaboration with the Department of Viral and Cellular Genetics of our Institute (Dr. F. Senigl).

Epigenetic agents, especially 5AC, are drugs intensively tested for anti-tumour therapy. However, their immunomodulatory effects are not fully understood. We have performed more studies dealing with the 5AC effects on selected immune cell lineages, namely MDSC (results are discussed below) and dendritic cells. We have investigated the effects of DNA methyltransferase inhibitor 5AC and histone deacetylase inhibitor trichostatin A (TSA) on the murine bone marrow-derived, as well as on the human monocyte-derived DC maturation. The major impact of 5AC and TSA on the DC maturation process consisted in the inhibition of unmethylated CpG oligodeoxynucleotide (CpG ODN) 1826- or LPS-induced activation of pro- and anti-inflammatory cytokine gene expression activation (*Stepanek et al., J Biol Regul Homeost Agents 2011*). Briefly, based also on *in vivo* experiments, in this study we demonstrated the potential of epigenetic agents to hamper the immune response induction through their inhibitory effects on DC. In this study, we collaborated with the laboratory of Dr. R. Spisek (Second Faculty of Medicine, Charles University, Prague), where experiments with human DC were performed.

2.3. Immune suppression in tumour development and therapy, focus on myeloid-derived suppressor cells

Local and systemic immune suppression represents another obstacle blocking the effective anti-tumour immune responses. Myeloid-derived suppressor cells (MDSC), a heterogenic population of immature cells of myeloid lineage that accumulates in lymphoid organs and blood during tumour growth, nowadays attract more and more attention as key players blocking anti-tumour immunity. Of note, MDSC cellularity and activity can be modulated, either increased or blocked, by distinct chemotherapeutic agents. We can suppose that impacts of particular chemotherapeutics on this cell population can substantially contribute to the therapeutic outcome. In the first study of our group published by Mikyskova et al. (*J Immunother 2012*), we investigated MDSC accumulation after cyclophosphamide (CY) administration. Spleen MDSC accumulating after CY therapy (CY-MDSC) were compared with those expanded in mice bearing HPV16-associated TC-1 carcinoma (TU-MDSC). Both CY-MDSC and TU-MDSC accelerated growth of TC-1 tumours *in vivo*; however, their phenotype and immunosuppressive function differed. CY-MDSC consisted of higher percentage of monocyte-like subpopulation, and this was accompanied by lower relative expression of immunosuppressive genes and lower suppression of T-cell proliferation. After IFNγ stimulation, the expression of immunosuppressive genes increased, but the suppressive ability of CY-MDSC did not reach that of CY-MDSC. The phenotype and function of MDSC obtained from mice bearing TC-1 tumours treated with CY was, in general, found

to lie between CY-MDSC and TU-MDSC. After *in vitro* cultivation of MDSC in the presence of IL-12, the percentage of $CD11b^+/Gr\text{-}1^+$ cells decreased and was accompanied by an increase in the percentage of $CD86^+/MHCII^+$ cells that are considered to be dendritic cells (DC). The strongest modulatory effect was noticed in the group of CY-MDSC. The susceptibility of CY-MDSC to all-trans-retinoic acid (ATRA) was also evaluated. *In vitro* cultivation with ATRA resulted in MDSC differentiation, and ATRA inhibited MDSC accumulation induced by CY administration. Our findings described differences between CY-MDSC and TU-MDSC and supported the rationale for utilization of ATRA or IL-12 to alter MDSC accumulation after CY chemotherapy with the aim to improve its anti-tumour effect.

In the second study we raised the question how DNA methyltransferase inhibitor 5AC can influence the MDSC phenotype (*Mikyskova et al., J Leukoc Biol 2014*). We found that the percentage of MDSC both in the TME and spleens of 5AC-treated mice bearing TRAMP-C2 or TC-1/A9 tumours decreased, compared to the untreated controls. The changes in the MDSC percentage were accompanied by a decrease in the *Arg-1* gene expression, both in tumour microenvironment and spleens. As described above, CY treatment of the tumours resulted in additional MDSC accumulation in the tumour microenvironment and spleens. This accumulation was subsequently inhibited by 5AC treatment. Combination of CY with 5AC led to the highest tumour growth inhibition. Further, *in vitro* cultivation of spleen MDSC in the presence of 5AC reduced the percentage of MDSC. This reduction was associated with increased percentage of $CD11c^+$ and $CD86^+/MHCII^+$ cells, which means the phenotype of dendritic cells. The observed modulatory effect on MDSC correlated with reduction of the *Arg-1* gene expression, VEGF production and loss of suppressive capacity. Similar, albeit weaker effects were observed when MDSC from the spleens of tumour-bearing animals were cultured with 5AC. Taken together, our findings indicate that, beside the direct anti-tumour effect, 5AC can reduce the percentage of MDSC accumulating in the TME and spleens during tumour growth and CY chemotherapy, which can be beneficial for the outcome of cancer therapy. To obtain clinically relevant data, we are now investigating the changes in the MDSC percentage in patients with myeloid dysplastic syndrome upon their treatment with 5AC (collaboration with Dr. Jonasova, First Faculty of Medicine, Charles University, Prague).

Besides MDSC, we have focused on the problem of T regulatory cell (Treg) elimination or inactivation using specific anti-CD25 antibodies, which in combination with immunostimulation is an attractive and widely studied modality especially in anti-tumour immunotherapy. Since CD25 is not expressed exclusively on Tregs but also on subpopulations of activated lymphocytes, the modulatory effects of the specific anti-CD25 antibodies can also be partially attributed to their interactions with the effector cells. In our setting, we analysed effector functions of activated invariant natural killer T (iNKT) cells in combination with anti-CD25 mAb PC61. Upon PC61 administration, α-galactosylceramide (α-GalCer)-mediated activation of iNKT cells resulted in decreased IFNγ but not IL-4 production. In order to determine whether mutual interactions between Tregs and iNKT cells take place, we compared IFNγ production after α-GalCer administration in anti-CD25-treated and "depletion of regulatory T cell" (DEREG) mice. Since no profound effects on IFNγ induction were observed in DEREG mice, deficient in $FoxP3^+$ Tregs, our results indicate that the anti-CD25 antibody acts directly on $CD25^+$ effector cells. In agreement with these findings, *in vivo* experiments demonstrated that although both α-GalCer and PC61 administration inhibited TC-1 tumour growth in mice, no additive/synergic effects were observed when these substances were used in combination therapy. This study was performed in our laboratory, we

collaborated with Dr. T. Sparwasser (Twincore, Hannover), who supplied us with the transgenic mice, and with H. Bontkes (Amsterdam), who helped us with the study design and evaluation.

2.4. Cellular stress, senescence, IFNγ and anti-tumour immunity

As continuation and further development of our interests in the immunological impacts of cancer chemotherapy, as well as in the (epigenetic) regulations of tumour cell escape from immune responses, we started to pay attention to the immunological impacts of the genotoxic stress and senescence induction in tumour cells. The basic prerequisites are that tumour cell interactions with the immune system are influenced by senescence-inducing chemotherapy and that senescent cells, or cells in genotoxic stress in general, contribute to the pro-tumorigenic and immunosuppressive microenvironment. On the other hand, the immune response can either eliminate senescent tumour cells or induce senescence of the proliferating tumour cells. Using preclinical models, our aim is to obtain clinically relevant data that will enable us to suggest effective combined chemoimmunotherapy of the minimal residual tumour disease that will be based on combinations of senescence-inducing chemotherapeutics, including epigenetic agents and subsequent immunotherapy. So far, we have obtained preliminary data indicating pro-tumorigenic and immunosuppressive effects of senescent cells. In a recent, already published study (*Hubackova et al., Oncogene, in print*) we have shown that IFNγ induced oxidative stress, DNA damage and tumour cell senescence in both murine and human tumour cell lines via TGFβ/SMAD signalling-dependent induction of Nox4 and suppression of ANT2. This publication is a result of our close collaboration with the Department of Genome Integrity. The study was performed mainly in this department, our contribution was concentrated into the murine cell lines studies.

2.5. Immunotherapy of MHC class I-deficient tumours

Down-regulation of MHC class I expression on the cell surface is a common mechanism by which tumour cells can escape from the T cell-mediated anti-tumour immunity. This down-regulation represents an obstacle for the efficacy of anti-tumour vaccines, which are mostly designed to elicit T cell-mediated specific immunity. For these reasons, MHC class I-deficient tumours and their immunotherapy remain our long-term field of interest. The mechanistic studies concerning regulation of the MHC class I expression on tumour cells has been discussed above, here the immunotherapeutic experiments will be mentioned.

First, we investigated the efficacy of prophylactic peptide and peptide-pulsed dendritic cell-based vaccines in a murine model of experimental MHC class I-deficient tumours (TC-1/A9), expressing E6/E7 oncogenes derived from HPV16, and compared the efficacy of particular vaccination settings to anti-tumour protection against parental MHC class I-positive TC-1 tumours in order to design an experimental vaccine effective against MHC class I-deficient tumours (*Reinis et al., Int J Oncol 2010*). The peptide vaccine based on the "short" peptide $E7_{49-57}$ harbouring solely the CTL epitope and co-administered to the C57BL/6 mice with CpG oligodeoxynucleotide (CpG ODN) 1826 was effective against MHC class I-positive but not -deficient tumours, while the "longer" peptide $E7_{44-62}$ (peptide 8Q, harbouring CTL and Th epitopes)-based vaccines were also effective against MHC class I-deficient

tumours. Unlike in the peptide immunization setting, treatment with dendritic cells pulsed with a "short" peptide resulted in the tumour growth inhibition, albeit weaker as compared to the immunization with the longer peptide. These data demonstrate that peptide and dendritic cell-based vaccines can be designed to elicit protective immunity against MHC class I-deficient tumours. In the second, mechanistic study we have examined the role of different immune cell lineages in the development of immunity against tumours of the same aetiology but with different MHC class I expression (*Indrova et al., Oncol Reports 2011*). *In vivo* depletion of $CD8^+$ cells but not of $CD4^+$ or NK 1.1^+ cells in the immunization period resulted in complete elimination of the protective effects of immunization with irradiated TC-1 cells (MHC class I-positive cell line) against the TC-1 tumour challenge. On the other hand, after immunization with irradiated TC-1/A9 or with MK16 tumour cells (MHC class I-deficient sublines), a remarkable dependence on the presence of $NK1.1^+$ cells was observed, while the tumour growth inhibition after $CD4^+$ or $CD8^+$ depletion was not efficient. These results were supported by the *ex vivo* analysis, also suggesting that the development of immunity against MHC class I-deficient tumours is highly dependent on the activity of the NK 1.1^+ cell population.

Genetically modified tumour cells producing cytokines such as IL-12 are potent activators of the anti-tumour immune responses and represent a promising therapeutic modality when combined with chemotherapy or other means of therapy. Several combination settings have already been discussed above. Here, the experiments have been completed with a study demonstrating that IL-12-producing cellular vaccine could augment the chemotherapy of human papilloma virus (HPV) 16-associated murine, both metastasizing and non- metastasizing tumours, distinct in expression of the MHC class I molecules, with cytostatic agent gemcitabine (*Mikyskova et al., Oncol Reports 2011*). Using the HPV16-associated MHC class I-deficient tumour models, we have also demonstrated synergic tumour-inhibitory effects of the IL-12 producing cellular vaccines and 12 carbon acyl chain β-galactosylceramide (C12 β-D-GalactosylCeramide), an activation ligand for the iNKT cell T receptor (*Simova et al., Int J Cancer 2010*).

2.6. Dendritic cell-based vaccine against prostate cancer (contractual research)

In 2013, we established collaboration (contract research) with the SOTIO Ltd. Company, which is developing a dendritic cell-based therapeutic vaccine against prostate cancer. This immunotherapy has already entered the Phase III Clinical Trial. In our laboratory, we have employed murine transgenic models for spontaneous prostate cancer, as well as a model based on transplantation of syngeneic prostate tumour cells for the proof-of-principle studies of the dendritic cell-based treatment combined with chemotherapy. The first results will probably be published this year.

2.7. Reviews

Within the last period, we have published mainly original articles. However, we published several review articles focused on the topics investigated at our Laboratory concerning immunotherapy of the MHC class I-deficient tumours (*Reinis, Future Oncol 2010*), animal models for dendritic cell-based therapy (*Pajtasz-Piasecka, Indrová, Immunotherapy 2010*) and IL-12 in cancer treatment (*Bubenik, Folia Biol 2010*).

2.8. References

(Authors and/or co-authors of the evaluated team are in bold; corresponding author from the evaluated team is marked by asterisk; titles of the papers published during the evaluation period are underlined)

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Research Report of the team in the period 2010–2014

Institute	Institute of Molecular Genetics of the CAS, v. v. i.

Scientific team	Laboratory of RNA Biology

2.1. Introduction

The long-term interest our team is pre-mRNA splicing. We have focused on two major topics – formation of splicing machinery in living cells and regulation of alternative splicing by chromatin modifications. In addition, our group studies snRNP mutations in human hereditary disease retinitis pigmentosa, a project that stemmed from the research of snRNP biogenesis.

2.2. Formation of splicing machinery in living cells

Spliceosomal small nuclear ribonucleoprotein particles (snRNPs) are the basic building blocks of the spliceosome, which catalyses pre-mRNA splicing. We recognize five major snRNPs named according to the snRNA they contain – U1, U2, U4, U5 and U6. Each snRNP contains one snRNA, a ring of seven Sm proteins (LSm in the case of U6) and a set of proteins specific for each snRNP. Biogenesis of snRNPs is a complex process that involves export of snRNAs to the cytoplasm and re-import into the nucleus. In this project, we focused on assembly of spliceosomal snRNP complexes and the spliceosome in living cells and the role of cellular compartments in this process.

2.2.1. To determine splicing kinetics, we utilized cell lines stably expressing beta-globin-based mini-gene reporter and GFP-tagged splicing factors. For GFP tagging, we utilized bacterial artificial chromosomes. This approach is more laborious but preserves endogenous promoters as well as exon-intron structure of the tagged gene. This prevents overexpression of tagged proteins, which often happens when widely used CM-promoter based vectors are employed for expression of tagged proteins. In collaboration with the group of Martin Hof from J. Heyrovsky Institute of Physical Chemistry AS CR we utilized Fluorescence Correlation Spectroscopy (FCS) to measure diffusion of snRNPs in the cell nucleus. We then applied Fluorescence Recovery After Photobleaching (FRAP) to determine interactions of snRNPs with pre-mRNA. We utilized the diffusion coefficient measured by FCS to fit FRAP recovery curves, which allowed us to determine average binding time of individual snRNPs with pre-mRNA. These data supported the step-wise assembly of the spliceosome on pre-mRNA and allowed us to estimate splicing kinetics *in vivo.* **Our results showed that *in vivo* splicing is accomplished within 20-30 s**. These results were published in (Huranova et al., 2010) and were one of the first determinations of splicing kinetics in living human cells.

2.2.2. Three out of five snRNPs join the spliceosome as a preassembled complex – the U4/U6•U5 tri-snRNP, which is assembled in a step-wise process. First U4 and U6 snRNPs are formed followed by addition of the U5 particle. We and others previously showed that both steps occur in nuclear structures called Cajal bodies (Schaffert et al., 2004; Stanek and Neugebauer, 2004; Stanek et al., 2003). Here, we measured the assembly rate of tri-snRNPs in Cajal bodes using the FRAP approach. In collaboration with Jan Malinsky from the Institute of Experimental Medicine AS CR and Petr Herman from the Faculty of

Mathematics and Physics, Charles University we created a complete kinetic model of tri-snRNP formation in the Cajal body. Next, we measured the residency time of proteins specific for individual snRNPs in Cajal bodies by FRAP and combined the measured FRAP recovery curves with the kinetic model. Using a unique combination of mathematical modelling and direct measurement of snRNP dynamics in living cells we were able **to estimate the absolute speed of the snRNP assembly reaction**. Our results also show that **accumulation of snRNP complexes within Cajal body increases the speed of tri-snRNP assembly by an order of magnitude** (Novotny et al., 2011).

2.2.3. While working on U4/U6 snRNP assembly and the role of LSm proteins 2-8 we noticed that depletion of U6-specific protein LSm8 caused delocalization of other LSm proteins to the cytoplasm, where they induced formation of processing bodies (P-bodies). In addition, we showed that LSm8 overexpression leads to P-body loss. We proposed a model where the balance between cytoplasmic LSm1 and nuclear LSm8 dictates cellular distribution of the remaining LSm2-7 proteins. If LSm8 concentration is lowered, **LSm2-7 move from the nucleus to the cytoplasm,** where together with LSm1 they create new binding sites for other P-body components and **nucleate new P-bodies** (Novotny et al., 2012).

2.2.4. We also studied localization and biogenesis of the spliceosomal U1 snRNP particle, which is essential for recognition of an intron and formation of early splicing complexes. We localized the U1-specific protein U1-70K into nuclear structures called Gems. We utilized Förster Resonance Energy Transfer (FRET) to show **that U1-70K interacts with the SMN complex** directly in Gems. Using co-immunoprecipitation of SMN with U1-70K deletion mutants we identified the N-terminal U1-70K domain to interact with the SMN complex. Finally we showed that **the U1-70K N-terminal domain is important for Gem integrity**. These results, which were published in (Stejskalova and Stanek, 2014), have a broader impact because similar behaviour (SMN binding and importance for Gems integrity) was identified as feature of several proteins whose mutations were implicated in the aetiology of amyotrophic lateral sclerosis, a progressive neurodegenerative disease. Our data thus potentially link U1-70K to amyotrophic lateral sclerosis.

2.2.5. Recently, we analysed the fate of defective or incomplete snRNPs in the nucleus. We provide evidence that **Cajal bodies serve as a checkpoint that controls final steps of snRNP formation**, namely U5 snRNP maturation and U4/U6 and U4/U6•U5 assembly. We show that incomplete or defective snRNPs are sequestered in Cajal bodies in a SART3-dependent manner. SART3 is a factor that was previously shown to bind U6 snRNA and enhance U4/U6 snRNP formation. Here we provide evidence that SART3 also controls U4/U6 and U4/U6•U5 assembly and tethers incomplete particles to coilin, the Cajal body scaffolding protein. We propose a model where SART3 monitors tri-snRNP assembly and sequesters incomplete particles in Cajal bodies, thereby allowing cells to maintain a homeostatic balance of mature snRNPs in the nucleoplasm.

In addition, we showed that inhibition of final snRNP maturation steps induced formation of Cajal bodies in cells that normally lack these nuclear compartments, suggesting Cajal body nucleation was triggered by an imbalance in snRNP assembly. We propose that a higher concentration of incomplete snRNPs together with SART3 can, directly or indirectly, induce coilin oligomerization, which leads to nucleation of microscopically visible Cajal bodies. As far we know this is the **first example showing that Cajal bodies are induced by an imbalance in the snRNP assembly pathway**. We previously

showed that accumulation of U4, U5 and U6 snRNPs in Cajal bodies increased di- and tri-snRNP assembly rates ten times (see above (Novotny et al., 2011)). Therefore, concentration of assembly intermediates in Cajal bodies enhances the assembly kinetics and could compensate for low levels of tri-snRNPs. Simultaneously, localization of immature snRNPs in an area of inactive splicing protects the cell against aberrant snRNPs, which could potentially interfere with mRNA processing. **Segregation of stalled assembly intermediates in Cajal bodies** provides cells with **a buffering system** that helps to **maintain snRNP homeostasis in the nucleoplasm.** We believe that these data are of a crucial importance for cell biology of the nucleus because they explain how a nuclear structure is formed based on metabolic needs of cells. These data were recently published in (Novotny et al., 2015).

2.3. Regulation of alternative splicing

Alternative splicing is an elegant way how cells increase the coding potential of their genome. It is estimated that 95 % of human multi-exon genes undergo alternative splicing. *In vivo* splicing is achieved when nascent RNA is still attached to RNA polymerase II (Pol II) and the DNA template. Consistent with this data, splicing factors have been shown to be recruited to the site of pre-mRNA synthesis and bind the nascent pre-mRNA, and 80 % of active spliceosomes are attached to chromatin. The main aims of this project were to analyse whether histone modification influences alternative splicing, identify target genes and determine the molecular mechanism underlying this regulation. One of the critical assumptions of the model that chromatin modifications modulate alternative splicing is that splicing occurs while pre-mRNA is still in the close vicinity of chromatin and attached to Pol ll. When the project started in 2010, the Pol II elongation rate in cells was determined by several groups and these measurements revealed transcription rate between 3-5 kb/min. However, almost nothing was known about *in vivo* splicing kinetics. Our kinetic measurements **showed that *in vivo* splicing is accomplished within 20-30 s** (Huranova et al., 2010), which together with Pol II transcription rate suggests that **most introns are removed co-transcriptionally, while pre-mRNA is still in the close vicinity of chromatin**.

2.3.1. Next we concentrated on the **role of histone acetylation in regulation of alternative splicing.** We inhibited cellular histone deacetylases (HDACs) by sodium butyrate and analysed alternative splicing using genome-wide exon array. We found 683 genes with an altered splicing pattern after HDAC inhibition. Thirteen of the top hits were further confirmed by conventional RT-PCR. One of the top hits was fibronectin, whose alternative splicing regulation is well described, and we chose to use this gene for further characterization of molecular details of splicing regulation via HDAC activity. Using three different inhibitors we confirmed that HDAC class I are mainly involved in alternative splicing regulation. We confirmed these results by targeted knockdown of two major members of class I family – HDAC1 and HDAC2. We showed that depletion of HDAC1 but not HDAC2 affects fibronectin alternative splicing and this effect can be rescued by expression of catalytically active HDAC1, while catalytically inactive HDAC1 mutant did not show any effect. These data showed that **that the catalytic activity of HDAC1 modulates alternative splicing**.

Next we concentrated on the **molecular mechanism of this regulation**. We analysed expression and acetylation of splicing regulators, namely SFRS5, which regulates fibronectin alternative splicing. HDAC inhibition did not change expression of SFRS5 or other splicing factors. Using mass spectrometry we were not able to detect any changes in acetylation of SFRS5. Therefore, we tested whether histone acetylation changes Pol II elongation rate along the genes. Using chromatin immunoprecipitation and *in vivo* measurement we showed that increased histone acetylation enhances the elongation rate of Pol II

along the gene, which negatively affects association of splicing regulators (SFRS5) with the nascent pre-mRNA. Together, these data suggested **that histone acetylation regulates alternative splicing via modulation of Pol II elongation speed, which influences interaction of the nascent pre-mRNA with splicing regulators**. These data together with genome-wide analysis and HDAC knockdowns were published in (Hnilicova et al., 2011).

2.3.2. To further characterize the **role of histone acetylation in splicing regulation** we concentrated our effort on **acetyl-histone binding proteins, namely Brd2**, which contains two bromodomains that interact with acetylated lysines in histone H4. Acetylation of H4 was, based on our previous experiments, one of the major suspects to regulate alternative splicing. Using RNAi we knocked down Brd2 and analysed splicing effects using exon microarrays. We identified ~300 genes that changed alternative splicing upon depletion of Brd2 and confirmed nine alternative splicing events by classical RT-PCR. To determine Brd2 interaction with acetylated histones we created a series of mutants that lack either the C-terminal domain or contain point mutations in bromodomains and we tested Brd2 interaction with chromatin by immunoprecipitation and live-cell fluorescent microscopy. Finally, we mapped Brd2 binding to target genes using chromatin immunoprecipitation. Together, our data showed that **Brd2 is an important factor in splicing regulation that modulates alternative splicing via binding to promoters of target genes** (Hnilicova et al., 2013).

2.3.3. Our previous data showed close connection between promoters, histone acetylation and alternative splicing, suggesting that promoters and histone modification can act synergistically to modulate alternative splicing. We decided to analyse this phenomenon in more detail. We created a fibronectin-based mini-gene splicing reporter and used a series of promoter deletions to identify promoter elements that are responsible for alternative splicing regulation. We identified the CRE element whose deletion or mutation significantly affected alternative splicing. We further showed that CRE deletion/mutation prevented binding of histone acetyl-transferase p300 to the promoter, which reduced histone acetylation both at the promoter and regions downstream of the mini-gene reporter. These data, which were published in (Duskova et al., 2014), suggest that **promoters regulate alternative splicing via histone acetylation,** which would be the first molecular explanation for the mechanism of promoter-regulated splicing.

2.4. Mutations causing retina degeneration

Human hereditary disease named retinitis pigmentosa (RP) is manifested by gradual loss of photoreceptor cells. RP is caused by mutations in ~70 different genes, most of them specifically expressed in the eye. However, mutations in five snRNP-specific proteins were described to cause RP, and together snRNP mutations represent the second largest set of mutations causing this disease.

Previously, we characterized a mutation in U4 snRNP-specific protein hPrp31 (HPRPF31) (Huranova et al., 2009). Here, we prepared two RP mutations in U5-specific protein hBrr2. We utilized the DNA recombination approach and tagged and mutated hBrr2 (SNRNP200) on bacterial artificial chromosome to preserve the endogenous promoter and exon intron structure. We did not identify any defects in snRNP or spliceosome formation. However, measuring hBrr2 mutant dynamics in living cells by FRAP revealed a potential alteration of splicing kinetics. However, we did not find any defects in splicing efficiency or alternative splicing of either housekeeping genes or reporters derived from retina-specific genes. hBrr2 is an RNA helicase, which must be tightly controlled during the splicing reaction,

and RNA helicases were suggested to be crucial components of the splicing-control mechanism. Therefore, we tested splicing fidelity using a β-globin gene-based reporter, which in its first exon contains a 5′-cryptic splice site 16 nt upstream of the normal splice site. Our data showed that **RP mutation in hBrr2 enhances utilization of the cryptic splice site and indicated that hBrr2 played a role in recognition of the optimal 5′-splice site,** and this ability was affected by RP mutations. These data pointing to a new role of hBrr2 in controlling splicing kinetics and splicing proofreading were published in (Cvackova et al., 2014).

2.5. Review publications

Based on our results on chromatin and alternative splicing we were invited to write a review on the mutual relationship between chromatin and splicing (Hnilicova and Stanek, 2011) and our expertise in FRET and pre-mRNA splicing resulted in a review on utilization of FRET for detection of RNA-protein interactions during RNA processing (Simkova and Stanek, 2012).

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Note: Authors and/or co-authors of the evaluated team are in bold; corresponding author from the evaluated team is marked by asterisk; titles of the papers published during the evaluation period are underlined. Only publications relevant to the evaluation period 2010-1014 are marked.

Research Report of the team in the period 2010–2014

Institute	Institute of Molecular Genetics of the CAS, v. v. i.

Scientific team	Laboratory of Viral and Cellular Genetics

The team consists of three pregradual students (the category under 25) and seven PhD students (25-30) supervised by four senior postdocs/young fellows and two research assistants. The PhD studies in our lab typically take five years. In a close future, two small teams are planned to split off the lab, which will decrease the number of senior staff. Two senior staff members are close to retirement and are on a soft-end track.

2.1. Introduction

The mission of the Laboratory of Viral and Cellular Genetics is the basic research in retrovirology with a particular focus on the interactions between retroviruses and host cells at the molecular level. Such interactions take place at all stages of the retrovirus replication cycle. Retroviruses enter cells via specific receptors, integrate into the host genome, and use the cell transcription machinery to express retroviral genes. Specific binding of retroviral envelope proteins to host cell receptors is the prerequisite for cell permissiveness to the infection. Retroviruses broaden their host range by mutations of the *env* gene, and vice versa, host cells develop resistance to retrovirus by mutations of genes encoding the specific receptors. Another defence mechanism used by the host cells is the inactivation (silencing) of integrated proviruses at the level of transcription via DNA methylation and modifications of adjacent histones. Epigenetic and epigenomic features of the host cell genome could be revealed using retroviruses as markers of various genomic loci. Transcriptional provirus silencing affects the outcome of retrovirus infection and results in retrovirus latency exemplified by HIV-1. Infectious retroviruses can become endogenous via integration into the germ line. We study the complex interplay of viral and host genomes by genomic screens for new endogenous retroviruses and epigenetic view at their expression. We also aim at translation of our results into biotechnology. Epigenetic silencing is an obstacle in using retroviruses as vectors for gene transfer and transgenesis and we studied the strategies of protection of retroviral vectors from CpG methylation. This effort led to an improvement of retrovirus-based vectors with relaxed integration pattern and long-term expression of transduced genes. Our results were published in 17 original research articles.

2.2. Genomic and epigenomic features of retrovirus integration and expression

Our team was the first all around the world who started to examine the retrovirus integration at the whole genome level at the beginning of the genomic era and we published a genomic profile of HIV-1 integration as soon as the human genome assembly appeared. The non-random integration of retroviruses and retrovirus-derived vectors into the genomes of host cells with specific preferences for transcribed genes, gene-rich regions, and CpG islands has been gradually described for most of retroviral species. During the evaluated period we studied the expression of individual proviruses and correlated it with genomic and epigenomic features of the integration loci. We cloned and characterized avian

sarcoma virus integration sites from virus-induced chicken tumours. Growing progressively, dependent on high and stable expression of the transduced v-*src* oncogene, these tumours represent clonal expansions of cells bearing transcriptionally active replication-defective proviruses. Therefore, integration sites in our study distinguished genomic loci favourable for the expression of integrated retroviruses and gene transfer vectors. Analysis of integration sites from avian sarcoma virus-induced tumours strikingly showed non-random distribution, with proviruses found prevalently within or close to transcription units, particularly in genes broadly expressed in multiple tissues but not in tissue-specifically expressed genes (Plachý et al., 2010). We infer that proviruses integrated in these genomic areas efficiently avoid transcriptional silencing and remain active for a long time during the growth of tumours. Defining the differences between unselected retroviral integration sites and sites selected for long-terminal-repeat-driven gene expression is relevant for retrovirus-mediated gene transfer and has ramifications for gene therapy.

Knowing that autonomous transcription of integrated retroviruses strongly depends on genomic, mostly suppressive effects at the integration loci, we addressed the role of epigenetic factors, DNA methylation, and histone modifications at the whole-genome-scale. We performed clonal analysis of provirus silencing with an avian leucosis/sarcoma virus-based reporter vector and correlated the transcriptional silencing with the epigenomic landscape of respective integrations. We found efficient provirus silencing in the human HCT116 cell line, which is strongly but not absolutely dependent on the *de novo* DNA methyltransferase activity, particularly of *Dnmt3b*. Proviruses integrated close to the transcription start sites of active genes into the regions enriched in H3K4 trimethylation display long-term stability of expression and are resistant to the transcriptional silencing after over-expression of Dnmt3a or Dnmt3b. In contrast, proviruses in the intergenic regions tend to spontaneous transcriptional silencing even in *Dnmt3a* (-/-) *Dnmt3b* (-/-) cells. The silencing of proviruses within genes is accompanied by DNA methylation of long terminal repeats, whereas silencing in intergenic regions is DNA methylation-independent (Šenigl et al., 2012). These findings indicate that the epigenomic features of integration sites are crucial for their permissiveness to proviral expression. Furthermore, this study exemplifies that retroviral expression can be used as a marker of transcriptional capacity of a given genomic locus and help us in the current effort of epigenome description.

We continue these studies by more detailed description of histone modifications at the site of integration of long-term transcriptionally active proviruses. The specific combinations of epigenetic marks define functional epigenomic states (heterochromatin, weak, strong, poised enhancers and promoters, gene bodies, PCG-inactivated euchromatin, insulators, etc.) and we demonstrate accumulation of active proviruses into active promoters and enhancers, which is an important conclusion for the gene therapy (Šenigl et al., unpublished).

2.3. Modification of retroviral vectors towards resistance against DNA methylation

We used the aforementioned findings in an original strategy to improve retroviral vectors and protect them against DNA methylation and transcriptional silencing. Our approach is based on CpG islands, particularly unmethylated genomic parts known to keep adjacent promoters transcriptionally active. In the CpG island adjacent to the adenosine phosphoribosyltransferase gene, the protection against transcriptional silencing can be attributed to the short CpG-rich core element containing Sp1 binding sites. We inserted this CpG island core element, IE, into the long terminal repeat of a retroviral vector derived from Rous sarcoma virus, which normally suffers from progressive transcriptional silencing in mammalian cells. IE insertion into a specific position between enhancer and promoter sequences led to

efficient protection of the integrated vector from silencing and gradual CpG methylation in rodent and human cells. Individual cell clones with IE-modified reporter vectors display high levels of reporter expression for a sustained period and without substantial variegation in the cell culture. The presence of Sp1 binding sites is important for the protective effect of IE, but at least some part of the entire anti-silencing capacity is maintained in IE with mutated Sp1 sites. We have described more relaxed integration patterns of long-term active proviruses of the modified vectors and weaker dependence on the vicinity to transcription start sites, CpG islands, and H3K4me3-enriched regions. The modified active proviruses accumulate particularly in enhancers and gene bodies (Šenigl et al., 2012; unpublished data). We suggest that this strategy of anti-silencing protection by the CpG island core element may prove generally useful in retroviral vectors.

2. 4. Receptors for retroviruses and the host cell permissiveness

A considerable part of our efforts was devoted to understanding the interplay between receptors for retroviruses and retroviral surface glycoproteins. The avian sarcoma and leukosis virus (ASLV) family of retroviruses contains five highly related envelope subgroups (A to E) thought to have evolved from a common viral ancestor in the chicken population. Three genetic loci in chickens determine the susceptibility of cells to infection by the subgroup A to E, and genetic defects in these loci were found in chicken lines resistant to ASLVs. Thus, Darwinian co-evolution of virus and host result in the broad scale of virus subgroups and receptor variants. In addition to classical resistance and susceptibility, in inbred lines of chickens we previously observed phenotypes that are somewhere in between either efficiently susceptible or resistant to infection by specific subgroups of ASLV. In the case of decreased susceptibility of the chicken P line to the A subgroup ASLV, we characterized a molecular defect in the *tva* receptor gene. We found two alleles of the *tva* receptor gene with similar intronic deletions comprising the deduced branch-point signal within the first intron and leading to inefficient splicing of *tva* mRNA. As a result, we observed decreased susceptibility to subgroup A ASLV *in vitro* and *in vivo*. These alleles were independently found in a close-bred line of domestic chicken and Indian red jungle fowl (*Gallus gallus murghi*), suggesting that their prevalence might be much wider in outbred chicken breeds. We identified defective splicing to be a mechanism of resistance to ASLV and conclude that such a type of mutation could play an important role in virus-host coevolution (Reinišová et al., 2012).

Our experience with receptors for ASLVs paid off in further studies of subgroup J avian leukosis virus (ALV-J). This virus is unique among the avian sarcoma and leukosis viruses in using the multimembrane-spanning cell surface protein Na(+)/H(+) exchanger type 1 (NHE1) as a receptor. The precise localization of amino acids critical for NHE1 receptor activity is key in understanding the virus-receptor interaction and potential interference with virus entry. Because no resistant chicken lines have been described so far, we compared the NHE1 amino acid sequences from permissive and resistant galliform species. In all resistant species, the deletion or substitution of W38 within the first extracellular loop was observed either alone or in the presence of other incidental amino acid changes. Using the ectopic expression of wild-type or mutated chicken NHE1 in resistant cells and infection with a reporter recombinant retrovirus of subgroup J specificity, we studied the effect of individual mutations on the NHE1 receptor capacity. We suggest that the absence of W38 abrogates binding of the subgroup J envelope glycoprotein to ALV-J-resistant cells. Altogether, we describe the functional importance of W38 for virus entry and conclude that natural polymorphisms in NHE1 can be a source of host resistance to ALV-J (Kučerová et al., 2013).

One of the important issues in retrovirus heterotransmission is related to cellular factors that prevent virus replication. Rous sarcoma virus (RSV), a member of the avian sarcoma and leukosis family of retroviruses, is able to infect and transform mammalian cells; however, such transformed cells do not produce infectious virus particles. Using the well-defined model of RSV-transformed rodent cells, we established that the lack of virus replication is due to the absence of chicken factor(s), which can be supplemented by cell fusion. Cell fusion with permissive chicken cells led to an increase in RNA splicing and nuclear export of specific viral mRNAs, as well as synthesis of respective viral proteins and production of virus-like particles. RSV rescue by cell fusion can be potentiated by *in trans* expression of viral genes in chicken cells. We conclude that rodent cells lack some chicken factor(s) required for proper viral RNA processing and viral protein synthesis.

2. 5. Endogenous retroviruses

Endogenous retroviruses, which constitute a great portion of vertebrate genomes, have traditionally been our scientific concern. In the last ten years, we reported that DNA methylation regulates transcription of endogenous retroviruses and retrotransposons L1 mobilize endogenous retroviruses of W family into polyA-containing processed pseudogenes. During the evaluated period, we continued our studies on syncytin-1 and -2, human fusogenic glycoproteins encoded by the env genes of endogenous retroviral loci ERVWE1 and ERVFRDE1, respectively, which contribute to the differentiation of multinucleated syncytiotrophoblast in chorionic villi. In non-trophoblastic cells, however, the expression of syncytins has to be suppressed to avoid potential pathogenic effects. We studied the epigenetic suppression of ERVWE1 and ERVFRDE1 5'-long terminal repeats by DNA methylation and chromatin modifications. Immunoprecipitation of the provirus-associated chromatin revealed H3K9 trimethylation at transcriptionally inactivated syncytins in HeLa cells. qRT-PCR analysis of non-spliced ERVWE1 and ERVFRDE1 mRNAs and respective env mRNAs detected efficient splicing of endogenously expressed RNAs in trophoblastic but not in non-placental cells. Pointing to the pathogenic potential of aberrantly expressed syncytin-1, we have found deregulation of transcription and splicing of ERVWE1 in biopsies of testicular seminomas. Finally, ectopic expression experiments suggest the importance of proper chromatin context for the ERVWE1 splicing (Trejbalová et al., 2011). Our results thus demonstrate that cell-specific retroviral splicing represents an additional epigenetic level controlling the expression of endogenous retroviruses.

Another type of endogenous retroviruses, porcine endogenous retroviruses (PERV), represent a major safety concern in pig-to-human xenotransplantation. To date, no PERV infection of a xenograft recipient has been recorded; however, PERVs are transmissible to human cells *in vitro*. Some recombinants of the A and C PERV subgroups are particularly efficient in infection and replication in human cells. Transcription of PERVs has been described in most pig cells, but their sequence and insertion polymorphism in the pig genome impede identification of transcriptionally active or silenced proviral copies. Furthermore, little is known about the epigenetic regulation of PERV transcription. Here, we report on the transcriptional suppression of PERV by DNA methylation *in vitro* and describe heavy methylation in the majority of PERV 5' long terminal repeats (LTR) in porcine tissues. In contrast, we have detected sparsely methylated or nonmethylated proviruses in the porcine PK15 cells, which express human cell-tropic PERVs. We also demonstrate the resistance of PERV DNA methylation to inhibitors of methylation and deacetylation. Finally, we show that the high permissiveness of various human cell lines to PERV infection coincides with the inability to efficiently silence the PERV proviruses by 5'LTR methylation. In conclusion, we suggest that DNA methylation is involved in PERV regulation,

and that only a minor fraction of proviruses are responsible for the PERV RNA expression and porcine cell infectivity (Matoušková et al., 2013).

In a broader international team, we studied the safety concerns of xenotransplantation. Acellular materials of xenogenic origin are used worldwide as xenografts, and phase I trials of viable pig pancreatic islets are currently being performed. However, limited information is available on transmission of porcine endogenous retrovirus (PERV) after xenotransplantation and on the long-term immune response of recipients to xenoantigens. In Prague, there is a unique group of burn patients who had received living pig-skin dressings for up to eight weeks. We analysed the blood of these patients for the presence of PERV as well as for the level and nature of their long term (maximum, 34 y) immune response against pig Ags. Although no evidence of PERV genomic material or anti-PERV Ab response was found, we observed a moderate increase in anti-αGal Abs and a high and sustained anti-non-αGal IgG response in those patients. Abs against the nonhuman sialic acid Neu5Gc constituted the anti-non-αGal response with the recognition pattern on a sialoglycan array differing from that of burn patients treated without pig skin. These data suggest that anti-Neu5Gc Abs represent a barrier for long-term acceptance of porcine xenografts. Because anti-Neu5Gc Abs can promote chronic inflammation, the long-term safety of living and acellular pig tissue implants in recipients warrants further evaluation (Scobie et al., 2013).

We also paid attention to the genomic aspects of endogenous retroviruses, which led to identification of a new type of lentiviral elements. In contrast to other retroviral genera, lentiviruses only rarely form ERV copies. Using the *in silico* strategy, we screened 104 publicly available vertebrate genomes for the presence of endogenous lentivirus sequences. In addition to the previously described cases, the search revealed the presence of endogenous lentivirus in the genome of Malayan colugo (*Galeopterus variegatus*). At least three complete copies of this virus, denoted ELVgv, were detected in the colugo genome, and approximately one hundred solo LTR sequences. The assembled consensus sequence of ELVgv had typical lentivirus genome organization including three predicted accessory genes. Phylogenetic analysis placed this virus as a distinct subgroup within the lentivirus genus. The time of insertion into the dermopteran lineage was estimated to be more than thirteen million years ago. We report the discovery of the first endogenous lentivirus in the mammalian order *Dermoptera*, which is a taxon close to the *Primates*. Lentiviruses have infiltrated the mammalian germline several times across millions of years. The colugo virus described here possibly represents the oldest documented endogenization event and its discovery can lead to new insights into lentivirus evolution. This is also the first report of an endogenous lentivirus in an Asian mammal, indicating a long-term presence of this retrovirus family in the Asian continent (Hron et al., 2014).

2. 6. Inbred lines of chicken as a research model

Most of the research done in our laboratory was based on the chicken model established at our institute in the 1950s and maintained here for almost 60 years. The inbred state of these chicken lines has proved to be crucial for basic discoveries in retrovirology and still provides an important advantage for receptor studies and many other aspects of our research. One of the first chicken inbred lines RH-C was established in 1932 in Reaseheath, England. After its arrival to Prague in 1958, the breeding programme has been conducted with the aim to establish congenic lines for immunogenetic studies. Historically, our system of chicken inbred lines contributed to several topics, mainly immunology, virology and viral disease resistance. The availability of MHC(B) congenic lines prompted a systematic search for genetic

recombinants resulting in a three-locus model – with B-F (class I), B-L (class II) and B-G (class IV), which was the first approximation of the MHC(B) structure. Further extensive studies revealed the detailed molecular structure of chicken MHC(B) genes on the basis of this framework. Many experimental examples of the association of particular MHC(B) haplotypes with resistance to tumour diseases induced by infectious viruses have been described, including different strains of Rous sarcoma virus (RSV) and Marek's disease virus. It turned out that only a single dominantly expressed class I gene is expressed in many chicken MHC(B) haplotypes. This contrasts with a typical mammalian MHC, which expresses several class I genes, thus extending the spectrum of recognizable (viral) antigens. This would explain the frequent directly observable MHC(B) – disease associations in the chicken and relative paucity of experimental examples of MHC-associated disease resistance in mammals. The model system of Prague congenic chicken lines CB and CC challenged with RSV or its oncogene v-*src* alone in the form of plasmid DNA could serve as an example. The v-*src* C-tail peptide517-524 (LPACVLEV) contains critical anchor amino acids (valine at positions 5 and 8) important for binding to the dominantly expressed class I (B-F12) molecule present in the MHC(B) haplotype of inbred line CB. A protective effect of the T cell-mediated immune response to LPACVLEV against RSV challenge was demonstrated in CB chickens immunized with peptides encapsulated in liposomes. The MHC(B) congenic chicken lines CB and CC (regressors and progressors of v-*src*-induced tumours, respectively) were also vaccinated with mutated, non-oncogenic v-*src* gene construct DNA. A high degree of vaccine protection against oncogenic v-*src* challenge was demonstrated in the CB chickens. Similarly, vaccination against infectious bursal disease virus (IBDV) protects some experimental chicken lines but not others, depending on the presence of particular MHC class I molecules that are able to bind IBDV-derived peptides.
During the evaluated period, the inbred lines of chickens were indispensable for our studies on retroviral receptors (Reinišová et al., 2012; Kučerová et al., 2013), provirus silencing (Plachý et al., 2010; Šenigl et al., 2012), cell permissiveness (Lounková et al 2014), and metastasis of v-*src*-induced tumours (Čermák et al., 2010; Kovářová et al., 2013). In addition to these topics, we contributed to several collaborative studies on transgenesis in the chicken (Trefil et al., 2010; Mucksová et al., 2013), Chagas disease (Teixeira et al., 2011; Guimaro et al., 2014), and evolution of sex chromosomes (Mořkovský et al., 2010).

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Research Report of the team in the period 2010–2014

Institute	Institute of Molecular Genetics of the CAS, v. v. i.

Scientific team	Laboratory of Immunobiology

An overarching theme of our research are cellular, molecular and signaling processes underpinning immune homeostasis. That mainly concerns (i) the mechanisms guiding the process of central and peripheral T cell tolerance and autoimmunity; (ii) initiation of T cell activation; and (iii) embryonic hematopoiesis.

Brief history of the lab. Our lab started in 2007 when I moved from my long-term postdoctoral study in Toronto to Prague and decided to continue study on molecular and signaling processes underlying T cell proximal signaling. Also, right from the beginning, based on several promising experimental observations, we have initiated two additional brand-new projects in the field of embryonic hematopoiesis and diabetes and autoimmunity. Approximately in 2011, our focus has gradually shifted from the topic of diabetes/autoimmunity to the field of central and especially peripheral immunological tolerance.

Publishing activity. After the initial period, when we built the team and lab infrastructure, in following years (2010-2015) we have significantly increased our publication activity in all three area of interest. As documented in the section **2.5. List of publications**, we have published 15 papers in international journals (with IF up to 13.97), 1 book chapter and one article in Czech national popularization magazine „Vesmir“ („Universe“). Three additional manuscripts are currently in a review/revision proces, see below).

Methodological approaches and equipment. At present time, the laboratory is well equipped for immunological work on molecular, cellular and organismal levels. We routinely use confocal and super-resolution microscopy, state of the art FACS and cell-sorters, cytofluorometers, qRT-PCR and other advance technologies. If needed, support is also provided by an experienced bioinformatics groups, microarray platform (Illumina and Affimetrix) and deep-sequencing technical support teams. For testing and verification of our new hypotheses and concepts, we routinely use various genetically modified (GM) mouse models. This approach has became a pinnacle of our experimental work. For this purpose, in the last few years, we have introduced or are currently introducing several state-of-art genome editing tools and protocols such as BACs, TALENs and CRISPRS, to generate transgenic and KO animals. Currently, we also possess many valuable mouse GM strains which allow us to delineate precise molecular and cellular mechanisms underpinning processes related to the main goal of our research.

Lab's personel. The head of the Department, Dominik Filipp, is also a senior lecturer in the graduate course *Innate immunity* and *Advances in Immunology* at the Department of Cell biology, Charles University, Prague and Comenius University in Bratislava, Slovakia. His group regularly publishes papers

in internationally well recognized immunological and biochemical journals. In addition, well-trained and experienced young scientists and students participate in the above indicated project: one postdoc (Balounova), 4 PhD students (Ballek, Dobes, Splichalova, Voboril), one research associate (Dobesova**)**, one graduate (Fellnerova) and two undergaduate students (Sukenikova, Brabec). It is important to mentioned that the research team is stabilized and we don't expect any major personal changes in the next one or two years.

Grant support. Currently, we are using several grants which financially support our research activities (two from GA ČR, one from TA ČR, three from GAUK, and one AVČR-DAAD travel grant for a bilateral collaboration with our German partner lab of Prof. Klein). Their biggest disadvantage is that they are rather of limited amount of money, and thus several of them is required to fully support our research. I consider the financial support as the weakest aspect of our research activities which must be fixed in the very near future. Towards this, we have already applied, and throuthout this year we will submit several grant proposals to secure the funding for years to come.

Collaboration with laboratories abroad. We have several very efficient collaborations with leading scientist all around the world: mainly with Prof. Ludger Klein, LMU Munich, Germany, with who we have regular phone/skype consultations, who's lab also participates in a short term student Exchange program between our laboratories and who has provided us with several mouse models originally generated in his lab to study processes underpinning immune central tolerance. Last year, Prof. Klein's and Filipp's labs were awarded a bilateral travel grant from German DAAD and Czech AVCR agencies for "Program of Project Based Personnel Exchange" for 2015-2016, to cover travel expenses associated with our collaborative effort in the field of immune peripheral tolerance. This will further strengthen our already established ties and prepare us for the rapid initiation of the proposed project. It is also of note that Jan Dobeš was in 2013 awarded with the travel grant from European Federation of Immunological Societes (EFIS) and spent more than 3 months in Prof. Klein's lab. In addition we have very informal working relationship with laboratories of Prof. P. Poussier and J.C. Zuniga-Pflucker (University of Toronto, Canada), Prof. Eystein Husebye, University of Bergen, Norway, Prof. Annamari Ranki and Prof. Kai Krohn, University of Helsinki, Finland, and Dr. Jakub Abramson, Weizmann Institute, Israel. All of them work in the field of autoimmunity and tolerance and often provide us with valuable advices, consultations and reagents. We also benefit from invaluable contacts and discussions with colleagues from a local Krc's campus and with laboratories from our institute.

2.2. Topic #1: Autoimmunity and immunological tolerance

The major focus of our research between 2010 and 2014 was to elucidate the mechanism guiding the process of (i) autoimmunity in diabetes and (ii) of central and peripheral tolerance.

2.2.1. Autoimmunity in Type 1 diabetes. Type 1 diabetes (T1D) is an autoimmune disease characterized by progressive destruction of insulin-producing pancreatic β-cells, leading to dependency on exogenous insulin. T cells are central to the mechanism of β-cell destructions. On the other hand, components of innate immune system have been considered only as collateral factors in the process of autoimmunity. This scenario is likely incorrect as infectious agents assumed to be involved in the initiation of T1D are primarily sensed by innate immune mechanisms. Thus, in collaboration with the clinical lab in the Motol

Hospital and 2nd Medical faculty of the Charles University, we set out to characterize the nature of innate immune mechanisms that conspire with adaptive ones to initiate the break of tolerance. Our effort have materialized in several publications in international journals focused on diabetes and clinical imunology (***section 2.5., publications #2, 3, 4, 7 and 8***).

2.2.2. Central and peripheral tolerance. In the last couple of years, our research refocused even more on the contribution of cellular and molecular factors controlling the process of central and chiefly peripheral tolerance (***section 2.5., publication #9***). We have shown that the physiological role of enteric α-defensin production in the thymus is critical for the maintenance of central tolerance in the small intestine. These molecules, expressed by Paneth cells in the crypts of small intestine, are also expressed by a sizable fraction of medullary thymic epithelial cells (mTECs) where their expression is dependent on the Autoimmune regulator (AIRE) transcription factor. The immunological consequences of defective enteric α-defensin expression in the thymus were confirmed by the presence of anti-HD5 autoantibodies in the sera of APECED patients who are deficient in AIRE function. Moreover, our new mouse model of APECED demonstrated that self-reactive enteric α-defensin-recognizing T cells alone are sufficient to drive the process of initiation of Paneth cell destruction, leading to intestinal microbiome dysregulation and enhanced Th17 responses which further amplify inflammatory autoimmunity in the intestine. The manuscript describing this work has been already accepted for publication in a prestigious journal ***Gastroenterology*** (***section 2.5., publication #14***). We also participated in research related to the Wnt signaling in the small intestine in health and cancer (***section 2.5., publication #15, 16***).

In addition, in close collaboration with Prof. Klein, we have characterized a functionally distinct lymph node cell population with capacity to delete self-reactive $CD8^+$ and $CD4^+$ T cells or mediate the conversion of the latter into Tregs (*manuscript in preparation*).

2.3. Topic #2: Lck, lipid rafts and TCR proximal signalling. We also continue in our effort to understand the earliest biochemical events leading to the activation of T cells. This mainly concerns the processes associated with the regulation of the proximal T cell signaling where two Src-family tyrosine kinases (SFK), Lck and Fyn, provide critical functions. Towards this end we have identified important parameters of the initiation of TCR signaling, subcellular compartments where preactivated pool of Lck resides and also critically re-evaluated the current, so called Lck-stand-by model of TCR signaling (***section 2.5., publications 5, 6, 13 and 17***). In addition, we have identified several candidate proteins involved in regulation of translocation of Lck to lipid rafts via linking this process to microtubular cytoskeletal network (The *manuscript was originally submitted to* ***Nature communication*** *where it underwent a full review process and is currently in revision*).

2.4. Topic #3: Toll-like receptors and embryonic hematopoiesis. For historical reasons and deeply entrenched curiosity, we are also very interested in the macrophage expression pattern and function of Toll-like receptors (TLRs) and other immune-related proteins during the early mammalian embryogenesis (***section 2.5., publications 10 and 11***). We have shown that TLRs expressed on embryonic macrophages couple inflammatory signals to iron metabolism during early ontogenesis. In addition, Toll-like receptor 2 (TLR2) seems to be a suitable surface marker which allows tracking the earliest haematopoietic progenitors in a precirculation embryo. Our newly generated transgenic mice which enable us to perform genetic lineage tracing experiments provided evidence that these early TLR2

expressing progenitors contribute not only to primitive but also definitive hematopoiesis (*the manuscript was originally submitted to **Cell Stem Cell**, where it underwent a full review process, and is currently in revision*).

We also extended the scope of our interest in innate immunity. In collaboration with Prof. Ferriere, Ecole Nationale Supérieure de Chimie de Rennes, Rennes, France and Ing. Chlubnova, his former Czech PhD student, we have, in 2010, initiated studies related to the immunomodulatory properties of newly synthetized oligofuranosides. These unusual sugar moieties, naturally occuring on the surface of intracellular pathogens, such as Leischmania or mycobacteria, display potent immunostimulatory activities (***section 2.5., publication #1, 12***). Our aim is to identify elements of oligofuranoside signaling, their role in induction of cytokine production and/or maturation of antigen presenting cells, and, most importantly, determine the specificity or redundancy of receptors in oligofuranoside signaling (the manuscript describing novel immunomodulatory activities of some of these oligofuranosides has been recently submitted).

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7. Stechova, K., Kolar, M. and **Filipp D**. ***2013.*** Lessons from the gene expression studies of immunocompetent cells in relationship to type 1 diabetes development. ***Chapter in the book: GeneExpression: New Research. Publisher: Nova Science***
8. J, Blatny R, Halbhuber Z, Kolar M, **Neuwirth A**, Petruzelkova L, Ulmannova T, Kolouskova S, Sumnik Z, Pithova P, Krivjanska M, **Filipp D**, Stechova K. The Effect of Diabetes-Associated Autoantigens on Cell Processes in Human PBMCs and Their Relevance to Autoimmune Diabetes Development. ***J Diabetes Res. 2013: 589451***.
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Research Report of the team in the period 2010–2014

Institute	Institute of Molecular Genetics of the CAS, v. v. i.

Scientific team	Laboratory of Molecular Pharmacology

2.1. Introduction

In the Laboratory of Molecular Pharmacology we are studying principles of signalling via G-protein Coupled Receptors for major neurotransmitters: Glutamate (Glu), Gamma-aminobutyric acid (GABA) and Cannabinoids, namely via Cannabinoid Receptor 1 (CB1R). We focus on description of the activation mechanism of Metabotropic Glutamate receptors (1-10), and GABAb receptors that belong to family C GPCRs (10-15). Receptors that fall into family C are known to be expressed on the cell surface in dimeric form. In our studies we documented that the dimeric structure of these receptors is crucial for their activation. Moreover, we brought evidence that the activation mechanism of these receptors is asymmetrical in respect of the subunit composition. This means that in active state, only one of the two transmembrane heptahelical domains (HD) reaches the active state that is further transmitted on the G proteins. Next, we studied the subunit composition of the mGluR1 dimers. We found that mGluR1 can assemble from two distinct splice variants. We also participated in a study of ILRAPL protein and its involvement in glutamatergic signalling that is directly relevant to pathophysiology. Other collaborations are more of technical aspects from our side and are oriented on the description of transcriptional regulations of various nuclear receptors (16, 17).

2.2. Signalling via Metabotropic Glutamate Receptors

Heterotrimeric guanine nucleotide-binding protein (G protein)-coupled receptors (GPCRs) are major players in cellular communication and comprise receptors for various stimuli, such as light, taste, and many small molecule neurotransmitters, as well as small peptide and large protein hormones. Many GPCRs act as monomers; notably, the light receptor rhodopsin, as well as the 2-adrenergic and -opiate receptors, can fully activate their effector G proteins in monomeric form. However, many GPCRs can also exist and function as dimers. Evidence for multimeric GPCRs is based on findings of physical association in recombinant, as well as native, systems and on functional data indicating interactions between two or more GPCRs. Some family C GPCRs, such as GABAB and mGluRs, form constitutive dimers and represent opportune models to decipher conformational changes in a GPCR dimer. Each subunit of a family C GPCR dimer is composed of a large N-terminal Venus flytrap domain (VFT) containing the agonist binding site, followed by a cysteine-rich domain, and the HD. For mGluRs, mutational analyses, as well as structural and modelling data, suggested that agonist binding to the VFT induces a relative movement between the two subunits, which may be accompanied by a conformational change in the HD, leading to G protein activation. In the case of the GABAB receptor, one subunit (GABAB1) mediates ligand binding, and the other subunit (GABAB2) couples to G proteins. Only the heterodimer comprising both GABAB1 and GABAB2 (GABAB1/2) triggers full transmembrane

signalling. The distinct functional deficits of each of the two subunits may explain why some family C GPCRs form obligatory dimers, but they also raise a number of important issues concerning their mechanisms of activation. It is unclear whether activation involves a conformational change in the HDs or whether G protein activation results only from the movement of the subunits relative to each other. If a conformational change in the HD is relevant for receptor activation, it is unknown whether this conformational change occurs on the same time scale (~50 ms) as in the case of family A GPCRs, for which agonists bind directly to the HD. It is also unclear whether conformational changes occur in the HDs in both subunits of the dimer and, if so, whether both conformational changes are necessary for G protein activation. In earlier studies, we provided evidence for G protein coupling after conformational change in a single HD in the mGluR dimer. Next, we directly examined the conformational changes in the HD and investigated how the kinetics of such conformational changes relates to the kinetics of other processes involved in family C GPCR activation.

Fluorescence resonance energy transfer (FRET) technologies are useful in studying conformational changes of proteins and protein-protein interactions in living cells. Fusion of fluorophores, such as cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP), or the small yellow label fluorescein arsenical hairpin binder (FlAsH) at different positions in the intracellular loops and C-terminal tail of a receptor allowed for measurement of FRET between different regions of the protein (intramolecular conformational changes). FRET measurements enabled determination of the kinetic parameters of activation for the receptors studied. Activation time constants ranged from ~40 to 100 ms for the a2A-, b1-, and b2-adrenergic receptors; adenosine A2A receptor; and various muscarinic receptors to ~1 s for the family B parathyroid hormone receptor. Tagging GPCRs and downstream signalling proteins with fluorescent proteins allowed temporal and spatial resolution of the signalling patterns of several GPCRs. FRET techniques have been used to study intermolecular events in dimeric GPCRs by placing a single fluorophore in each subunit of a GPCR dimer. For mGluR1a receptors, Kubo's group showed an agonist-dependent increase in FRET between the second intracellular (i2) loops of the HDs and a decrease between the first intracellular loops of the HDs within several seconds after adding agonists. A similar study on mGluR1b receptors (a C-terminally shortened splice variant of mGluR1) reported only small increases in FRET between the i2 loops. However, no intramolecular conformational changes were observed in either of these FRET studies. These studies suggested that, unlike the family A GPCRs in which intramolecular conformational changes in the HD represent the activation mechanism, the dimeric family C GPCRs might be primarily activated by intermolecular movements of the two subunits relative to each other.

These findings led us to investigate and compare inter- versus intramolecular activation-dependent changes in fluorescently labelled mGluR1 receptors. We created sensors for each process by placing CFP and YFP at suitable positions within the mGluR1 receptor subunit and used these to analyse the sequence of events during dimer activation and the crosstalk between the two subunits.

Our studies elucidate the dynamics of inter- and intramolecular conformational changes in the mGluR1 dimer using FRET measurements and an antagonist (AIDA) and exhibited properties consistent with a key role in the importance of the VFT for transmission of agonist binding to the HD.

The availability of both inter- and intramolecular sensors for the mGluR1 dimer allowed us to establish the sequence of events in the activation of this receptor. After the binding of an agonist to the VFT domain of one subunit, the two subunits appear to move closer to each other before there is a change

within the HD. The rate of each step increased with the concentration of the agonist but saturated at high agonist concentrations, suggesting that the maximal kinetics approached the actual rates of the relevant steps. The intersubunit step occurred first with a time constant of ~35 ms, whereas the time constant for the intrasubunit step was ~52 ms. The rate of the intersubunit step was much faster than the rate determined by Tateyama form Kubo's lab, who did not use a fast superfusion device, but it was slower than the rate determined by the group of M. Knopfell. The latter have designed their experiments specifically to be able to monitor rapid effects and observed a rate constant of ~10 ms, but their signal amplitude was only 1 %, which in our hands makes it difficult to obtain reliable signals.

The rates of the intrasubunit changes are in the same range as those measured for many family A GPCRs, including the 2A-, 1-, and 2-adrenergic receptors; the A2A-adenosine receptor; and various muscarinic receptors. Thus, the overall principle of GPCR activation may be similar across different families of GPCRs. The rates of deactivation were similar for the inter- and the intrasubunit sensors and were in the range of 300 ms. This suggests that either the two deactivation processes occurred in a closely linked manner or, under our conditions, the two processes were limited by agonist dissociation and any potential kinetic differences were too small to be detected. Several studies indicate that in many GPCR dimers, only one of the two subunits becomes activated. We have also reported that in the dimeric mGluR1, only one subunit activates G proteins. In line with these data, the agonist-induced signal of the A-sensor was suppressed by the presence of various mGluR1 constructs. However, only mGluR1 constructs that were capable of coupling to G proteins suppressed the A-sensor activation signal, whereas those that were not capable of coupling to G proteins did not.

Our data suggest that the agonist-induced activation of mGluR1 dimers occurs in a sequential coordinated manner, and this may be true of other family C GPCRs. After agonist binding to the VFT domain of one subunit, the two subunits move relative to each other within ~30 ms. This intermolecular conformational change is followed by a conformational change in the HD of one of the two subunits within ~50 ms, which results in G protein activation. The latter time constant is similar to those reported for family A GPCRs. Because family A and family C receptors exhibit similar kinetics of receptor conformational changes, this suggests that differences in their rates of signalling are likely related to differences in the kinetics of the downstream events of recruiting, interacting with, and activating G proteins.

The study was initiated in the Laboratory of Molecular Genetics at IMG, ASCR; complete cDNA recombination of receptor/fluorophore chimeras was performed here, including controls of expression, trafficking and function of the receptor subunits; the pharmacological characterization of key mutants was performed in CNRS, Montpellier during the stay of Veronika Hlavaáčková there. Initial FRET measurements were also done at IMG. Final FRET measurements, including fast detection, was done at the University of Wurzburg by Veronika Hlaváčková, who in the meantime finished Ph.D. studies and obtained a prestigious EMBO fellowship that we applied for, to conclude the study in Wurzburg.

2.2. Molecular structure of Metabotropic Glutamate Receptor 1

Alternative splicing governs the properties of many synthesized proteins, including several neurotransmitter receptors, both metabotropic and ionotropic. RNA splicing is tightly regulated spatially and in time, and the distinct protein variants often have unique and functionally relevant characteristics.

Alternative splicing of GRM1 gene transcripts results in the expression of at least three mGluR1 variants (a,b,d) in rodents; however, the expression of variants other than mGluR1a and mGluR1b in the human brain is uncertain. The mGluR1 variants have identical VFTs and HDs and short common adjacent sequences including a basic RRKK motif following the transmembrane region, but differ in their following intracellular C-termini. The long variant, mGluR1a, is characterized by a unique sequence of 313 amino acids while the short variant, mGluR1b, has only 20 specific residues. For mGluR1b targeting, the RRKK motif is responsible for endoplasmic reticulum retention when this subunit is expressed alone. For mGluR1a, an undetermined sequence within the extensive C-terminus masks its own retention signal. Previously, we reported that in transfected HEK293 cells, mGluR1a and mGluR1b readily combine in receptor complexes and the long C-tail of mGluR1a neutralizes the retention effect of not only its own RRKK motif, but also of that from the connected mGluR1b, and thus mGluR1a/b heterodimers are trafficked to the cell membrane.

Targeting of receptors and signalling molecules to the correct cellular compartments within neurons is essential for their specific functions in inter-neuronal communication. Several motifs within the mGluR1a C-termini are required for protein interactions with trafficking partners that are involved in sorting to dendrites and clustering at postsynaptic portions of dendritic spines.

In the literature there is accumulating indirect evidence for possible heterodimerization of the two splice variants. The indications include the overlapping pattern of mGluR1a and mGluR1b distribution in vivo, along with the finding that mGluR1b distribution might be dependent on association with the mGluR1a variant. However, direct evidence in an in vivo system has not yet been shown. Here we found that the incorporation of the mGluR1b subunit into mGluR1a/b dimers in the cerebellum is common, and both variants co-localize in native Purkinje cells. These data incited our interest in understanding targeting of the mGluR1b variant in tandem with mGluR1a in neurons.

Dimerization is a prerequisite for the family C GPCR function. Heterodimerization is well documented and recognized as a receptor formation requirement for GABAb and sweet and umami taste receptors. In the case of mGluRs, the concept (until recently) was that dimeric complexes are assembled uniquely as homodimers. Our initial study indicating otherwise was carried out in heterologous expression systems and revealed formation of receptor complexes containing short and long mGluR1 splice variants. Also, it was shown that distinct mGluRs might combine in receptor complexes in a heterologous system. Several studies by other groups suggest possible dimer formation between Group I mGluRs, mGluR1 and mGluR5, or association between subunits that fall into Groups II and III. Recently, pharmacological evidence of heterodimerization between members of the latter groups was presented. The mGluR2 and mGluR4 in vivo assembly into complexes with novel pharmacological properties was confirmed. In our study, we show new evidence for mGluR heterodimerization, i.e. endogenously expressed mGluR1a and mGluR1b variants readily assemble into dimeric receptor complexes.

The intracellular carboxyl-termini of GPCRs mediate interactions with trafficking, regulatory and scaffold proteins. Due to GRM1 gene alternative splicing these C-tails vary between long (mGluR1a) and short (mGluR1b) variants. Distribution of mGluR1 variants was studied previously in transfected neurons. The authors of the earlier studies used transfected primary neuronal cultures with either mGluR1a or mGluR1b variants, but never in tandem, possibly assuming that only homodimers form functional receptors. In a study that employed retinal neurons, the transfected mGluR1a was observed in cell bodies and in dendrites. The mGluR1b variant expressed alone showed a different distribution

compared to mGluR1a, as it was excluded from dendrites and directed to axons. In other studies, (including ours) the axonal targeting of mGluR1b variant was not observed. This may be due to the use of distinct neuronal cultures. However, in certain neuronal population, notably in striatal neurons, mGluR1a was also described in the presynaptic compartments. Therefore, it might well be that when describing the targeting properties of molecules, the specific pattern in distinct cell types has to be taken into account. The outcomes of a study exploiting mGluR1b transgenic mice in the background of mGluR1 knockout led the authors to speculate that mGluR1b might be associated with mGluR1a in wild type animals, as the distribution of the mGluR1b subunit was altered in the absence of mGluR1a. This possibility was also discussed beforehand by authors of a careful study that revealed an mGluR1b subcellular distribution pattern reminiscent of that of mGluR1a.

In our most recent publication, we present data that demonstrate the association between mGluR1a and mGluR1b in vivo. We also show that endogenously expressed mGluR1a and mGluR1b can form heterodimeric mGluR1a/b complexes. First, we used the SDS soluble fraction from rat brain tissue homogenates for immunoprecipitation. This detergent solubilization disrupts non-covalent protein-protein interactions, but does not disturb covalently linked mGluR1 subunits associated within a single receptor complex. Therefore, only the mGluR1a covalently linked to mGluR1b would co-precipitate in further manipulation of the SDS-detergent soluble fraction. Indeed, we detected not only co-precipitation of mGluR1a with mGluR1a, but also mGluR1b with mGluR1a, and vice versa (mGluR1a with mGluR1b). The use of strong ionic detergent prior to immunoprecipitation also minimizes the possibility of pulling down mGluR1a dimers with mGluR1b dimers. Not only would oligomeric formation of non-covalently interacting complexes be disrupted by our detergent solubilization protocol, but also higher oligomeric formation is unlikely in the case of the mGluRs.

Subcellular fractionation revealed that mGluR1b homodimers are detected in relatively low amounts, so that a rather minor portion of mGluR1b were incorporated into homodimers when compared with the amounts incorporated into mGluR1a/b dimers, and these marginal mGluR1b homodimers are almost excluded from the synaptosomal fraction. The mGluR1a/b heterodimer was the major form of mGluR1b detected, including in synaptic membrane fractions, where it was enriched over the relative amounts found in the microsomal fractions. We propose that mGluR1b homodimers are not trafficked in the same way as mGluR1a/b dimers, but are rather destined for degradation after retention within the ER.

Immuno-histochemical staining using antibodies specific for mGluR1a and mGluR1b also show that the two splice variants have overlapping distributions, especially in Purkinje cells in the cerebella of two species. Moreover, electron microscopy with a double labelling strategy for the first time allowed the side-by-side detection of the two variants in discrete perisynaptic membrane regions of dendritic spines. Thus, suggestions of possible mGluR1 heterodimer formation from previous studies have now been documented using the direct approaches that we employed here.

As our results indicate that mGluR1b distribution overlaps with that of mGluR1a in vivo, next we addressed the question of the assembly of mGluR1a/b dimers in neurons and its consequence on targeting of the heterodimer. When we transfected primary cortical neurons with the mGluR1a subunit, it was readily trafficked to distal dendrites in accordance with the expected distribution. On the other hand, mGluR1b was retained mainly in the soma and only a small portion was found in dendrites. However, when the mGluR1b subunit was co-expressed with mGluR1a, its distribution showed a similar

pattern to that of mGluR1a. Thus, mGluR1b is co-transported to distal dendrites within heterodimeric mGluR1a/b complexes and these heterodimers are most likely formed already in the neuronal soma. We present several lines of evidence for this statement, notably data showing the trafficking of mGluR1b with mGluR1a upon co-expression in transfected primary neurons and their association in subcellular fractions from the cerebellum.

On the one hand, mGluR1a/b heterodimerization might be of functional importance since two distinct intracellularly oriented C-termini combine in one receptor complex. On the other hand, in the mGluR1a/b dimer, there will be a numerical loss of motifs that confer such important interactions as those seen with Homer proteins or Tamalin, compared to the mGluR1a homodimer. The mGluR1b C-tail was also reported to have distinct interacting partners and the mGluR1a/b heterodimer is thus expected to have some unique functional properties, but their relevance within the heterodimers described herein remains to be clarified.

Our results show that mGluR1a heterodimerization with mGluR1b has implications to trafficking. The mGluR1a variant within the mGluR1a/b receptors has a dominant effect on trafficking of these novel complexes.

The study was performed mainly in the Laboratory of Molecular Genetics at IMG, ASCR, except for electron microscopy study that was performed at NIDCD/NIH, Bethesda MD, USA and tissue analysis in CNRS in Montpellier, France.

2.3. Molecular composition of Metaboropic Glutamate Receptor signalosome

Interleukin-1 receptor accessory protein-like 1 (IL1RAPL1) gene mutations are associated with cognitive impairment ranging from nonsyndromic X-linked mental retardation to autism. IL1RAPL1 belongs to a novel family of Toll/IL-1 receptors, whose expression in the brain is upregulated by neuronal activity. Very little is known about the function of this protein. In this study, we showed in collaboration with several other teams that IL1RAPL1 is present in dendritic spines where it interacts with PSD-95, a major component of excitatory postsynaptic compartment. PSD-95 was shown to interact with both ionotropic (NMDA) and metabotropic glutamate receptors. Interaction of PSD95 with guanylate-kinase-associated protein connects Shank protein with long forms of Homer proteins that associate with mGluR1a and mGluR5.

Using gain- and loss-of-function experiments in neurons, it was demonstrated that IL1RAPL1 regulates the synaptic localization of PSD-95 by controlling c-Jun terminal kinase (JNK) activity and PSD-95 phosphorylation. Mice carrying a null mutation of the mouse Il1rapl1 gene show reduction of both dendritic spine density and excitatory synapses in the CA1 region of the hippocampus. These structural abnormalities are associated with specific deficits in hippocampal long-term synaptic plasticity. The interaction of IL1RAPL1 with PSD-95 discloses a novel pathophysiological mechanism of cognitive impairment associated with alterations of the JNK pathway leading to mislocalization of PSD-95 and abnormal synaptic organization and function. In conclusion, the finding of PSD-95 as a novel partner of IL1RAPL1 and the implication of the JNK/PSD-95 pathway in the IL1RAPL1 KO phenotype disclose a novel pathophysiological mechanism for mental retardation associated with IL1RAPL1 mutations.

The major part of the study was performed in the partners' laboratories; mainly in the CNR Neuroscience Institute and Department of Pharmacology, University of Milan, Italy, but also in the INSERM and CNRS, France, University of Turin, Italy, Institut des Neurosciences Cellulaires et Integratives, Dulbecco Telethon Institute, 20129 Milano, Italy, Institut de Genetique et de Biologie Moleculaire et Cellulaire-Institut de la Souris, Illkirch cedex, France, Neuromuscular Diseases and Neuroimmunology, Neurological Institute Foundation ''Carlo Besta'', Milan, Italy. We prepared and characterized novel antibodies not available commercially that were crucial for the study and did use them in protein chemistry experiments.

2.4. Protein SGIP1 modulates Cannabinoid Receptor 1 signalling

Presynaptically localized Cannabinoid receptor 1 (CB1R) is a seven transmembrane domain receptor coupled to Gi/Go-proteins and also to some other signalling pathways such as extracellular signal-regulated kinases1 and 2 (ERK1/2). CB1R is activated by endogenous cannabinoids (e.g. anandamide or 2-arachidonylglycerol) synthesized post-synaptically and also by a range of non-endogenous compounds including the *L,* (-)-trans-9-tetrahydrocannabinol (THC), the psychoactive compound from the plant *Cannabis sativa*. CB1R activation leads to attenuation of either inhibitory or excitatory synaptic transmission in various central nervous system (CNS) structures including the hypothalamus, the hippocampus, the nucleus accumbens, the prefrontal cortex, and the cerebellum. Molecular interactions of the endocannabinoid (eCB) system should be understood in detail for therapeutic implementation of this neurotransmitter system. Possible pharmacological targets include treatment of various ailments such as energy imbalance disorders and obesity, drug addiction, pain, emesis, insomnia, eating disorders (fasting) and psychiatric conditions.

Intracellular C-termini of GPCRs, including that of CB1R, play an important role in desensitization and internalization of the receptors, processes related to fast development of tolerance to drugs acting at CB1R. CB1R internalization via clathrin-coated pits is fast and massive when CB1R is expressed *in vitro* in heterologous systems. However, internalization of the CB1R located in pre-synaptic segments of neurons are resistant to internalization, while CB1R on cell bodies do internalize much more readily upon activation.

In order to deeply investigate the role of intracellular proteins affecting CB1R signalling we employed yeast-two hybrid system (Y2H) to identify proteins that interact with the C-terminus of CB1R. We detected the Src homology 3-domain growth factor receptor-bound 2-like (endophilin) interacting protein 1 (SGIP1) as a potential novel CB1R associated molecule.

Whilst there is little published data regarding SGIP1, the information available is stimulating. SGIP1 is highly conserved across species. Originally, it was identified as a protein with elevated levels of expression in the brains of obese Israeli Sand Rat (*Psammomys obesus*). Of interest, mutations in the gene coding for SGIP1 were associated with energy balance disturbances also in humans. SGIP1 interacts with phospholipids, Eps15 and intersectin 1 – proteins involved in clathrin-mediated endocytosis.

We analysed the impact on CB1R signalling imposed by SGIP1. We depict SGIP1 as a novel intrinsic molecule that modifies CB1R signalling in a pathway-specific manner and that SGIP1 acts as a signalling biased regulator of G protein-coupled receptors (GPCR).

SGIP1 is expressed almost exclusively in the central nervous system (CNS). Using fluorescence microscopy, we studied the distribution of SGIP1 in primary neurons derived from the prefrontal cortex and its co-localization with the CB1R. Using cellular compartment specific markers MAP2, Tau or Bassoon we describe strong co-localization of SGIP1 with the axonal (Tau) and pre-synaptic (Bassoon) markers and only poor co-localization with the dendritic marker MAP2. Our interpretations of SGIP1 targeting to axons, including strong pre-synaptical staining, correlates with that of CB1R. We confirm the co-localization of CB1R and SGIP1 using labelling of both proteins in these structures. Recently, SGIP1 was identified as one of major pre-synaptic proteins, which is in agreement with our findings.

We used the commonly employed CB1R agonist WIN55,212-2. We show that SGIP1 mediates cell surface stability of the activated CB1R in transfected HEK 293 cells. This, together with the overlapping distribution in pre-synaptic portions of neurons of the two molecules *in vivo*, might clarify discrepancies between observations from studies describing massive CB1R internalization in heterologous systems and its lack *in vivo* in pre-synaptic regions. From our results, it is apparent that SGIP1 stabilizes the activated receptor on the cell surface and prevents its clathrin-coated pit-mediated internalization by a so far unknown mechanism. SGIP1 endorses stability of the receptor on the cell surface, and thus next we asked what is the effect of SGIP1 on CB1R signalling.

Our data show that SGIP1 does not significantly modify the G protein pathway. We measured direct activation of Gαi1 and Gαo proteins as a change in BRET efficiency between alpha and gamma subunits, and in dose response measurements using G□qi fusion protein and consecutive Ca^{++} mobility assay. Hence, there was no significant difference in the acute activation of these G proteins by the CB1R receptor in the presence or absence of SGIP1. Moreover, there was no difference in assays with the different tested G-alpha proteins. This likely means that desensitization of the receptor, which precedes the internalization, influences the Gi/o-protein coupling profile with the activated CB1R. The G protein coupling is therefore likely to be regulated predominantly by G protein receptor-coupled kinases (GRKs) and other enzymes and not by internalization.

While CB1R-mediated Gi/o-protein signalling is only marginally influenced by SGIP1, the ERK1/2 signalling is altered profoundly. The ERK1/2 activation by CB1R upon WIN application was enhanced and lasted for longer time in the presence of SGIP1. For ERK1/2 activation with or without SGIP1, the G protein-elicited signlaling was required, as PTX treatment did abolish the ERK1/2 pathway in both situations. How can SGIP1 exhibit such profound switch in ERK1/2 signalling while leaving the G protein coupling basically unaffected? SGIP1 causes enhanced and long-lasting association of the activated CB1R with β-arrestin 2. There are several rationalizations for the enhanced stability of the CB1R-arrestin complex elicited by the receptor activation in the presence of SGIP1. A possible explanation might be in lack of sequestration of the CB1R-β-arrestin 2 complex that would occur upon receptor internalization and post-endocytic processing, which is abolished by SGIP1.

Biased signalling was recognized for GPCR ligands that are likely to promote or stabilize specific conformations of transmembrane heptahelical domains. Distinct G proteins or other pathways including ERK 1/2 signalling may be activated preferentially by biased agonists. Novel molecules that adjust GPCR signalling by a mechanism different from the ligand-dependent stabilization of specific conformation have been described. Such molecules change signalling elicited by a given ligand selectively to particular pathways. One group includes artificial peptides derived from sequences of the intracellular loops connecting transmembrane alpha-helices, other molecules act via interactions within intracellular

regions of the heptahelical domains, away from the ligand-binding sites. The latter are single-domain antibodies (nanobodies) that are directed against intracellular regions of the receptor and stabilize it in its active or inactive states. Intrinsic modulator of CB1R signalling pregnenolone, acting as a ligand-independent bias signalling adaptor, was shown to act as an ERK-pathway specific inhibitor leaving CB1R G protein coupling unmodified. Recent discoveries in CB1R pharmacology might bring lost momentum in search of clinical exploration of this system. Could side effects seen with Rimobamant be minimized if pathway specific antagonist or partial agonist of CB1R were used instead? Results of the current studies might point to a possibility of revealing which signalling pathway activated by CB1R is involved in energy balance regulation and whether the pathway(s) can be pharmacologically modulated separately apart from those involved in psychic well-being safeguarding.

The study was performed mainly in the Laboratory of Molecular Genetics at IMG, ASCR, except for some of the pharmacological assays that were performed in CNRS in Montpellier, France by the team members of IMG, namely Alena Hajkova, who acquired the knowledge and technical skills of these technologies during her stay in the laboratory of L. Prezeau and then introduced the technology at IMG, ASCR.

2.5. New research tools and methods

Several new technologies were implemented in collaboration in house; others were acquired in our partners' labs. The in house collaborations mainly include use of microscopy facilities for fluorescent microscopy and development of animal models using the TALEN technology of gene editing that were introduced by others and we gladly use them. We implemented new methods of measurements of functional pharmacological responses of GPCRs using FRET and BRET approaches.

2.6. Educational publications

J. Blahos as a member of a team of authors participated in the textbook "Biopharmaceuticals" (Biologická léčiva) (Grada Publisher, 2012) by contributing several chapters. Also, we participated in the discussion about the safety, possible pharmacological benefits and danger of cannabis medicinal use (published in Czech in the journal "*Česká a slovenská neurologie a neurochirurgie*" in 2013).

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Research Report of the team in the period 2010–2014

Institute	Institute of Molecular Genetics of the CAS, v. v. i.

Scientific team	Laboratory of Molecular and Cellular Immunology

Abbreviations used: B10: C57BL/10; CCL: chemokine (C-C motif) ligand; DPI: diphenyleneiodonium; IgE: immunoglobulin E; IL: interleukin; *Ir2*: Immune response 2; *Lmr*: *Leishmania major* response; *Ltr*: *Leishmania tropica* response; Mb: megabase; NO: nitric oxide; PCR: polymerase chain reaction; PCT: patent cooperation treaty; QTL: quantitative trait locus; RC: recombinant congenic; RPCI: Roswell Park Cancer Institute; SLA: soluble *Leishmania* antigen; *Tbbr*: *Trypanosoma brucei brucei* response; TBEV: tick-borne encephalitis virus; Th: T helper cell

2. 1. Introduction

The research programme of the laboratory aims to identify genes and molecular mechanisms involved in the control of immune response and susceptibility to complex infectious diseases. Complex diseases, in contrast to diseases caused by mutations in a single gene, are responsible for the largest part of human morbidity and mortality. These diseases are controlled by multiple genes – QTL (quantitative trait loci) and hence their pathogenesis cannot be explained by effects of a single gene with omission of others. The variation in these QTLs causes a large part of inter-individual differences in the course and severity of the disease. These differences strongly affect individual susceptibility to a disease, manifestation of specific pathogenic pathways and symptoms in individual patients, individual responsiveness to therapy, and the final outcome of the process. Modern immunology provided knowledge and tools to analyse systemic, cellular, and molecular aspects of these diseases. In parallel, progress in genetics, genomics, and determination of gene expression levels opened the possibilities to understand the large individual differences. Leishmaniasis is a prototypical complex disease and it has served as a major paradigm of immune response to an infectious agent. It is caused by intracellular protozoan parasites of the genus *Leishmania* and transmitted to vertebrates by phlebotomine sand flies. Infection can be asymptotic or lead to a large spectrum of clinicopathological manifestations. The basis of these differences is not well understood, but a large part of this variation is likely genetic. Therefore, we mapped the genes controlling the development of this disease and analysed the functional pathways associated with it. We established that susceptibility to *Leishmania major* is multigenically controlled (Demant et al. 1996) and detected 21 *Lmr* QTLs out of a total of 30 detected worldwide (*Lmr = Leishmania major* response) that regulate the response to infection by *L. major* (reviewed in Lipoldová and Demant, 2006; Kurey et al., 2009).

We were the first to combine the genetic mapping of susceptibility to leishmaniasis with a detailed analysis of pathology, immunology, and parasite load of individual mice. This revealed that surprisingly, the effects of individual *Lmr* loci do not simply increase or reduce the expression of immunological and pathological parameters in the direction of progression or healing, but each locus affected changes in a

subset of parameters, whereby these changes did not necessarily shift all parameters towards progression or healing, but the directional changes of individual parameters caused by the same gene could frequently point in different directions (reviewed in Lipoldová and Demant, 2006; Kurey et al., 2009). This discovery of the complex control of the individual disease phenotype, including more than ten different immunological and pathological phenotypes, has been a major contribution of the group to understanding the genetic variation of individual disease phenotypes. More recently, we have shown that this non-additivity of the genotypic effects on the disease phenotype is also very strongly visible in global gene expression studies, where introduction by crossing of a relatively limited set of genes from one strain into the genome of another strain causes in the so developed novel strain an expression pattern that is not a sum of specific parts of the expression patterns of the two original strains but leads to massive changes in the expression of genes and pathways that were not activated in either of the two parental strains.

In our recent research we concentrated on more precise definition of genetic and functional control of susceptibility to *L. major* (Section 2.3.1), finding potential links between the mechanisms influencing susceptibility to *L. major* and closely related species *L. tropica* and *Trypanosoma brucei brucei* (Section 2.3.2) and on using the obtained data (Section 2.3) and techniques learned or newly developed (Section 2.2) for translational research (Section 2.4).

A robust tool for high-resolution genetic analysis

We use a special tool for study of complex diseases: the recombinant congenic (RC) mouse strains. Each of 20 CcS/Dem RC strains carries a different random subset of 12.5 % of genes of the strain STS (resistant to *Leishmania major*) on the BALB/c (susceptible) background. By this way, RC strains compartmentalize randomized subsets of immune response genes, and so each RC strain exhibits a very different, unique, and reproducible pattern of immune response and symptoms of the disease. In part of experiments we also used a series of O20/A-c-C57BL/10-*H2*^{pz} (OcB/Dem) RC strains. The genetic composition of RC strains to be used here has been exactly defined by genotyping with > 500,000 SNPs (single-nucleotide polymorphisms) (Sohrabi et al., unpublished data). This increases the statistical power of genetic mapping and functional studies. We used this system for studies of activation of lymphocytes (**Lipoldová et al. 2010**) and susceptibility to infection to *L. major* (Demant et al. 1996; Lipoldová and Demant 2006), *L. tropica* (**Kobets et al. 2012a; Sohrabi et al. 2013**), *Trypanosoma brucei brucei* (**Šíma et al. 2010**) and tick-borne encephalitis virus (**Palus et al. 2013**). RC strains revealed a surprising nature of the genetics of inter-strain variation of gene expression patterns after infection with *L. major*. Comparison of these changes in the parental strains BALB/c (susceptible) and STS (resistant) and RC strain CcS-20 that is highly resistant in spite of carrying 87.5% of genes of the BALB/c strain and only 12.5% from STS has shown that the patterns of gene expression and activity of ontological pathways in CcS-20 are not a combination of responses observed in BALB/c and STS, but represent an entirely novel pattern that cannot be deduced from the observations on the two parental strains. This results in consistent activation of several related ontological families of pathways that were not activated at all in BALB/c or in STS. Understanding the mechanisms of induction of these non-additive patterns of effects of genetic variation is essential for correctly interpreting the role of genetic effects of genes when located in different genetic backgrounds.

The RC strains have been provided to us by Dr. Peter Demant, Roswell Park Cancer Institute [RPCI], Buffalo, USA.

2.2. New research tools and methods

Mapping of genes controlling *Leishmania* parasite numbers have been difficult due to the lack of a simple, sensitive method that allows simultaneous testing of a large number of samples. We have therefore designed a highly sensitive PCR-ELISA for detection and quantification of *Leishmania* parasites in host tissues. Unlike other DNA-based assays, the present method uses digoxigenin- and biotin-labelled primers. This eliminates the need for a separate step of hybridization of the PCR product with labelled probes. The PCR product is detected using sandwich ELISA with anti-digoxigenin detecting antibodies. The sensitivity of this technique is 0.3 fg of parasite DNA/reaction (**Kobets et al. 2010**). It enabled us to map genes that control parasite numbers in genome-wide search and to analyse their relationship with other disease parameters (**Sohrabi et al. 2013**), as well as to analyse the effectivity of vaccination (**Rohoušová et al. 2011**) and drug treatment (**Patent Cooperation Treaty (PCT)/CZ2014/000103**). It was subsequently adopted by other laboratories to detect other pathogens. The whole study was performed in the Laboratory of Molecular and Cellular Immunology. M. Svobodová from the Department of Parasitology, Faculty of Science, Charles University, Prague helped us in the early stages of experiments with parasite cultures.

Analysis of susceptibility to *L. tropica* (see Section 2.3.2) was difficult due to the inability to find a suitable animal model and to optimize conditions of infection. We have therefore, in collaboration with the Department of Parasitology, Charles University and Hospital Bulovka, adapted and optimized cultivation of *Leishmania* promastigotes on a biphasic SNB-9 (saline-neopeptone-blood 9) medium that was originally developed for *Trypanosoma* cultivation and combines the advantages of biphasic and liquid media (**Grekov et al. 2011**).

2.3. Mapping and functional analysis of genes controlling complex infectious diseases

2.3.1. Control of susceptibility to *Leishmania major*

A unique model for analysis of epistasis

We developed a unique model for analysis of epistasis – a two-way interaction between genomes of two resistant strains, O20 and C57BL/10 (B10). Transfer of a small number of genes of strain O20 into the genetic background of strain B10 generated a new inbred strain B10.O20, which is highly susceptible. In the opposite direction, transfer of limited numbers of B10 genes to O20 genetic background occurred in the OcB RC strains, each of which carries a small random set of 6.2 % or 12.5 % of the genes of strain B10 on the genetic background of strain O20. Two of these strains also exhibit significant susceptibility. These two sets of data show that a susceptible phenotype can develop in mice carrying specific combinations of genes originating from two resistant strains. Therefore, both susceptibility and resistance can be encoded by different combinations of genes, and hence are likely functionally heterogeneous. As a basis for further mechanistic studies of these epistatic effects, we characterized the basic immunologic and genetic parameters of these models.

1. Strain B10.O20 is highly susceptible to *L. major,* with large skin lesions, high parasite numbers in the skin and lymph nodes, and massive infiltration of $CD11b^{+}Gr1^{+}$ cells in the spleen. Splenocytes of infected B10.O20 mice after stimulation with soluble *Leishmania* antigen (SLA) produce more Th1, Th2 and Th17 cytokines than B10 and O20, suggesting a chronic inflammation with imbalance of several axes of immune response. In contrast, splenocytes of highly resistant O20 mice lacked response to SLA by cytokine production and their intraperitoneal macrophages responded to SLA by IL-12 production but not by production of NO, suggesting a novel mechanism of resistance (Sohrabi et al. submitted manuscript). As B10.O20 carries only 4.2 % of O20-derived genes located on eight short genomic segments on different chromosomes, it offers a unique opportunity to define the genetics, mechanisms, and pathogenesis of this strong epistasis.

2. We determined susceptibility to *L. major* in the strains O20 and B10.O20, and 15 OcB RC strains which carry different and only minimally overlapping small sets of 6.2 % or 12.5 % of B10-derived genes (Mamm Genome 7: 55, 1996). The unique combinations of B10 and O20 genes present in the strains OcB-11 and OcB-31 led to skin lesion development. The lesion size of OcB-11 and OcB-31 at 8 weeks post-infection differ significantly from O20 (P = 0.040, and 0.006, respectively). To test whether these differences are genetically controlled, we analysed the genetics of difference between OcB-31 and O20, using also the OcB-31 substrain OcB-43. In F_2 hybrids between OcB-31 (or OcB-43) and O20 we detected three susceptibility loci. Two loci on chromosomes 3 and 15 control parasite numbers in the liver. B10 alleles are associated with higher parasite numbers. Development of skin lesions is controlled by an interaction between loci on chromosomes 2 and 3; the largest lesions were in homozygotes for B10 alleles on chromosome 2 and heterozygotes on chromosome 3.

Combining detailed genetic analysis of these models with analysis of immunological and pathological parameters of infected mice and gene expression studies will provide a powerful tool to describe different mechanisms of resistance and susceptibility.

2.3.2. Searching for common and species-specific control of susceptibility

During the evolution, the pathogens developed different mechanisms how to evade host immunity. Some of these mechanisms are common for groups of pathogens, the other differ even in the closely related species. This is mirrored by the fact that some genes, for example *Slc11a1* (solute carrier family 11 (proton-coupled divalent metal ion transporters), member 1) or *Lyst* (lysosomal trafficking regulator)/beige have been found to control susceptibility to several pathogens (reviewed in Lipoldová and Demant, 2006), whereas other are species-specific. Several *Lmr* map to the same chromosomal regions as some loci influencing response to other infectious agents such as *Mycobacterium tuberculosis*, *Salmonella typhimurium*, *Borrelia burdorferi*, *Listeria monocytogenes*, *Trypanosoma congolense*, *Leishmania donovani* and *Trypanosoma cruzi* (Lipoldová and Demant, 2006). This indicates that some *Lmr* genes may control susceptibility to several pathogens. In order to search for these common as well as species-specific genes, we have extended our studies to closely related species *Leishmania tropica* and *Trypanosoma brucei brucei*.

First genetic model of susceptibility to *L. tropica* and genetic architecture of response

Although the *Leishmania tropica*-induced cutaneous disease has long been known, its potential to visceralize in humans was recognized only recently. As nothing was known about the genetics of host responses to this infection and their clinical impact, we developed the first defined animal model suitable for genetic, pathological and therapeutic studies in *L. tropica* infection. Comparison of *L. tropica* and *L. major* infections indicated that the strain patterns of response are partly species-specific, with different sex effects and largely different host susceptibility genes (**Kobets et al. 2012a**). Therefore, analysis of genes affecting the host's responses to this infection can elucidate the characteristics of individual host-parasite interactions. On the basis of the observed strain differences we performed linkage analysis of the responsible genes. We have selected strain CcS-16 that is highly susceptible to both *L. major* (Vladimirov et al. 2003) and *L. tropica* (**Kobets et al. 2012a**), but also exhibits marked differences in systemic response to these two parasites. In F_2 hybrids between BALB/c and CcS-16 we detected and mapped eight gene-loci, *Ltr1-8* (*Leishmania tropica* response 1-8) that control various manifestations of the disease: skin lesions, splenomegaly, hepatomegaly, parasite numbers in spleen, liver, and inguinal lymph nodes, and serum level of CCL3, CCL5, and CCL7 after *L. tropica* infection. These loci are functionally heterogeneous – each influences a different set of responses to the pathogen. *Ltr3* that controls parasite numbers in the spleen exhibits the recently discovered phenomenon of transgenerational parental effect on parasite numbers in the spleen. The most precise mapping (4.07 Mb) was achieved for *Ltr1* (chromosome 2), which controls the parasite numbers in lymph nodes.

Comparison of genetic control of response to *L. tropica* and *L. major* might indicate some common and some distinct mechanisms in response to these two parasites. We compared the genetic relationship between the *Ltr* (**Sohrabi et al. 2013**) and *Lmr* (Vladimirov et al. 2003; Havelková et al. 2006) loci detected in the strain CcS-16. Five *Ltr* loci co-localize with the previously described loci that control susceptibility to *L. major*, three are species-specific. *Ltr2* co-localizes not only with *Lmr14* (*Leishmania major* response 14), but also with *Ir2* influencing susceptibility to *L. donovani* and might therefore carry a common gene controlling susceptibility to leishmaniasis (**Sohrabi et al. 2013**).

The whole study was performed in the Laboratory of Molecular and Cellular Immunology except for collection of promastigote secretory gel from *Phlebotomus sergenti*, which was undertaken by M. Svobodová (Department of Parasitology, Faculty of Science, Charles University). RC strains were provided by P. Demant (RPCI, USA). T. Jarošíková (Faculty of Biomedical Engineering, Czech Technical University in Prague) participated in DNA typing.

Susceptibility to *Trypanosoma brucei brucei*

We performed the first genetic analysis and mapping of genes for susceptibility to *T. brucei brucei* infection using the RC strain CcS-11 and mapped four loci *Tbbr* (*Trypanosoma brucei brucei* response) 1-4 controlling survival. While mapping in F_2 hybrids of inbred strains usually has a precision of 40–80 Mb, in RC strains we mapped *Tbbr2* to a 2.15 Mb segment containing only 26 genes, which will enable an effective search for the candidate gene (**Šíma et al. 2010**). *Tbbr2* might be potentially also involved in the control of *L. major*, as it overlaps with locus *Lmr22*, which in interaction with *Lmr5* controls serum IL-4 in *L. major*-infected mice (Kurey et al. 2009). Definition of susceptibility genes will improve the understanding of pathways and genetic diversity underlying these two diseases and may lead to new

strategies to overcome the active subversion of the immune system by *T. b. brucei* (**Šíma et al. 2010**) and *L. major*.

The study was performed in the Laboratory of Molecular and Cellular Immunology. L. Quan (RPCI) and A.P.M. Stassen (Maastricht University, The Netherlands) helped us with statistical analysis, M. Svobodová (Department of Parasitology, Faculty of Science, Charles University) selected suitable *Trypanosoma* ssp., RC congenic strains were provided by P. Demant (RPCI). T. Jarošíková (Faculty of Biomedical Engineering, Czech Technical University in Prague) participated in DNA typing.

Susceptibility to tick-borne encephalitis virus (TBEV) infection

The RC system was also successfully used to analyse the susceptibility to TBEV. We have found that BALB/c mice exhibit intermediate susceptibility to the infection of TBEV, STS mice are highly resistant, whereas the RC strain CcS-11, carrying 12.5 % of the STS genome on the background of the BALB/c genome, is even more susceptible than BALB/c and is characterized with shorter survival time and higher expression of CCL2 and IP-10 (interferon-γ-inducible protein-10) /CXCL10 in the brain. Importantly, CcS-11 and BALB/c carry identical alleles at TBEV susceptibility genes *Oas1b* (2'-5'-oligoadenylate synthetase 1B), *Cd209*, *Tlr3* (toll-like receptor 3) and *Ccr5* (chemokine (C-C motif) receptor 5), and thus their phenotypic difference is caused by a presently unknown gene (**Palus et al 2013**). We have mapped this unknown gene and are currently sequencing a potential candidate gene. Genetic studies have been performed by our group; functional studies have been done by the laboratory of D. Růžek (Institute of Parasitology, Biology Centre of the Czech Academy of Sciences, České Budějovice).

2.4. Development of translational applications

2.4.1. Novel compound against *Leishmania* and *Trypanosoma* parasites

Parasites of the genus *Leishmania* threaten the population of 98 countries on five continents. In the last 10 years, due to climatic and environmental changes, wars, and other unknown effects the endemic areas have been heavily extended and the number of reported cases increased. In Europe, for example, leishmaniasis was previously restricted to the Mediterranean region, but now it has spread to northern Italy and southern Germany, where dozens of cases have been reported in people who did not travel outside the area (**Kobets et al. 2012b**), and recently autochthonous cases of infected animals were reported from Hungary and Switzerland. Unfortunately, safe and effective human vaccine against the disease does not exist. Similarly, there is no suitable and simple treatment without side effects (**Kobets et al. 2012b**). We have screened the library of 2448 chemical compounds for growth inhibition of *L. major*. This primary screening resulted in the identification of diphenyleneiodonium (DPI) as an effective inhibitor. Further tests revealed that DPI kills Leishmania parasites both *in vitro* and *in vivo* more effectively than the current drugs such as amphotericin B. Moreover, DPI is also effective in killing *T. b. brucei* (**PCT/CZ2014/000103**). The effective concentrations of the compound were non-toxic to tested human cell lines. These studies were performed in collaboration with the group of P. Bartůněk (Laboratory of Cell Differentiation, Institute of Molecular Genetics, ASCR), which performed high-throughput screening and toxicity tests.

2.4.2. Vaccination by sand fly bites

We have analysed the potential use of the saliva of transmitting insect vector of leishmaniasis for vaccination and found that the protective effect is limited to short-term exposure (**Rohoušová et al. 2011**). This study represented a collaborative project between the Department of Parasitology, Faculty of Science, Charles University and the Laboratory of Molecular and Cellular Immunology, IMG. The Department of Parasitology performed vaccination experiments and analysed the immune response, the Laboratory of Molecular and Cellular Immunology was responsible for detection and quantification of *Leishmania* parasites in mouse tissues.

2.5. Review publications

We have described both our genetic (Gusareva et al. 2009a; Gusareva et al. 2009b, Havelková et al. 2006; Kurey et al. 2009; Lipoldová et al. 2000) and functional (Gusareva et al. 2006; Gusareva et al. 2008; **Kobets et al. 2012a**; **Rohoušová et al. 2011**) studies in the context of current knowledge (**Gusareva et al. 2014; Kobets et al. 2012b**).

The review of **Gusareva et al. (2014)** in a high-impact journal describes in detail immunoglobulin E (IgE) and its important role in the defence against helminths and parasitic infection and in pathological states including allergic reactions, anti-tumour defence and autoimmune diseases. We summarize progress in the genetics of regulation of the IgE level in human diseases and show that integration of different approaches and use of animal models synergistically enhance generation of new knowledge about both protective and pathological roles of this important antibody class.

The invited review of **Kobets et al. (2012b**) discusses the approaches used for the vaccination against leishmaniasis, the progress that has been achieved in *Leishmania* detection, and the drugs used to treat leishmaniasis. Unfortunately, no safe and effective vaccine currently exists against any form of human leishmaniasis and the available antileishmanial drugs have serious side effects. We suggest that the application of novel strategies based on advances in genetics, genomics, advanced delivery systems, and high throughput screenings for leishmanicidal compounds would lead to improvement of prevention and treatment of this disease.

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Research Report of the team in the period 2010–2014

Institute	Institute of Molecular Genetics of the CAS, v. v. i.

Scientific team	Laboratory of Cell and Developmental Biology

2.1. Introduction

The Laboratory of Cell and Developmental Biology was established in January 2007. The prospective scientific goal of the laboratory is to elucidate the signalling mechanisms leading to cellular transformation and cancer. In direct link to our previous research, the group focused in the past five years on several interconnected topics. All these topics were related to the Wnt signalling pathway, in particular to the Wnt pathway components and molecular mechanisms. Importantly, we started to move from studies performed predominantly *in vitro* (in culture cells; see e.g. **Valenta et al., 2003**) or in simple model organisms (e.g. *C. elegans*; **Asahina et al., 2006**) to experiments performed in mice. We applied advanced molecular biology techniques such as chromatin immunoprecipitation with DNA microarray analysis (ChIP-on-chip) and expression profiling to identify genes regulated by physiological or aberrant Wnt signalling in the mouse gastrointestinal epithelia or in human intestinal cancer cells. Based on such experiments, we generated genetically modified mice allowing conditional inactivation of the "gene of interest" in adult tissues. Furthermore, we produced transgenic mice expressing the Cre recombinase gene from the studied loci. The latter animals were employed – in combination with various "reporter" mouse strains – for lineage tracing experiments in mouse tissues. Since Wnt signalling is frequently deregulated in many types of cancer, we also identified and characterized novel chemical inhibitors of the Wnt signalling pathway. We screened (in collaboration with several other laboratories) large collections of chemical compounds that included thousands of different "small molecules". The impact of selected chemicals on growth of intestinal tumours in mice and proliferation of human cancer cells (growing either in cultures or as xenotransplants in immunodeficient animals) was tested. In addition, the correlation between suppression of aberrant Wnt signalling and antineoplastic effects of the particular inhibitor was examined. Finally, to "translate" our results to human medicine we set up collaboration with two clinical laboratories. We obtained a good access to various samples of human tumours and matched healthy tissue. This enabled us to conclude whether findings based on the data obtained in experimental mice or cancer cells are relevant to the conditions observed in human patients.

2.2. Novel components of the Wnt pathway and their role in healthy adult tissues and cancer

The body of a multi-cellular organism is a highly organized structure of cells, tissues and organs. The Wnt pathway, initiated by secreted Wnt proteins, controls a remarkably diverse array of processes that include cell proliferation, differentiation, cell migration, and cell polarity. The best studied so-called canonical pathway depends on beta-catenin as its key effector. Besides its structural engagement in E-cadherin-based adherens junctions, beta-catenin associates with DNA-binding proteins of the lymphoid

enhancer-binding factor/T-cell factor (LEF/TCF) family to modulate expression of context-specific target genes. In the absence of a Wnt stimulus, cytosolic beta-catenin is marked for degradation by a cytoplasmic protein complex called the beta-catenin destruction complex that includes casein kinase 1 alpha (CK1α) and glycogen synthase kinase 3-beta (GSK-3β). Scaffolding of the kinases and beta-catenin is mediated by structural proteins Axin 1/2 and adenomatous polyposis coli (APC) tumour suppressors. Ultimately, N-terminally phosphorylated beta-catenin is ubiquitinated by the F-box-containing beta-transducin repeat containing (beta-TrCP) E3 ubiquitin protein ligase and subsequently destroyed by the proteasome. Beta-catenin contains a transactivation domain, and thus TCF/beta-catenin complexes act as bipartite transcriptional activators of target genes such as c-myc, Cyclin D1, CD44 and Axin2.

The epithelial lining of the gastrointestinal (GI) tract represents one of the most intensively self-replenishing organs in mammals. With a rate of entire renewal every 3-5 days, this dynamic and organized cell turnover represents an attractive paradigm for tissue maintenance studies. The single-cell epithelial sheet of both the small and large intestine penetrates into the underlying connective tissue of lamina propria to form tubular glands called crypts. In addition, luminal protrusions of the mucosa termed villi are present in the small intestine to further increase the surface area. The homeostasis is sustained by crypt-resident multipotent intestinal stem cells. Numerous studies conducted both *in vivo* and *in vitro* have firmly established the role of Wnt signalling in preservation of stem cell proliferation and pluripotency. Detailed description of Wnt and other signalling pathways involved in intestinal homeostasis and tumorigenesis can be found in our two recent review articles (**Krausova and Korinek, 2012**; **Krausova and Korinek, 2014**). In these reviews we summarized advances in characterization and regulation of epithelial stem cells in the gut. In addition, we presented novel experimental approaches utilized to investigate the epithelial cell signalling circuitry *in vivo* and *in vitro*.

The involvement of Wnt signalling in self-renewal, regeneration and transformation of the intestinal epithelia make this tissue a suitable cellular system to search for novel Wnt signalling pathway components or to perform functional tests of newly identified molecules. As described above, in canonical Wnt signalling the association of Wnt ligand with its corresponding receptors leads to the stabilization and accumulation of beta-catenin. Subsequently, beta-catenin enters the cell nucleus and together with the LEF/TCF transcriptional regulators activates expression of Wnt target genes. To identify genes regulated by Wnt l pathway effector TCF4, we performed ChIP-on-chip assay on chromatin isolated from cultured colorectal cancer-derived cells. One of the most prominent targets of the TCF/beta-catenin complexes was the promoter region of the tumour necrosis factor receptor superfamily, member 19 (TNFRSF19, alternative name TROY) gene. In subsequent experiments we confirmed that TROY is a genuine target of canonical Wnt signalling. To study the identity of the Troy-producing cells in detail, we used bacterial artificial chromosome (BAC) carrying the mouse Troy locus to generate transgenic mice (designated Troy-CreERT2) expressing tamoxifen-inducible Cre enzyme inserted in frame at the translation initiation codon of the Troy gene. We intercrossed Troy-CreERT2 with Rosa26R “reporter” mice and performed cell labelling experiments in the intestinal tissue. Rosa26R animals produce bacterial beta-galactosidase (lacZ) mRNA from the ubiquitously active Rosa26 allele. The mRNA is not translated (and the enzyme produced) unless a transcriptional stop signal flanked (“floxed”) by a pair of loxP sites is removed from the genome by Cre-mediated excision. These lineage tracing experiments revealed that Troy is produced specifically in intestinal stem cells. Subsequently, we focused on clarifying the possible molecular function(s) of TROY. We used cell lines with inducible expression of TROY. Moreover, we tested the effect of TROY knockdown in cells with aberrant Wnt

signalling. Based on these experiments we concluded that Troy inhibits the Wnt pathway at the level or upstream of the beta-catenin degradation complex and the inhibition involves the stability and/or the phosphorylation status of the Wnt co-receptor LRP. We also discovered that Troy physically interacts with another (unique) stem cell marker, Lgr5, and modulates its function. Finally, to address the impact of TROY on Wnt signalling in a more physiological context, we employed Troy-deficient mice. In particular, the Troy function was tested in defined primary cultures of the intestinal crypts called organoids. The growth of these "little guts" is fully dependent on the exogenous Wnt or R-spondin ligands. Of note, R-spondins bind the Lgr4/5/6 receptors and function as Wnt signalling agonists. Importantly, these experiments convincingly showed the crosstalk of Troy and Wnt/R-spondin signalling. These results were published in the prestigious scientific journal Gastroenterology (**Fafilek et al., 2013**). Recently, 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 can serve as the cell-of-origin for gastric cancer. The described results were included into a manuscript submitted to the respected Cell journal (**Hayakawa et al., submitted**). As the referees requested additional experiments, we expect re-submitting the revised version of the manuscript in the course of the year 2015.

Our ChIP-on-chip analysis revealed several other (next to TROY) potential TCF/beta-catenin target genes. One of these genes, naked cuticle homolog 1 (Nkd1), displayed a very similar expression pattern to TROY. The activity of the Wnt pathway undergoes complex regulation to ensure proper functioning of this principal signalling mechanism during development and in adult tissues. The regulation may occur at several levels and includes both positive and negative feedback loops. Similarly to Troy, the Nkd1 gene encodes a negative regulator of Wnt signalling. Interestingly, hybridization of mRNA *in situ* using an anti-sense Nkd1 probe revealed robust staining of Nkd1 mRNA in the intestinal crypts. We therefore decided to follow these observations and characterize Nkd1-expressing cells in more detail. We applied the BAC recombineering strategy (as used for the production of TROY-CreERT2 animals) and generated Nkd1-CreERT2 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. Interestingly, in contrast to analogous experiments using the Troy-CreERT2 "reporter" transgenic mouse, Nkd1-CreERT2 marked all cells located at the bottom part of the crypts (i.e. stem and Paneth cells and transit amplifying cells). Moreover, since Nkd1 and Troy expression marks tissue regions surrounding the hepatic centrilobular vein (this particular area of the liver displays active Wnt signalling), we employed the Cre-producing mice to sort Nkd1-positive hepatocytes and perform their expression profiling. The assay confirmed that Nkd1 represents a robust marker of the perivenous hepatic regions. To gain more insight into the role of NKD1 in human neoplasia 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 tumours with deregulated Wnt signalling. The results were recently published in an article in the Cellular Signalling journal (**Stancikova et al., 2015**).

2.3. Hypermethylated in cancer 1 (HIC1) and its role in intestinal carcinogenesis

Recently, we identified two proteins, Dazap2 and HIC1, acting as modulators of Wnt signalling in mammalian cells. Both modulators associate with the nuclear effector of Wnt signalling, the TCF4 transcription factor. Dazap2 increases affinity of TCF4 for its DNA recognition motif and functions as a positive modulator of Wnt signalling (**Lukas et al., 2009**). In contrast, HIC1 functions as Wnt signalling antagonist (**Valenta et al, 2006**). HIC1 represents a prototypic tumour suppressor gene frequently inactivated by DNA methylation in many types of solid tumours. The gene encodes a sequence-specific transcriptional repressor involved in the cycle or stress control. HIC1 potentiates the p53 stability (and function); however, the precise tumour-suppressive mechanisms of HIC1 remain elusive. In the mouse, Hic1 is essential for embryonic development and exerts an anti-tumour 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. In addition, we used gene targeting to replace a sequence encoding Hic1 by citrine fluorescent protein cDNA. We demonstrated that the distribution of Hic1-citrine fusion polypeptide corresponds to the expression pattern of wild-type Hic1 (**Pospichalova et al., 2011**). Hic1-citrine "reporter" mice were employed to monitor the activity of the Hic1 locus in the adult gut. In both small and large intestine, native citrine fluorescence was observed in epithelial cells. Subsequently, production of Hic1 protein in differentiated epithelial cells was confirmed by immunoblotting and quantitative PCR. Additionally, we employed Hic1 conditional allele-based screen to identify genes directly 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 signalling. Interestingly, in the chemical carcinogenesis model, larger and more proliferative colonic tumours develop in Hic1 mutant mice when compared to wild-type animals. In summary, our results indicated that the tumour-suppressive function of Hic1 might be related to its inhibitory action on pro-proliferative signals mediated by Tlr2 present on tumour cells (**Janeckova et al., in press**).

2. 4. Signaling properties of Wnt ligands

Extracellular Wnt ligands act as morphogens initiating specific cellular responses in relation to the concentration of a particular Wnt in the extracellular space. The majority of 19 mammalian Wnt proteins contain two fatty acyl groups and multiple N-linked-glycosylations. Nevertheless, the biological significance of these posttranslational modifications, especially their role in the formation of the Wnt concentration gradient, was elusive. We examined - using various experimental systems - how glycosylations and acylations contribute to the secretion, extracellular movement and signalling activity of mouse Wnt1 and Wnt3a ligands. In a published study we showed that the level of acylation is crucial for Wnt1- and Wnt3a-initiated signalling. Interestingly, although double-acylation of Wnt1 was indispensable for signalling in mammalian cells, in Xenopus embryos the S-palmitoyl-deficient protein retained the signalling activity. Originally it was presumed that the lipidic adducts mediate tighter association of the ligand with the cell surface. However, our results revealed that the opposite is true and acylation promotes Wnt release into the extracellular space (**Doubravska et al., 2011**). Such detailed examination of biochemical bases of signalling properties of Wnt ligands was performed for the first time.

2. 5. Aberrant Wnt signalling as a therapeutic target in cancer

Cancer affecting colon and rectum [colorectal carcinoma (CRC)] constitutes one of the most commonly diagnosed neoplasia in developed countries. It is estimated that more than one million patients are clinically diagnosed every year with a mortality rate exceeding 30 %. The pathogenesis of CRC is linked to the aberrant activity of the Wnt signalling cascade. In about 90 % of sporadic CRC the hyperactivation of Wnt signalling occurs via mutations in APC and AXIN1/2 tumour suppressors or in beta-catenin. This results in locally uncontrolled cell proliferation and formation of polyps progressing into malignant carcinoma. Along with the intestinal cancer, hyperactive Wnt signalling causes many other types of solid tumours and leukaemia. These findings suggest that inhibitors of the Wnt pathway have the potential to be utilized as antitumorigenic agents. In the years 2012 and 2013 the laboratory participated in two high-throughput screens using large collections of chemical compounds to identify inhibitors of the Wnt signalling pathway. Interestingly, several chemical compounds that inhibited Wnt signalling with high potency and specificity in different experimental setups were tankyrase (TNKS) 1/2 inhibitors. TNKS controls protein stability by adding a secondary modification called poly(ADPribosyl)ation to substrate proteins. TNKS 1/2 play a fundamental role in Wnt signalling by controlling protein stability and turnover of Axin1/2 in the context of the beta-catenin destruction complex. The poly(ADPribosyl)ation chain formed by TNKS creates a recognition signal for E3 ubiquitin ligase RNF146 that polyubiquitinates Axin1/2 (and other substrate proteins), leading to its subsequent degradation in the 26S proteasome. We mainly participated in elucidation of the molecular mechanisms of Wnt signalling inhibition. Importantly, it was shown that AXIN protein stabilization by TNKS inhibition is sufficient to impact tumour growth in cells harbouring mutated APC. These results established antitumor efficacy for TNKS inhibitors in APC-mutant cancers. The collaboration resulted in two articles published in Cancer Research (**Waaler et al., 2012**; **Lau et al., 2013**). In these studies we mainly participated in the cell-based assays (reporter gene assays, quantitative RT-PCR). We also designed (and employed) a new strategy based on genetically modified mice to test the anticancer effect of the discovered compounds. Next, we performed “our own” screening experiment for novel chemical inhibitors of Wnt signalling using STF cells. The genome of these cells (derivative of human embryonic kidney cells HEK293) harbours integrated copies of the reporter firefly luciferase gene whose expression is driven by the Wnt-responsive promoter. Three large libraries of bioactive chemical compounds were screened in STF cells for a putative Wnt pathway modulatory effect. We identified monensin, a natural antibiotic, as a potent inhibitor of Wnt signalling and a prospective anticancer drug. The inhibitory effect of monensin on Wnt/beta-catenin signalling was tested in mammalian cells, in zebrafish, and in Xenopus embryos. In the mouse model of intestinal cancer, monensin suppressed progression of the intestinal tumours (**Janeckova et al., 2014**).

2.6. References

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Research Report of the team in the period 2010–2014

Institute	Institute of Molecular Genetics of the CAS, v. v. i.

Scientific team	Laboratory of Mouse Molecular Genetics

2.1. Introduction

One of the fundamental questions of speciation deals with the genetic and molecular mechanisms ensuring reproductive isolation between nascent species. Sterility of interspecific hybrids is the most studied but still poorly understood postzygotic form of reproductive isolation, which restricts gene flow between closely related species. Most of what we know about hybrid sterility came from the studies of fruit fly (*Drosophila*) species hybrids, but in the last decade, house mouse (*Mus musculus*) emerged as an excellent mammalian model. Forty years ago, our laboratory localized the first mammalian hybrid sterility locus *Hst1* on the mouse genetic map (**Forejt and Ivanyi 1974**) and more recently, we were also the first to identify the *Hst1* locus with *Prdm9* (PR-domain containing 9) gene encoding a meiotic histone 3 methyltransferase (**Mihola et al., 2009**; for review see **Forejt et al. 2012**). An important prerequisite for these studies was the construction of suitable, genetically defined tools. In the past we derived two inbred strains, PWD/Ph and PWK/Ph, from wild mice of *Mus m. musculus* subspecies (for review: **Gregorova and Forejt, 2000**) and more recently, we constructed a series of 27 chromosome substitution strains, in which individual C57BL/6J (*Mus m. domesticus*) chromosomes of the recipient strain were substituted by PWD chromosomes (*Mus m. musculus*) (**Gregorova et al. 2008**). These inbred and chromosome substitution strains were deposited at the World's reference centre of genetically defined laboratory mice at The Jackson Laboratory, Bar Harbor, ME and are accessible to research community worldwide. The PWK/Ph strain belongs to the panel of 17 mouse inbred strains re-sequenced by the Wellcome Trust Sanger Institute (**Keane et at. 2011**) and to the eight parental strains selected (out of >400) for the Collaborative Cross, a genetic reference population generated by the international community effort (**Churchill et al. 2004**). In the last two years, we sold two temporary licenses for the C57BL/6J-Chr 9PWD/Ph/ForeJ chromosome substitution strain to GENENTECH, San Francisco, CA, a leading biotech company.

In the period 2010-2014, our team focused on genetics of gametogenesis, in particular on the role of the *Prdm9* gene in hybrid sterility and on the genetics of meiotic chromosome pairing and sex chromosome inactivation in sterile hybrids.

2.2. Genetic resources and SYSGENET

Our team participated in the COST action SYSGENET, European systems genetics network for the study of complex genetic human diseases using mouse genetic reference populations. The aim of the consortium was to map the European infrastructure for multiple phenotypic studies on diverse mouse models as a prerequisite of systems genetics approach to complex traits. Our team contributed the

information on our panel of chromosome substitution strains and we organized the 8th SYSGENET MC and WG Meeting / Workshop about future activities in Prague, June 17-18, 2013. Two joint publication summarized the SYSGENET activities (**Schughart et al., 2010, 2013**). In another collaborative project, we contributed our panel of consomic strains to study the size and shape of mandible as a complex trait model (**Boell et al. 2011**).

2.3. *Prdm9*, hybrid sterility and genetic mechanisms of meiotic asynapsis

Hybrid sterility 1, the first candidate for a mammalian speciation gene was identified as *Prdm9* (**Mihola et al. 2009**), but the mechanism causing sterility has remained unknown. In the next paper, we showed that the meiotic progress and fertility of hybrid males can be improved by removal (by a *Prdm9* gene knockout) as well as by overexpression (by a *Prdm9*-containing BAC transgene) of the C57BL/6J allele of *Prdm9*. The results showed that the *Prdm9* incompatibilities which are absent in the parental species appear in hybrids and play a role in hybrid sterility (**Flachs et al. 2012**). The following study provided direct evidence that the middle region of *Mus m. musculus* Chr X carries another major hybrid sterility gene(s) interacting with *Prdm9.* The findings excluded other potential candidates such as Chr Y, imprinted genes, or mitochondrial DNA. Overall, the results revealed the Dobzhansky-Muller type of incompatibility of *Prdm9* and the X-linked hybrid sterility gene, and indicated the oligogenic nature of F1 hybrid sterility, which should be amenable to reconstitution by proper combination of chromosome substitution strains (**Dzur-Gejdosova et al. 2012**).

Before we can explore the molecular mechanisms of the first example of Dobzhansky-Muller gene incompatibility, we need to identify the *Prdm9*-interacting X-linked hybrid sterility gene. As the first step of the positional cloning we set up high resolution genetic mapping, combining a QTL approach and construction of consomic strains with the PWD/B6 recombinant X chromosomes. We localized the hybrid sterility locus, designated Hybrid sterility X2, *Hstx2*, into 4.7 Mb interval on chromosome X. As possible *Hstx2* candidates, seven protein-coding genes appropriately expressed in time and space were identified in the region, together with a cluster of miRNA genes (**Bhattacharyya et al. 2014**). The Dobzhansky-Muller model implies that the incompatibilities evolve randomly, unless a particular gene or nongenic sequence diverges much faster than the rest of the genome. In our next paper (**Bhattacharyya et al. 2013**) we proposed that asynapsis of heterospecific chromosomes in meiotic prophase provides a recurrently evolving trigger for the meiotic arrest of interspecific F1 hybrids. We observed extensive asynapsis of chromosomes and disturbance of the sex body in >95% of pachynemas of *Mus m. musculus* x *Mus m. domesticus* sterile F1 males. Asynapsis was not preceded by a failure of double-strand break induction, and the rate of meiotic crossing over was not affected in synapsed chromosomes. The sensitivity of homeologous chromosomes to asynapsis was under the tight control of *Prdm9* and *Hstx2* in male hybrids. In hybrid females, about half of the pachtene oocytes were affected by asynapsis, independently of their *Hstx2/Prdm9* genotype.

The role of *Prdm9* and *Hstx2* gene is not limited to the interaction of PWD and B6 genomes, since by using another *Mus m. musculus* derived inbred strain, PWK/Ph, we found that *Prdm9* and Chromosome X control the partial meiotic arrest and reduced the sperm count in (PWKxB6) F1 males. Asynapsis of heterosubspecific chromosomes and semisterility were partially suppressed by removal of the B6 allele of *Prdm9*. Polymorphisms between PWK and PWD on Chromosome X but not in the *Prdm9* region were responsible for the modification of the outcome of *Prdm9* - Chromosome X F1 hybrid incompatibility.

Our results showed extended functional consequences of *Prdm9* - Chromosome X incompatibility on the fertility of hybrids (**Flachs et al. 2014**).

To further investigate the interspecific differences in genetic control of spermatogenesis, testicular transcriptomes were compared between two closely related mouse species, *Mus musculus* and *Mus spretus*. An excess of differentially regulated non-coding RNAs was found on Chromosome 2 including the intronic antisense RNAs, intergenic RNAs and premature forms of Piwi-interacting RNAs (piRNAs). Moreover, a striking difference was found in the expression of X-linked *G6pdx* gene, the parental gene of autosomal retrogene *G6pd2* (**Homolka et al. 2011**).

Trisomy for any intact autosome is embryonic lethal in the mouse. Partial trisomy of the proximal 30 Mb of chromosome 17 is viable and the males are semisterile. We used this model aneuploidy to study the meiotic silencing of unsynapsed chromatin, comparing genome-wide transcriptional profiles of liver cells and male germ cells. Although the 1.6-fold change in expression of triplicated genes reflected the gene dosage in somatic cells, the extra copy genes were compensated in early pachytene spermatocytes, showing 1.18-fold increase. The dosage compensation of trisomic genes was concordant with the incidence of HORMAD2 protein and histone γH2AX markers of unsynapsed chromatin. Our results indicate that the silencing of unsynapsed chromatin is the major factor driving the gene dosage compensation in primary spermatocytes (**Jansa et al. 2014**).

2.4. Review publications

Two reviews were published on hybrid sterility in mice (**Forejt et al., 2012**, **Forejt 2013**). A few paragraphs on the use of C57BL/6J-Chr#PWD chromosome substitution strains were contributed to a review article of Joe Nadeau (**Nadeau et al. 2012**).

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Research Report of the team in the period 2010–2014

Institute	Institute of Molecular Genetics of the CAS, v. v. i.

Scientific team	Laboratory of Signal Transduction

2.1. Introduction

The main research interest of the Laboratory of Signal Transduction has been traditionally focused on understanding the molecular mechanisms governing signal transduction from plasma membrane receptors, specifically the high-affinity IgE receptor (FcεRI) and KIT, to the cytoplasm. In the evaluated period we performed functional studies on the role of protein tyrosine kinases (PTKs), protein tyrosine phosphatases (PTPs), transmembrane adaptor proteins, stromal interacting molecule 1 (STIM1), and tetraspanins in the regulation of such important FcεRI- and KIT-mediated activation events as tyrosine phosphorylations and dephosphorylations of the receptor and other kinase and phosphatase substrates, calcium responses, degranulations, production of cytokines and chemokines, and/or chemotaxis. We used numerous state-of-the-art technologies to determine structure-function relationships of the molecules under study. We focused on the mouse system, in which we could use unique mouse strains defective in selected surface molecules and bred at the IMG. We adopted and modified new methods for manipulating expression of the molecules of our interest. Part of our effort was also directed toward development of new research tools, which resulted in production of unique recombinant proteins, monoclonal antibodies, DNA aptamers, and methods for gene manipulation and genotyping. The results were published in 12 original research articles and four review articles (in bold in the text below). New methods and probes with commercial potential have been commercialized in business-oriented partners, spin-off companies of IMG.

2.2. FcεRI-mediated signalling and phosphatases

At the beginning of the evaluated period we tested the hypothesis that changes in the activity and/or topography of PTKs and PTPs could play a major role in the FcεRI-induced cell activation (**Heneberg et al., 2010**). These experiments partly represented continuation of our previous studies initiated almost 20 years ago in which we described the role of SRC-family kinase LYN in activation of mast cells by aggregation of the glycosylphosphatidylinositol-anchored molecules Thy-1 (Dráberová and Dráber, 1993). At the time when the experiments started, it was clear that the binding of multivalent antigen to the IgE-FcεRI complexes induces tyrosine phosphorylation of the receptor subunits. However, it was not clear how the binding is communicated across the plasma membrane to the cellular interior and two major models had been considered, the "transphosphorylation model" and the "lipid raft model". The "transphosphorylation model" was based on observations that a small fraction of LYN kinase is constitutively bound to FcεRI in the absence of immunoreceptor tyrosine-based activation motif (ITAM) phosphorylation. When IgE-FcεRI complexes are aggregated by multivalent antigen, LYN bound to one receptor could phosphorylate ITAMs on the adjacent receptor and initiate the signalling pathway. The

alternative "lipid raft model" postulated that LYN kinase is not pre-associated with FcεRI but instead is separated from it into membrane microdomains called lipid rafts; this prevents LYN-mediated FcεRI phosphorylation in non-activated cells. After activation, the aggregated FcεRI associates with lipid rafts, and only this pool of the FcεRI is tyrosine phosphorylated. This model was supported by experiments showing that LYN kinase

Both these theories were challenged by experiments in which we examined activation of rat basophilic leukaemia cells (RBL) or mouse bone marrow-derived mast cells (BMMCs) by phosphatase inhibitors, H_2O_2 or pervanadate. These compounds induced phosphorylation of the FcεRI β and γ subunits, similarly as FcεRI aggregation, but in sharp contrast to antigen-induced activation, neither H_2O_2 nor pervanadate induced any changes in the association of FcεRI with detergent-resistant membranes and in the topography of FcεRI detectable by electron microscopy on isolated plasma membrane sheets. In cells stimulated with pervanadate, H_2O_2, or antigen, enhanced oxidation of active site cysteine of several PTPs was detected. Interestingly, most of the oxidized phosphatases bound to the plasma membrane were associated with actin cytoskeleton. Several PTPs (SHP-1, SHP-2, HePTP and PTP-MEG2) showed changes in their enzymatic activity and/or oxidation state during the activation. Based on these and other data we proposed that down-regulation of enzymatic activity of PTPs and/or changes in their accessibility to the substrates play a key role in initial tyrosine phosphorylation of the FcεRI. We conclude that the “transphosphorylation model” and “lipid raft model” do not explain all experimental data and we proposed a new model, “PTK-PTP” equilibrium model, suggesting that a fraction of LYN kinase in resting cells is associated with FcεRI and phosphorylates it. This activity is counterbalanced by the action of PTPs. After FcεRI triggering there is a shift in the PTK-PTP steady state in favour of PTKs. This model is supported by some other studies using various experimental approaches and other immunoreceptors [reviewed in (**Bugajev et al., 2010**)]. This work was done exclusively in our laboratory, except for the mass spectrometry, which was done at the Institute of Microbiology.

To determine the role of FcεRI subunits in the initial stages of mast cell activation, we previously produced a variant of RBL cells deficient in the expression of the FcεRI-γ subunit. No FcεRI was detectable on the surface of such cells (Dráberová and Dráber, 1995). In collaboration with B.A. Helm, University of Sheffield, UK we transfected various mutant FcεRI-γ subunits into the FcεRI-γ-deficient RBL cells and analysed surface expression of the FcεRI and FcεRI-induced degranulation. We found that mutant FcεRI-γ subunits with polar or non-polar amino acids at position T22 are capable of restoring surface expression of the FcεRI and degranulation in FcεRI-deficient RBL cells (**Rashid et al., 2012**). The data supported the concept that the mechanism of assembly of the FcεRI tetramer (αβγ2) is different from T cell receptor and some other immunoreceptors in which the assembly is based on the 3-helix assembly model. Our laboratory provided mutant cells and participated in analysis and writing of the manuscript (Pe. Draber was the corresponding author). The laboratory in Sheffield prepared mutant FcεRI-γ subunits and performed transfection and functional studies.

We also participated in a study focused on elucidating the role of Stat5 in FcεRI signalling (**Pullen et al., 2012**). Previous studies indicated that Stat5 is required for mast cell development, survival, and IgE-mediated function. Stat5 tyrosine phosphorylation is swiftly and transiently induced by activation of

FcεRI. However, the mechanism for this mode of activation was unknown. We found that Stat5 co-localizes with FcεRI in antigen-stimulated mast cells. We confirmed this membrane localization by depleting lipid rafts, which ablated Stat5 tyrosine phosphorylation. Through the use of various pharmacological inhibitors and murine knock-out models, we found that IgE-mediated Stat5 activation is dependent upon FYN kinase and independent of SYK, LYN, PI3K, AKT, BTK, and JAK2. Immunoprecipitation experiments revealed that unphosphorylated Stat5 pre-associates with FYN, and that this association is diminished significantly during the activation. SHP-1 tyrosine phosphatase deficiency enhanced Stat5 phosphorylation. This effect was more apparent with the knock-out of Gab2, a scaffolding protein that docks with multiple negative regulators including SHP-1, SHP-2, and Lyn. Targeting of Stat5A or B by gene deletion or specific siRNA pools revealed that IgE-mediated mast cell cytokine production is selectively dependent upon the Stat5B isoform. The combined data implicated that FYN is the major positive mediator of Stat5 after FcεRI engagement, and demonstrated distinct roles for Stat5A and Stat5B in the mast cell function. Most of the experiments in this study were performed at Virginia Commonwealth University, Virginia, US with the help of five other research institutes from US, Japan and Czech Republic. We contributed by providing unique reagents and cells, design of some experiments, evaluation of data and writing the manuscript.

2.3. FcεRI signalling and STIM1

Aggregation of FcεRI leads to tyrosine phosphorylation of numerous substrates followed by calcium response regulated by a sophisticated machinery of molecules for release of calcium from cytoplasmic stores and calcium influx from extracellular space. The key regulatory role in this process is played by STIM1 [reviewed in (Draber and Draberova, 2005). To understand the role of STIM1 in mast cell signalling we prepared monoclonal antibodies specific for STIM1 and analysed the role of STIM1 in mast cell signalling (**Hájková et al., 2011**). We found that activation of BMMCs induced by FcεRI aggregation or treatment with pervanadate or thapsigargin resulted in generation of protrusions containing microtubules. Formation of these protrusions depended on the influx of extracellular Ca^{2+}. Changes in cytosolic Ca^{2+} concentration also affected microtubule plus-end dynamics detected by microtubule plus-end tracking protein EB1. Experiments with knock-down or re-expression of STIM1 confirmed the important role of STIM1 in the formation of microtubule protrusions. Although STIM1 in activated cells formed puncta associated with microtubules in the protrusions, relocation of STIM1 to the close proximity of the cell membrane was independent of the growing microtubules. In accordance with the inhibition of Ag-induced Ca^{2+} response and decreased formation of microtubule protrusions in BMMCs with reduced STIM1, the cells also exhibited impaired chemotactic response to Ag. Based on these data we proposed that rearrangement of microtubules in activated mast cells depends on STIM1-induced store-operated calcium entry, and that Ca^{2+} plays an important role in formation of microtubule protrusions in BMMCs. It is possible that calcium-sensitive microtubule protrusions might be involved in the sensing of external chemotactic gradients of antigen or other signals reaching mast cells at inflammatory sites. This study was a joint project between two laboratories at IMG: our laboratory, focused on production of mast cells deficient in STIM1 and characterization of their degranulation, calcium response and chemotaxis, and the Laboratory of Biology of Cytoskeleton focused on analysis of cytoskeleton in STIM1-deficient and control cells. L. Janáček from the Department of Biomathematics, Institute of Physiology of the AS CR participated in evaluation of time-lapse images.

2. 4. New roles of transmembrane adaptor protein NTAL in FcεRI signalling

A considerable part of our efforts was devoted to understanding the role of the transmembrane adaptor protein NTAL (Non-T cell activation linker), which was discovered at our Institute (Brdička et al., 2002). Previously we found that NTAL-deficient cells exhibit enhanced degranulation (Volná et al., 2004). Next we examined the possible cross-talk of NTAL with cytoskeleton in FcεRI- and stem cell factor (SCF)-activated mast cells (**Tumova et al., 2010**). We found that BMMCs from NTAL-deficient mice, responding to antigen alone or in combination with SCF, exhibit reduced spreading on fibronectin, enhanced filamentous actin depolymerization and enhanced migration towards the antigen when compared to wild-type cells. No such differences between wild-type and NTAL-deficient BMMCs were observed when SCF alone was used as an activator. We also examined activities of two small GTPases, Rac and Rho, important regulators of actin polymerization. Stimulation with antigen and/or SCF enhanced the activity of Rac(1,2,3) in both NTAL-deficient and wild-type cells. In contrast, the RhoA activity decreased and this trend was much faster and more extensive in NTAL-deficient cells, indicating a positive regulatory role of NTAL in the recovery of RhoA activity. After restoring NTAL in NTAL-deficient cells, both spreading and actin responses were rescued. This study was the first report of a crucial role of NTAL in the signalling, via RhoA, to the mast cell cytoskeleton. The study was completely produced by our group.

Next we focused on the cross-talk between NTAL and tetraspanin CD9 in FcεRI- and c-Kit-activated cells (**Hálová et al., 2013**). To this end we prepared a new monoclonal antibody specific for tetraspanin CD9. Binding of the antibody to BMMCs triggered activation events which included cell degranulation, Ca^{2+} response, dephosphorylation of ezrin/radixin/moesin (ERM) family proteins and potent tyrosine phosphorylation of the NTAL adaptor protein, but only weak phosphorylation of another transmembrane adaptor protein, the linker for activation of T cells (LAT). Phosphorylation of NTAL was observed with the whole antibody but not with its $F(ab)_2$ or Fab fragments. This indicated involvement of the Fcγ receptors. As documented by electron microscopy of isolated plasma membrane sheets, CD9 colocalized with FcεRI and NTAL but not with LAT. Further tests showed that both anti-CD9 antibody and its $F(ab)_2$ fragment inhibited mast cell chemotaxis towards the antigen. Experiments with BMMCs deficient in NTAL and/or LAT revealed different roles of these two adaptors in antigen-driven chemotaxis. The combined data indicated that chemotaxis towards the antigen is controlled in mast cells by a crosstalk among FcεRI, tetraspanin CD9, transmembrane adaptor proteins NTAL and LAT and cytoskeleton-regulatory proteins of the ERM family. The entire study was performed in our group.

In previous studies we reported that NTAL was a negative regulator of FcεRI signalling in mouse mast cells (see experiments described above), whereas in other studies, using mast cells from other species we found that it was a positive regulator of FcεRI-mediated activation in human and rat mast cells (Tkaczyk et al., 2004; Draberova et al., 2007). To determine whether different methodologies of NTAL ablation [knockout in mouse cells vs knockdown in human and rat mast cells] have different physiological consequences, we compared, under well-defined conditions, FcεRI-mediated signalling events in mouse BMMCs with NTAL knockout or knockdown (**Polakovicova et al., 2014**). BMMCs with both NTAL knockout and knockdown exhibited enhanced degranulation, calcium mobilization, chemotaxis, tyrosine phosphorylation of LAT and ERK, and depolymerization of filamentous actin. These data provide clear evidence that NTAL is a negative regulator of FcεRI activation events in murine

BMMCs, independently of possible compensatory developmental alterations. To gain further insight into the role of NTAL in mast cells, we examined the transcriptome profiles of resting and antigen-activated BMMCs with NTAL knockout or knockdown and corresponding control cells. Through this analysis we identified several genes that were differentially regulated in non-activated and antigen-activated NTAL-deficient cells when compared to the corresponding controls. Some of the genes seemed to be involved in the regulation of cholesterol-dependent events in antigen-mediated chemotaxis. The combined data indicated multiple regulatory roles of NTAL in gene expression and mast cell physiology. These studies were performed exclusively in our laboratory.

2. 5. New roles of transmembrane adaptor protein PAG in FcεRI signalling

Our previous studies with murine bone marrow-derived mast cells showed that FcεRI triggering induces transient hyper-phosphorylation of LYN kinase on its C-terminal regulatory tyrosine (Tyr 487), leading to the formation of a closed inactive conformation where the SRC homology (SH)2 domain interacts with phospho-Tyr 487 and transiently decreases LYN enzymatic activity (Tolar et al., 2004). This finding was surprising because in T cells the corresponding SRC family kinase (SFK), LCK, showed decreased tyrosine phosphorylation of the C-terminal regulatory tyrosine and enhanced enzymatic activity after activation through T cell immunoreceptor. Phosphorylation of the C-terminal inhibitory tyrosine in SFKs is catalysed by the C-terminal SRC kinase (CSK), a cytoplasmic PTK that can be anchored through its SH2 domain to the transmembrane adaptor protein PAG (phosphoprotein associated with glycosphingolipid-enriched microdomains). The role of PAG as a negative regulator of immunoreceptor signalling has been examined in several model systems, but no functions *in vivo* have been determined [reviewed in (**Draber et al., 2012**)].

In our recent publication we examined the activation of mouse BMMCs with PAG knockout, PAG knockdown, and corresponding controls (**Draberova et al., 2014**). Our data showed that PAG-deficient mast cells exhibit impaired antigen-induced degranulation, extracellular calcium uptake, tyrosine phosphorylation of several key signalling proteins (including FcεRI subunits, SYK, and phospholipase C), production of several cytokines and chemokines, and chemotaxis. The enzymatic activities of the LYN and FYN kinases were increased in non-activated cells, suggesting the involvement of LYN- and/or FYN-dependent negative regulatory loops. When BMMCs from PAG knockout mice were activated via KIT receptor, enhanced degranulation and tyrosine phosphorylation of the receptor were observed. *In vivo* experiments showed that PAG is a positive regulator of passive systemic anaphylaxis. The combined data indicated that PAG can function both as a positive and negative regulator of mast cell signalling depending upon the signalling pathway involved. The entire study was performed in our laboratory, except for production of mice with PAG knockout, which were produced within the framework of collaboration with the Center for Computational and Integrative Biology, Broad Institute of Harvard University and Massachusetts Institute of Technology Boston, Massachusetts, USA (R.J. Xavier and B. Seed).

2. 6. New research tools and methods

Genotyping and gene expression analyses by polymerase chain reaction (PCR) have been extensively used on 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 (Kovarova and Draber, 2000; Shaik et al., 2008). To determine whether the assays can be further simplified and whether it is possible to find conditions for quantitative PCR (qPCR) genotyping from the whole blood in the absence of DNA purification, we analysed new enhancers and their combinations (**Horáková et al., 2011**). We found that several DNA dyes (SGI, SYTO-9, SYTO-13, SYTO-82, EvaGreen, LCGreen or ResoLight) exhibited optimum qPCR performance in buffers of differing salt composition. Fidelity assays demonstrated that the observed differences were not caused by changes in Taq DNA polymerase-induced mutation frequencies in PCR mixes of different salt composition or containing different DNA dyes. In search for a PCR mix compatible with all the DNA dyes, and suitable for efficient amplification of difficult-to-amplify DNA templates, such as those in whole blood, of medium size and/or GC-rich, we found excellent performance of a PCR mix supplemented with 1,2-propanediol and 0.2 M trehalose (TP PCR mix). These two additives together decreased the DNA melting temperature and efficiently neutralized PCR inhibitors present in blood samples. They also made possible more efficient amplification of GC-rich templates than betaine and other previously described additives. Furthermore, amplification in the presence of the TP PCR mix increased the robustness and performance of routinely used qPCRs with short amplicons. The entire study was performed in our laboratory. At present, the PCR mix containing trehalose and 1,2-propanediol is commercially available (http://www.top-bio.cz).

Reliable genotyping also requires use of hot-start PCR in which the enzymatic activity of DNA polymerase is reversibly blocked e.g. by antibody. In our previous studies we prepared a hybridoma cell line producing a monoclonal antibody specific for Taq DNA polymerase and used it for hot-start PCR (Shaik et al., 2008). However, antibodies isolated from cultured cells are suboptimal for hot-start PCR. In the evaluated period, we decided to prepare synthetic DNA aptamers as a better tool for the hot-start PCR. From the library of random DNA oligonucleotides we selected new DNA aptamers that specifically block the enzymatic activity of Taq DNA polymerase. When compared to previously prepared anti-Taq antibody, the aptamer has several advantages, including (1) reversible denaturation at temperatures up to 100^{o}C, (2) low molecular weight, (3) well-defined composition without contaminants from mammalian cells (aptamers are prepared synthetically), (4) resistance to the action of proteases (possibility to prepare and store PCR mixes at room temperature). Anti-Taq aptamers were selected and completely characterized in our laboratory. At present, the new anti-Taq aptamer is used in most PCR assays performed in our laboratory as well as for hot-start PCRs in commercially available products (http://www.top-bio.cz).

Our studies also require simple and sensitive methods for detection of low concentrations of various cytokines and some other proteins in complex biological fluids. This is especially important when monitoring the immune responses under various physiological and pathophysiological conditions *in vivo* or following production of these compounds in *in vitro* systems. Cytokines and other immunogenic molecules are predominantly detected by enzyme-linked immunosorbent assays (ELISA) and newly also by immuno-PCR (iPCR). To simplify iPCRs we developed a new assay in which antibodies are connected

with multiple DNA templates through gold nanoparticles (Au-NPs) to form a new class of detection reagents (**Potuckova et al., 2011**). We compared functionalized Au-NP-based iPCR (Nano-iPCR) with standard ELISA and iPCR for the detection of interleukin (IL)-3 and SCF. The same immunoreagents (IL-3- and SCF-specific polyclonal antibodies and their biotinylated forms) were used throughout the assays. The obtained data showed that both Nano-iPCR and iPCR were superior in sensitivity and detection range than ELISA. Furthermore, Nano-iPCR was easier to perform than the other two methods. Nano-iPCR was used for monitoring changes in the concentration of free SCF during growth of mast cells in SCF-conditioned media. The results showed that growing cultures gradually reduced the amount of SCF in culture supernatants to 25 % after 5 days. The data indicate that Nano-iPCR assays may be preferable for rapid detection of low concentrations of cytokines in complex biological fluids. The commercial availability of monoclonal antibodies suitable for detection of cytokines and other targets by Nano-iPCR and the easy preparation of functionalized Au-NPs reduce the expenses for target protein quantification at least 10 times when compared to assays based on commercial ELISA kits. The entire study was performed in our laboratory. Later, in collaboration with other partners who provided clinical samples, we validated the method for detection of Tau protein and confirmed that Nano-iPCR is superior in sensitivity and detection range to ELISA for tau protein detection (**Stegurova et al., 2014**). The sensitivity of the assay was also confirmed in another study performed in collaboration with the Laboratory of Biology of Cytoskeleton, IMG (**Dráberová et al., 2013**). Our laboratory was involved in Nano-iPCR analysis.

An integral part of our research is production of unique probes (recombinant proteins and monoclonal antibodies) recognizing signal-transduction molecules. For example, we prepared new monoclonal antibodies specific for STIM1 (**Hájkova et al., 2013**) or CD9 (**Hálová et al., 2013**). Based on business agreement, the hybridoma cell lines were transferred to a spin-off company of IMG, Exbio, a.s., for commercial use of the antibodies.

2.7. Review publications

Most publications of our laboratory prepared in the evaluated period were research papers. However, we also published several review articles. They partly reflected our joint effort in the COST EU activities. Probably the most important was a review article in JACI, in which we and investigators from six other laboratories summarized our thoughts and data about molecular targets on mast cells and basophils for novel therapies. This article deals with molecular targets in their complexity and involves not only intracellular signalling pathway targets (described by two authors from our laboratory, Pe. Draber and I. Polakovicova), but also stimulatory or inhibitory receptors on the plasma membrane and soluble mediators released from mast cells and basophils, described by other authors (**Harvima et al., 2014**).

We also published an extensive review on mast cell chemotaxis, chemoattractants and signalling events (**Halova et al., 2012**), a review on transmembrane adaptor proteins in the FcεRI signalling (**Draber et al., 2012**), and a review on the molecular mechanisms of FcεRI-induced cell activation (**Bugajev et al., 2010**). All these reviews were produced exclusively in our laboratory with the exception of a review on

transmembrane adaptor proteins, in which one co-author (F. Levi-Schaffer) was from Israel. Finally, we produced a review on microscopy assays for evaluation of mast cell migration and chemotaxis (**Bambouskova et al., 2014**). In this review we described, for the first time, a new method developed in our laboratory for chemotaxis in homemade chambers. This method allows characterization of the directional movement of cells towards a chemoattractant embedded in an agarose cone and slowly released from the cone to the medium. After attachment of cells to a fibronectin-coated surface, the chemoattractant concentrated in the agarose cone is added. Movement of cells towards the cone (weighted down with glass beads) starts after an initial lag period of sensing the concentration gradient of the chemoattractant. As a relatively simple and cheap alternative to expensive commercial systems this method is newly used in our laboratory for observation of the cell behaviour and changes in morphology during chemotaxis. This review is a joint effort of our laboratory (M. Bambousková and Pe. Dráber), focusing on chemotaxis, and the Laboratory of Biology of Cytoskeleton focusing on microscopy techniques.

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(Authors and/or co-authors of the evaluated team are in bold; corresponding author from the evaluated team is marked by asterisk; titles of the papers published during the evaluation period are underlined)

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Abbreviations used: Au-NPs, gold nanoparticles; BMMC, bone marrow-derived mast cell; CSK, C-terminal SRC kinase; ELISA, enzyme-linked immunosorbent assay; ERM, ezrin/radixin/moesin, FcεRI, high affinity IgE receptor; IL-3, interleukin; iPCR, immuno-PCR; ITAM, immunoreceptor tyrosine-based activation motif; LAT, linker for activation of T cells; NTAL, Non-T cell activation linker; PAG, phosphoprotein associated with glycosphingolipid-enriched microdomains; PCR, polymerase chain reaction; PTK, protein tyrosine kinases; PTP, protein tyrosine phosphatase; qPCR, quantitative PCR; RBL, rat basophilic leukaemia cells; SCF, stem cell factor; SFK, SRC family kinase, SH, SRC homology; STIM1, stromal interacting molecule 1; TP PCR mix, PCR mix supplemented with 1,2-propanediol and 0.2 M trehalose.

And FcεRI are located, respectively, in low-density and high-density fractions of sucrose gradient after ultracentrifugation of lysates from non-activated cells solubilized with Triton X-100. After activation the majority of both LYN and FcεRI from cells analysed as above was found in low-density fractions [reviewed in **(Bugajev et al., 2010**)].

Research Report of the team in the period 2010–2014

Institute	Institute of Molecular Genetics of the CAS, v. v. i.

Scientific team	Laboratory of Cell Signalling and Apoptosis

2.1. Introduction

The main focus of the Cell Signalling and Apoptosis group in the past not only five years has been the initiation, regulation and possible exploitation of signalling from activated death receptors from the TNFR family with a more detailed focus on TRAIL receptors. In respect of TRAIL-triggered signalling we analysed the role of proximal events such as activation of caspase-8, participation of endocytosis and receptor-specific signalling in the activation of pro- and non-apoptotic pathways. We also focused on unravelling new drugs and approaches that can enhance TRAIL-induced apoptotic signalling in resistant cancer cells. Last but not least, we also analysed the activation and mechanism of TRAIL-induced signalling in human embryonic stem cells. In addition to TRAIL and its receptors, we also investigated the molecular and functional properties of another member of the death receptor family, still a bit mysterious Death Receptor 6 (DR6), and uncovered its prominent posttranslational modification and expression in activated T cells.

2.2. Uncovering novel mechanisms in TRAIL receptor signalling

We focus on assessment of the proximal events in TRAIL-induced pro-apoptotic signalling. In order to contribute to the still unclear role of endocytosis and post-endocytic signalling we evaluated the possible regulatory role of endosomal acidification in TRAIL-induced, DISC-mediated activation of caspase-8. In our paper published in the FEBS Journal (Horova et al., 2013) we documented that blocking the endosomal acidification by specific inhibitors of the vacuolar ATPase (vATPase) bafilomycin A1 or concanamycin markedly suppressed and delayed TRAIL-induced DISC formation, activation of caspase-8, and thus pro-apoptotic signalling. This suppression was enhanced by blocking the mitochondrial amplification loop in Bax-deficient cells and was not accompanied by significant changes in the levels of sphingolipids. Neither was this suppression of TRAIL-induced pro-apoptotic signalling related to the late endocytosis or to destabilization of lysosomes. We, however, observed profound suppression of the formation of multivesical bodies in the cells with blocked endosomal acidification, which could be related to inefficient activation of caspase-8. With the exception of sphingolipid assays and analysis of Rab5/7 roles, we performed all other experiments.

Still focusing on caspase-8 activation, we in collaboration with J.C. Martinou lab (University of Geneva) analysed the effect of TRAIL-activated caspase-8 in TRAIL-resistant colorectal cancer cells (Somasekharan et al., 2013). We discovered that activated caspase-8 in these cells, instead of inducing apoptotic signalling, activates ROCK1 kinase and thus contributes to transient blebbing and to the

migratory phenotype of the affected cells. In this joint project, we performed apoptotic assays and determined expression of TRAIL receptors and DISC composition.

In respect of yet unknown expression and function of death, particularly TRAIL, receptors, in collaboration with A. Hampl group (MU Brno) we also analysed the status and function of these receptors in human embryonic and induced pluripotent stem cells (hESCs and hIPSC) (Vinarsky et al., 2013). We uncovered that in both types of these stem cells, both pro-apoptotic TRAIL receptors but not Fas/CD95 and only low levels of TNFR1 are mainly expressed and functional. Although both hESCs and hIPSCs are intrinsically resistant to TRAIL-induced apoptosis, they can be sensitized to it by some known sensitizers (such as by us uncovered homoharringtonine, HHT). The resistance of hESCs is mainly related to the increased expression of anti-apoptotic Mcl-1, as its shRNA-mediated downregulation sensitized hESCs to TRAIL. We performed apoptosis assays, some Western blots and shRNA-mediated downregulation of Mcl-1.

Human TRAIL is unique among the death receptors, having two pro-apoptotic receptors DR4 and DR5 that are to a variable extent expressed in all somatic cells. Currently, there are also controversies about the cell type and condition-related roles of either of these receptors in transducing pro- and non-apoptotic signalling. In order to contribute to the resolution of their specific functions, we have prepared novel variants of DR4- and DR5-specific ligands and in detail analyse their signalling in several types human cancer cells. So far, we have uncovered that despite similar expression of DR4 and DR5 in colorectal and pancreatic cancer cells, the pro-apoptotic activation of caspase-8 as well as non-apoptotic activation of NF-kB signalling is predominantly mediated by DR5. Interestingly, the receptor DISC formation and the initial DISC-mediated processing of caspase-8 is similar for DR4 and DR5, indicating a post-DISC enhancement of DR-triggered signalling (manuscript in preparation).

2.3. Enhancing TRAIL-induced apoptosis in resistant cancer cells

The acquired resistance of primary tumour cells to TRAIL-induced apoptosis is often a hurdle in plausible use of the recombinant TRAIL or TRAIL-related pro-drugs in anti-cancer therapy. Thus one of the currently widely investigated possibilities is identification and characterization of either currently used or developing drugs that could function as sensitizers, enhancing TRAIL-induced apoptosis in resistant cancer cells. In our lab, as well as in several collaborative projects, we uncovered and functionally described several of these potential sensitizers.

In collaboration with P. Bartunek group here at IMG AS CR, by high-throughput screening (HTS) we picked homoharringtonine as a very potent enhancer of TRAIL-induced apoptosis of resistant colorectal cancer cells HT-29 (Beranova et al., 2013). Homoharringtonine (HHT, also under trademark Omacetaxine used for therapy of chronic myeloid leukaemia) in 50-100 nM concentration strongly enhanced TRAIL-induced apoptosis of HT-29 and of several other TRAIL-resistant cells of not only colorectal origin (RKO, SW620, Panc-1, PaTu and other) and its co-application with TRAIL basically led to elimination of these resistant cells both in vitro and in vivo (tumours implanted into immunodeficient mice). One of the main though not exclusive sensitizing functions of HHT is rapid downregulation of the expression of anti-apoptotic proteins cFLIP and Mcl-1 (HHT as a translation inhibitor blocks translation of these short-lived proteins). However, individual shRNA-mediated suppression of either cFLIP or Mcl-1 in HT-29 cells did not lead to such efficient TRAIL-induced growth suppression as with HHT, indicating that

either simultaneous downregulation of both proteins is required or HHT triggers some additional anti-apoptotic signalling. In this study, we performed all apoptotic assays, analysis of DISC, preparation of HT-29 cells with suppressed expression of Mcl-1 and cFLIP, and some growth suppression assays.

In the frame of other mutual collaborations, we helped to analyse the effect of several other agents that could sensitize resistant cancer cells to TRAIL-induced apoptosis. Together with A. Pintzas lab (IBRB, Athens) we tested BRaf inhibitor PLX4720 together with Hsp90 inhibitor 17-AAG in their sensitization of resistant colorectal cancer cells harbouring mutated BRaf to TRAIL-induced apoptosis (Oikonomou et al., 2011). Both agents, especially when combined, sensitized HT-29 and RKO cells to TRAIL-mediated apoptosis at least in part by upregulation of the DR5 receptor expression. We performed several apoptosis assays, Western blots and analysis of TRAIL receptor DISC.

In collaboration with P. Klener group (Charles University, First Faculty of Medicine, Prague) we characterized CDK inhibitor roscovitine as a potent enhancer of TRAIL-induced apoptosis in leukaemia and lymphoma cell lines and in primary cells from patients (Molinsky et al., 2013). We found that roscovitine treatment of TRAIL-resistant K562 or partly resistant Ramos cells markedly sensitized them to TRAIL-induced apoptosis, but did not affect TRAIL DISC formation or caspase-8 activation in these cells. However, roscovitine caused pronounced downregulation of anti-apoptotic proteins Bcl-XL and Mcl-1 and rewired ratios between pro- and anti-apoptotic proteins in mitochondria. In this communication, we analysed TRAIL receptor expression, DISC formation, and expression of proteins from the Bcl-2 family.

In several other reports our contribution was less pronounced - we provided reagents, consulted results or ran some minor experiments. These papers recently resulted from joint collaboration with I. Kim in Seoul pointing to curcumin, CGP74514A or emetine as variously efficient sensitizers of resistant cancer cells to TRAIL-induced apoptosis (Han et al., 2014; Park et al., 2013; Park et al., 2014), B. Zhivotovsky (KI Stockholm) assessing the role of calcium chelators in suppressing cFLIP-S expression, thus contributing to TRAIL-induced apoptosis of resistant lung cancer cells (Kaminskyy et al., 2013), or A. Kozubik (IBF AS CR, Brno) characterizing novel cisplatin derivative LA-12 in sensitizing colorectal and prostate cancer cells to TRAIL (Vondalova Blanarova et al., 2011).

At present, we prepare a manuscript describing Streptomyces metabolite manumycin A as a potent enhancer of TRAIL-triggered apoptosis in resistant cancer cells of different origin (Klima et al, in preparation). Manumycin A-dependent sensitization to TRAIL-induced apoptosis is likely connected with its probable function as mitocan (i.e. a compound that via targeting mitochondria eliminates cancer cells or at least sensitizes them to apoptosis) and uncoupling-mediated increased production of reactive oxygen species (ROS).

2.4. Molecular and functional characterization of Death receptor 6

Death receptor 6 belongs to one of the least described receptors from the death receptor family and its inactivation in mice is connected with mild hematopoietic phenotype (enhancement of B cell proliferation and activation; Schmidt CS et al., J Exp Med. 197(1):51-62, 2003). In order to contribute to uncovering the molecular and functional properties of DR6, in 2006 we started its characterization.

Initially, we were puzzled by the apparent discordance between its calculated molecular weight (80 kDa) and its apparent migration in SDS polyacrylamide gels (120-130 kDa). In subsequent studies, we discovered that this shift in apparent molecular weight is largely caused by heavy posttranslational modification, mainly glycosylation of its extracellular part (Klima et al., 2009). The extracellular part of DR6 is both N- and O-glycosylated and we mapped N-glycosylation to all six asparagines. The major site for mucine-like O-glycosylation was associated with the "stalk" domain juxtaposed to the cysteine-rich domains (CRDs). We also found that the intracellular membrane-proximal Cys368 is palmitoylated, but its palmitoylation is apparently in contrast to N-glycosylation not required for DR6 targeting to lipid rafts. We performed the majority of experiments; just the analysis of DR6 palmitoylation was carried out in collaboration with V. Korinek group at IMG AS CR.

In the subsequent study, we focused on DR6 expression and its possible role in hematopoietic cells. For the first time we showed that both DR6 mRNA and protein expression is upregulated in activated both CD4+ and CD8+ T cells (Klima et al., 2011). However, upregulation of DR6 expression in either mouse or human T cells is just transient and is apparently dependent on TCR-mediated activation of both NF-kB and NF-AT signalling pathways. Interestingly, TCR activation in DR6-positive T cell leukaemia cells Jurkat leads to strong suppression of DR6 expression at the transcription level. DR6 expression in T cells could thus serve for fine tuning of T cell activation. With the exception of the preparation of mouse T cells, the majority of the experimental studies were done in our lab.

2.5. Other collaborative projects (Petrickova et al, Leahomshi et al, Makrodouli et al)

In addition to the mainstream focus of our group, which is death receptor-mediated signalling, we also collaborated on several more or less related projects. We participated in the analysis of colabomycine E, another metabolite of Streptomyces that likely suppresses inflammation by blocking IL-1b release, but in contrast to manumycin A could not either induce or enhance apoptosis of cancer cells (we performed the apoptosis assays) (Petrickova et al., 2014). In collaboration with P. Klener group we analysed the molecular differences between resistant K562 erythroleukaemia cells and their TRAIL-sensitive clones. We helped to uncover that in TRAIL-sensitive clones, both pro-apoptotic TRAIL receptors and several key components of the pro-apoptotic signalling, such as caspase-8, Bid or Bim are upregulated. We performed analysis of the TRAIL DISC composition and determined the expression of TRAIL receptors (Leahomschi et al., 2013). Aiding A. Pintzas group we also participated in the analysis of oncogenic BRaf and HRas in cancer cell migration and prepared colorectal cancer cells with ncreased and suppressed expression of these oncogenes (Makrodouli et al., 2011).

2.6. References

(Authors and/or co-authors of the evaluated team are in bold; corresponding author from the evaluated team is marked by asterisk; titles of the papers published during the evaluation period are underlined)

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Research Report of the team in the period 2010–2014

Institute	Institute of Molecular Genetics of the CAS, v. v. i.

Scientific team	Laboratory of Molecular Immunology

2. Research Report of the team in 2010–2014

Characteristics of main results achieved by the team in the evaluated period. In the description of the result achieved in collaboration with other teams, the share of the team in its creation must be clearly specified (i.e. what specific activity the team contributed to the result). Maximum length of 10 pages.

2.1. Introduction

The main research interest of the Laboratory of Molecular Immunology has been traditionally aimed at understanding the molecular mechanisms of signal transduction by leukocyte plasma membrane receptors, with special emphasis on the involvement of membrane raft microdomains in these processes. To this aim, we have been using standard biochemical, molecular and cell biological approaches, including preparation of specific research tools – monoclonal and polyclonal antibodies, and also relevant mouse models, including gene knock-outs.

The results were published in 18 original research articles and four review articles (indicated in bold in the text below). Some of the by-products of our basic research with commercial potential, namely monoclonal antibodies, have been commercialized by biotech companies originated as IMG spin-off's.

In the evaluated period the Laboratory of Molecular Immunology has undergone major organizational changes – since 1. 1. 2013 its key junior researcher, Dr. Tomáš Brdička, became the head of a newly established junior research group (Laboratory of Leukocyte Signalling - http://www.img.cas.cz/research/tomas-brdicka/). At about the same time a new junior researcher joined our team (Dr. Meritxell Alberich-Jorda), who also brought a new research topic, namely molecular mechanisms of leukemogenesis, nicely complementary with our traditional topics. Since 1. 1. 2015 Dr. Alberich-Jorda also became independent as an "IMG Fellow", with her small junior research group (Laboratory of Haematooncology - http://www.img.cas.cz/research/meritxell-alberich-jorda/).

The research of the Laboratory of Molecular Immunology has been supported by research grants from the Czech Science Foundation, Ministry of Education, Youth and Sports, and Grant Agency of the Ministry of Health.

2.2. Membrane rafts and their involvement in leukocyte receptor signalling

Our interest in leukocyte membrane rafts started more than 25 years ago; we were among the first groups that demonstrated association of Src-family kinases with these microdomains (Stefanova et al., 1991) and we have discovered a number of raft-associated transmembrane adaptor proteins and GPI-

anchored proteins. An intensely discussed topic in this field has been the relationship between “detergent-resistant microdomains” (DRMs) and native membrane microdomains before exposure to detergents. A study published in 2006 claimed that association of transmembrane adaptor protein LAT with T-lymphocyte raft microdomains may not be essential to its signalling function. We explained this paradoxical result in our paper (**Otahal et al., 2010**) in which we demonstrated existence of a new type of detergent-resistant membrane rafts ("heavy rafts", missed in previous studies) containing a number of biologically important leukocyte membrane signalling proteins. The detergent-resistant fragments corresponding to these microdomains share some properties with "classical" rafts but differ from them in other aspects, presumably because of different protein composition and higher ratio of proteins to lipids. The study was performed entirely in the Laboratory of Molecular Immunology.

In a follow-up study, we identified the transmembrane domain of a heavy raft-associated transmembrane adaptor protein LAX as critical for the association with this particular type of membrane microdomains (**Hrdinka et al., 2012**); the study was also performed entirely in the Laboratory of Molecular Immunology.

In the following studies (**Otahal et al., 2011**), we examined the functional importance of targeting of a key Src-family kinase regulator, cytoplasmic tyrosine kinase Csk, into various compartments of T-cell plasma membrane ("classical" rafts, the newly described "heavy rafts" and non-raft membrane). Only all the Csk constructs targeting Csk into classical rafts profoundly inhibited TCR signalling, which provides strong functional evidence for the biological importance of membrane rafts, independent of the criticized biochemical (detergent-based) approaches. These results have also important implications for a current model of Lck involvement in TCR signalling. The study was performed entirely in the Laboratory of Molecular Immunology.

2.3. Novel transmembrane adaptor proteins associated with membrane microdomains

In previous years we discovered (sometimes in parallel with our competitors) several functionally more or less important palmitoylated transmembrane adaptor proteins (TRAPs) associated with leukocyte membrane raft microdomains (LAT, NTAL, LIME) and collaborated on characterization of two non-raft ones (SIT, TRIM) – for review see Horejsi et al. 2004. In recent five years we have been dealing with several other proteins of this family, discovered mostly by *in silico* screening for predicted gene products corresponding to TRAPs. One of them is PRR7, a highly evolutionarily conserved protein found by us in T-lymphocytes, previously described only in a single paper in neurons. Our results indicate involvement of this signalling protein present in a so far poorly characterized type of membrane microdomains in the regulation of TCR signalling, T cell activation and apoptosis of activated T cells (**Hrdinka et al., 2011**). The study was performed entirely in the Laboratory of Molecular Immunology; J. Paces helped with the *in silico* screening.

Another interesting palmitoylated transmembrane adaptor protein expressed in B cells and other professional antigen-presenting cells (APCs) discovered by us was named SCIMP (**Draber et al., 2011).** It is localized in raft-like tetraspanin-enriched microdomains and quantitatively relocalizes to immunological synapse after the contact between T cell and APC. The protein is inducibly associated with key signalling molecules (kinase Lyn, adaptor SLP65, kinase Csk). The results suggest that SCIMP is involved in signal transduction after MHC-II stimulation, and therefore serves as a regulator of antigen

presentation and other APC functions. This project now continues in the new Laboratory of Leukocyte Signalling led by T. Brdicka. Substantial part of the study was performed in the Laboratory of Molecular Immunology at IMG, AS CR; it was a continuation of a project started by T. Brdicka during his postdoctoral stay in the laboratory of A. Weiss (UCSF), with participation of M. Yeung, who are therefore also among the co-authors.

Finally, we for the first time thoroughly characterized protein LST1/A (encoded by a gene located in MHCIII locus) as a palmitoylated transmembrane adaptor protein expressed specifically in leukocytes of the myeloid lineage, where it localizes to the tetraspanin-enriched microdomains and binds SHP-1 and SHP-2 phosphatases in a phosphotyrosine-dependent manner, facilitating their recruitment to the plasma membrane. These data suggest a role for LST1/A in negative regulation of signalling by a so far unidentified myeloid cell receptor (**Draber et al., 2012).** The study was performed entirely in the Laboratory of Molecular Immunology.

2.4. The roles of membrane-associated protein tyrosine kinases and phosphatases in immunoreceptor signalling

Another area of our research was represented by studies of a so far insufficiently functionally characterized transmembrane phosphatase CD148 (**Stepanek et al., 2011a).** This study for the first time thoroughly analysed the so far controversial involvement of this membrane-associated enzyme in the regulation of Src-family tyrosine kinases, in relation to the much better known transmembrane phosphatase CD45. Our data suggested that differential effects of CD148 in T cells and other leukocyte subsets are due to its dual inhibitory/activatory function and specific expression pattern. Major part of the study was performed in the Laboratory of Molecular Immunology at IMG, AS CR; it was a continuation of a project started by T. Brdicka during his postdoctoral stay in the laboratory of A. Weiss (UCSF); a part of the cytofluorometric studies on human thymocytes was performed at the Department of Pediatric Haematology and Oncology, Second Faculty of Medicine, Charles University (T. Kalina, K. Svojgr), who are therefore among the co-authors.

In addition, we set out to contribute to elucidation of the so far controversial question of mutual roles of Src-family kinases (SFK) vs. Syk kinase in initiation of the B-cell receptor signalling (**Stepanek et al., 2013).** We addressed this issue by analysing controlled BCR triggering *ex vivo* on primary murine B cells and on murine and chicken B cell lines. Chemical and Csk-based genetic inhibitor treatments revealed that SFKs are required for signal initiation and Syk activation. In addition, ligand and anti-BCR Ab-induced signalling differ in their sensitivity to the inhibition of SFKs. Our results bring important advancement in the understanding of the molecular mechanisms underlying BCR signalling, as well as the B cell–specific effects of pharmacological Src and Syk inhibitors used, tested, or considered for the treatment of various malignant and immune diseases. The study was performed entirely in the Laboratory of Molecular Immunology.

2.5. Interaction of late apoptotic and necrotic cells with vitronectin

Our previous studies indicated that late apoptotic cells are brightly stained with monoclonal antibody 2E12 produced in the laboratory of P. Stockbauer (Inst. of Haematology and Blood Transfusion, Prague). As this might identify a practically useful apoptotic marker, we decided to identify the molecule

recognized by the antibody. Using immunoisolation followed by mass spectrometry, we identified the antigen recognized by 2E12 antibody as blood serum protein vitronectin (**Stepanek et al., 2011b).** We determined that vitronectin binds to the late apoptotic and necrotic cells in cell cultures *in vitro* as well as in murine thymus and spleen *in vivo*. Confocal microscopy revealed that vitronectin binds to an intracellular cytoplasmic structure after the membrane rupture. Thus, vitronectin could serve as a marker of membrane disruption in necrosis and apoptosis for flow cytometry analysis. Moreover, vitronectin binding to dead cells may represent one of the mechanisms of vitronectin incorporation into the injured tissues. The study was performed in the Laboratory of Molecular Immunology; mass spectrometric identification was performed by P. Man (Institute of Microbiology AS CR); P. Stockbauer provided the 2E12-producing hybridoma.

2. 6. Studies of myeloid leukaemia cell biology

This topic was introduced by M. Alberich-Jorda after her joining the lab at the end of 2012 as a continuation of her previous postdoctoral work at Harvard University.

C/EPBα proteins, encoded by the CCAAT-enhancer-binding protein α gene (*CEBPA*), play a crucial role in granulocytic development, and defects in this transcription factor have been reported in acute myeloid leukaemia. Our recent study **(Liss et al., 2014**) defined the C/EBPα signature characterized by a set of genes up-regulated upon C/EBPα activation and identified histone deacetylase inhibitors as potential candidates for the treatment of certain leukaemias characterized by down-regulation of the C/EBPα signature. Our laboratory performed RT-PCR and ChIP experiments and the entire paper was written here.

M. Alberich-Jorda (with IMG affiliation) also co-authored an important Nature study on a novel RNA arising from the *CEBPA* gene locus that is critical in regulating the local DNA methylation profile (**Di Ruscio et al., 2013**).

2.7. Chimeric antigen receptors (CARs)

A member of our lab, Pavel Otahal (employed mainly at the First Faculty of Medicine, Charles University, with part-time employment at IMG) has been working with his student on construction of chimeric antigen receptors (CARs) for future clinical use. CARs using scFv specific to antigens CD19 and CD20 were constructed and their biological functions were tested *in vitro* (after lentiviral transfection into primary human T cells or Jurkat T cell line) with encouraging results. This project had so far no publication or other output and will be continuing.

2. 8. Collaborative studies with relatively minor participation of the Laboratory of Molecular Immunology

(1) We participated in elucidation of the role of Btk kinase as a positive regulator in a signalling pathway initiated by the TREM-1/DAP12 receptor complex (**Ormsby et al., 2011**). In this study, performed mostly in the laboratory of H. Cerwenka in DKFZ Heidelberg, we participated in some of the biochemical

experiments (immunoprecipitation, Western blotting) and in the study design and manuscript preparation.

(2) We participated in a study demonstrating that actin-bundling protein L-plastin is involved in recruitment of the NK-cell receptor NKG2D into lipid rafts and in NKG2D-mediated NK cell migration (**E. Serrano-Pertierra et al., 2011**). This study was performed mostly in the laboratory of C. López-Larrea, (Hospital Universitario Central de Asturias, Oviedo). The first author, a student from the Spanish lab, spent two months in our lab and learnt some methods for isolation of detergent-resistant rafts and other biochemical techniques, and with our participation performed some of the relevant experiments.

(3) We participated in a collaborative study demonstrating high expression of cytoskeletal protein drebrin in TEL/AML1-positive B-cell precursor acute lymphoblastic leukaemia (**Vaskova et al. 2011**). Our laboratory developed and characterized the novel monoclonal antibody recognizing the protein drebrin, most of the experiments were performed by our collaborators at the Second Faculty of Medicine, Charles University Prague.

(4) We participated in a collaborative study demonstrating that the adaptor protein NTAL enhances proximal signalling and potentiates corticosteroid-induced apoptosis in T-acute lymphoid leukaemia (**Svojgr et al. 2012**). Our laboratory developed Jurkat cell line transfectants expressing the NTAL protein and performed part of the biochemical signalling experiments. Most of the experiments were performed by our collaborators at the Second Faculty of Medicine, Charles University Prague.

(5) We participated in a study demonstrating association of leukocyte transmembrane protein CD99 with MHC proteins and tetraspanin protein CD81 and its recruitment to immunological synapse (**Pata et al., 2011**). Most of the experiments were performed by our collaborators at Chiang Mai University, Chiang Mai, Thailand. A part of the study (immunoprecipitations, western blottings) was performed in our lab during the 2-year stay of the first author (a PhD student) in our laboratory.

(6) We provided substantial amounts of antibody reagents for two additional studies (**Cai et al., 2011; Nachmani et al., 2014**)

2.9. Review publications

Based on our extensive experimental work in the field of membrane rafts and raft-associated proteins, in the evaluated period we also published four extensive reviews, namely on the key transmembrane adaptor protein of T cells LAT (**Horejsi et al., 2010**), broadly expressed transmembrane adaptor protein PAG (also known as Cbp) (**Hrdinka and Horejsi, 2014**), leukocyte palmitoylated transmembrane adaptor

proteins (**Stepanek et al., 2014**), and on membrane microdomains and their roles in immunoreceptor signalling (**Horejsi and Hrdinka**, **2014**).

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2.11. Teaching and popularization activities, organizing conferences

In addition to the research activities described above, the members of the team (Hořejší, Brdička, Drbal, Otáhal) have been strongly involved in teaching undergraduate and graduate students in semestral courses at the Faculty of Science, Charles University, namely the basic course of **Immunology** (attended every year by approx. 170 students), **Molecular Immunology** (approx. 30 students), **Advances in Immunology** (approx. 30 students). We (Hořejší, Brdička) also repeatedly updated a Czech textbook of immunology (**Basic Immunology,** currently already 5th edition) which is being widely used by students of various Czech universities.

In the evaluated period, we (Hořejší, Brdička, Otáhal) also published over 20 **popularization articles** on immunological topics, mostly in the Czech magazine Vesmír (analogous to Scientific American).

V. Hořejší has been the main organizer of an important bi-annual international conference (**EFIS-EJI Tatra Immunology Conference,** taking place in Slovakian mountains since 1994), always with truly excellent European and American speakers.

Research Report of the team in the period 2010–2014

Institute	Institute of Molecular Genetics of the CAS, v. v. i.

Scientific team	Laboratory of Biology of Cytoskeleton

The main research interest of the Laboratory of Biology of Cytoskeleton has been traditionally focused on understanding the molecular mechanisms governing organization of microtubules in cells under normal and pathological conditions. The organization of dynamic microtubules, formed by αβ-tubulin dimers, is controlled by microtubule organizing centres (MTOCs). One of the key components of MTOCs is γ-tubulin, which is necessary for nucleation of microtubules. In the evaluated period we performed functional studies on the role of protein tyrosine kinases, stromal interacting molecule 1 (STIM1), p21-activated kinase interacting exchange factor (βPIX), G protein-coupled receptor kinase-interacting protein 1 (GIT1) and Ca^{2+} in regulation of microtubules in activated mast cells. We analysed new functions of γ-tubulin and microtubule proteins in cancer cells. For these studies we used methods of molecular biology, biochemistry and immunology as well as a variety of microscopic techniques, including TIRF microscopy, super resolution microscopy, live cell imaging and quantification of microtubule nucleation. Part of our effort was also directed toward development of new research tools, which resulted in production of unique recombinant proteins, monoclonal antibodies and methods for quantification of microtubule proteins in body fluids. The results were published in 14 original research articles and five review articles (in bold in the text below). New probes with commercial potential have been commercialized in business-oriented partners, spin-off companies of IMG.

2.2. Microtubule reorganization in activated mast cells

Activation of mast cells by aggregation of the high-affinity IgE receptors (FcεRI) initiates signalling events leading to the release of inflammatory and allergic mediators stored in cytoplasmic granules. A key role in this process is played by changes in concentrations of intracellular Ca^{2+} controlled by store operated Ca^{2+} entry (SOCE). Although microtubules are also involved in the process leading to degranulation (Sulimenko et al., 2006), the molecular mechanisms that control microtubule rearrangement during activation are largely unknown. We found that activation of bone marrow-derived mast cells (BMMCs) induced by FcεRI aggregation or treatment with pervanadate or thapsigargin resulted in generation of protrusions containing microtubules (microtubule protrusions). Formation of these protrusions depended on the influx of extracellular Ca^{2+}. Changes in cytosolic Ca^{2+} concentration also affected microtubule plus-end dynamics detected by microtubule plus-end tracking protein EB1. Experiments with knock-down or re-expression of STIM1, the key regulator of SOCE, confirmed the important role of STIM1 in the formation of microtubule protrusions. Although STIM1 in activated cells formed puncta associated with microtubules in the protrusions, relocation of STIM1 to a close proximity of the cell membrane was independent of growing microtubules. In accordance with the inhibition of antigen (Ag)-

induced Ca^{2+} response and decreased formation of microtubule protrusions in BMMCs with reduced STIM1, the cells also exhibited impaired chemotactic response to Ag. We have proposed that rearrangement of microtubules in activated mast cells depends on STIM1-induced SOCE, and that Ca^{2+} plays an important role in the formation of microtubule protrusions in BMMCs (**Hájková et al., 2011**). The study was performed primarily in our laboratory, in collaboration with the Laboratory of Signal Transduction at IMG that provided STIM1-deficient and control cells. Software for evaluation of time-lapse images was developed at the Institute of Physiology, AS CR.

Based on these findings we have concentrated on the possibility that changes in cytosolic Ca^{2+}concentration could affect microtubule nucleation. We found that in BMMCs γ-tubulin interacts with βPIX and GIT1. Immunoprecipitation and pull-down experiments revealed that the enhanced level of free cytosolic Ca^{2+} affects γ-tubulin properties and stimulates the association of γ-tubulin with GIT1 and γ-tubulin complex proteins (GCPs). Microtubule nucleation was also affected by the Ca^{2+} level. Microtubule regrowth experiments showed that depletion of βPIX stimulated microtubule nucleation, while depletion of GIT1 led to the inhibition of nucleation when compared to control cells. Phenotypic rescue experiments confirmed that βPIX and GIT1 represent, respectively, negative and positive regulators of microtubule nucleation in BMMCs. Live-cell imaging disclosed that both proteins are associated with centrosomes. Moreover, in activated BMMCs, γ-tubulin formed complexes with tyrosine-phosphorylated GIT1. To our knowledge, this study for the first time provided a possible mechanism for the concerted action of tyrosine kinases, GIT1/βPIX proteins and Ca^{2+} in the propagation of signals leading to regulation of microtubule nucleation in activated mast cells (Sulimenko et al., 2015). The study was performed primarily in our laboratory, except for degranulation and chemotaxis assays, which were done at the Laboratory of Signal Transduction at IMG.

2.3. New functions for γ-tubulins

Mammalian γ-tubulin is assumed to be a typical cytosolic protein necessary for nucleation of microtubules from MTOCs. On the other hand, in acentriolar cells γ-tubulin is also associated with nuclei, as we demonstrated previously (Binarová et al., 2000; Dryková et al., 2003). Using immunolocalization and cell fractionation techniques in combination with siRNAi and expression of FLAG-tagged constructs, we obtained evidence that γ-tubulin is present in the nucleoli of mammalian interphase cells of diverse cellular origins. Immunoelectron microscopy revealed γ-tubulin localization outside fibrillar centres where transcription of ribosomal DNA takes place. γ-Tubulin was associated with nucleolar remnants after nuclear envelope breakdown and could be translocated to nucleoli during mitosis. Pretreatment of cells with leptomycin B did not affect the distribution of nuclear γ-tubulin, making it unlikely that rapid active transport via nuclear pores participates in the transport of γ-tubulin into the nucleus. This finding was confirmed by heterokaryon assay and time-lapse imaging of photoconvertible protein Dendra2 tagged to γ-tubulin. Immunoprecipitation from nuclear extracts combined with mass spectrometry revealed an association of γ-tubulin with tumour suppressor protein C53 located at multiple subcellular compartments including nucleoli. The notion of an interaction between γ-tubulin and C53 was corroborated by pull-down and co-immunoprecipitation experiments. Overexpression of γ-tubulin antagonized the inhibitory effect of C53 on DNA damage G_2/M checkpoint activation. The combined results indicate that aside from its known role in microtubule nucleation, γ-

tubulin may also have nuclear-specific function(s) (**Hořejší et al., 2012).** The study was performed in our laboratory, except for ultrastructural localizations, which were done at the Laboratory of Biology of the Nucleus at IMG. Quantification of tubulin expression in glioblastoma cells was partially done at Drexel University College of Medicine, Philadelphia.

In mammals, two γ-tubulin genes encode proteins which share 97% sequence identity. Previous analysis of *Tubg1* and *Tubg2* knock-out mice has suggested that γ-tubulins are not functionally equivalent. *Tubg1* knock-out mice died at the blastocyst stage, whereas *Tubg2* knock-out mice developed normally and were fertile. It was proposed that γ-tubulin 1 represents ubiquitous γ-tubulin, while γ-tubulin 2 may have some specific functions and cannot substitute for γ-tubulin 1 deficiency in blastocysts. The molecular basis of the suggested functional difference between γ-tubulins remained unknown. We found that exogenous γ-tubulin 2 was targeted to centrosomes and interacted with GCP2 and GCP4. Depletion of γ-tubulin 1 by RNAi in U2OS cells caused impaired microtubule nucleation and metaphase arrest. Wild-type phenotype in γ-tubulin 1-depleted cells was restored by expression of exogenous mouse or human γ-tubulin 2. Further, we showed at both mRNA and protein levels using RT-qPCR and 2D-PAGE, respectively, that in contrast to *Tubg1*, the *Tubg2* expression was dramatically reduced in mouse blastocysts. This indicates that γ-tubulin 2 cannot rescue γ-tubulin 1 deficiency in knock-out blastocysts, owing to its very low amount. The combined data suggest that γ-tubulin 2 is able to nucleate microtubules and substitute for γ-tubulin 1. We proposed that mammalian γ-tubulins are functionally redundant with respect to the nucleation activity (**Vinopal et al., 2012**). The study was performed in our laboratory, except for preparation of mouse oocytes and embryos, which was done at the Laboratory of Epigenetic Regulations at IMG.

In subsequent studies we demonstrated that human γ-tubulins can be discriminated according to their electrophoretic properties and we unravelled, through in vitro mutagenesis, that the differences originate in the C-terminal regions of the γ-tubulin molecules. Using epitope mapping we identified an antigenic determinant recognized by an anti-peptide monoclonal antibody that is specific for human γ-tubulin 1. We have shown both at the mRNA and protein levels that γ-tubulin 1 is the predominant isotype in primary neurons derived from human foetal cerebral cortex. Comparable amounts of both γ-tubulin isotypes were found in autopsy samples from non-pathologic cerebral cortex of human adults. Localization of γ-tubulin 1 in mature cortical neurons was confirmed by immunohistochemistry performed on surgically resected clinical samples containing non-lesional cerebral neocortex. During all-trans retinoic acid-induced neuritogenesis in neuroblastoma SH-SY5Y cells, expression of human TUBG2 was upregulated, while the expression of TUBG1 was basically unchanged. Our data suggest that while both human γ-tubulins are nucleation competent, differences in their properties and accumulation of γ-tubulin 2 in mature neurons, in the face of predominant γ-tubulin 1 expression in these cells, may reflect additional γ-tubulin 2 function(s) in neurons (Dráberová et al., Mol. Biol. Cell 25: 108-109, Abstract P144, 2014). The study was performed in our laboratory, except for immunohistochemistry, which was done at Drexel University College of Medicine.

2.4. Microtubule proteins in cancer cells

Based on our original findings that overexpression and ectopic γ-tubulin cellular distribution is linked with tumour progression in gliomas, the most prevalent brain tumours (Katsetos et al., 2006), we have examined expression and distribution of γ-tubulin in other cancer types. Both in medulloblastomas (**Caracciolo et al., 2010**) and non-small cell lung cancer (**Maounis et al., 2012**), the expression of both γ-tubulin genes was increased. In these collaborative works with Drexel University College of Medicine, we performed immunofluorescence of cell lines and quantification of transcripts. Our results together with findings from other laboratories suggest that γ-tubulin may potentially serve as a prognostic/predictive biomarker for some cancer types.

In gliomas and glioblastoma cell lines we also analysed expression of the microtubule-severing enzyme spastin. Compared to normal mature brain tissues, spastin expression and cellular distribution was significantly increased in neoplastic glial phenotypes, especially in glioblastoma multiforme. Interestingly, an enrichment of spastin in the leading edges of cells was observed in cultured T98G glioblastoma cells and in populations of neoplastic cells from surgically excised tumours. Real-time PCR and immunoblotting experiments revealed increased levels of spastin mRNA and protein expression in all glioblastoma cell lines analysed, compared to normal human astrocytes. Spastin depletion resulted in reduced cell motility and higher cell proliferation. Taken together, these results indicate that increased expression of spastin in glioblastomas might be linked to tumour cell motility and invasion. Targeting spastin may offer a promising therapeutic strategy directed against glioma cell invasion with a potentially added benefit on tumour cell response(s) to conventional cytotoxic or tubulin-targeted treatments (**Dráberová et al., 2011**). This study was a joint project between our laboratory that focused on characterization and function of spastin in cell lines (immunofluorescence, RT-qPCR, RNAi, cell proliferation, cell motility) and Drexel University College of Medicine that focused on histochemistry. Analysis of spastin isoforms was performed at the University of Illinois Chicago. The panel of gliomas was provided from University Bergen, Norway. Software for evaluation of time-lapse images was developed at the Institute of Physiology, AS CR.

2.5. Tubulin epitope exposure on microtubules

The function of the cortical microtubules is linked to their organizational state, which is subject to spatial and temporal modulation by environmental cues. The role of tubulin posttranslational modifications in these processes is largely unknown. Although antibodies against small tubulin regions represent a useful tool for studying molecular configuration of microtubules, data on the exposure of tubulin epitopes on plant microtubules are still limited. Using homology modelling we generated a microtubule protofilament model that served for the prediction of surface exposure of five β-tubulin epitopes as well as tyrosine residues. Peptide scans newly disclosed the position of epitopes detected by antibodies 18D6 (β1-10), TUB2.1 (β426-435) and TU-14 (β436-445). Experimental verification of the results by immunofluorescence microscopy revealed that the exposure of epitopes depended on the mode of fixation. Moreover, homology modelling showed that only tyrosines in the C-terminal region of β-tubulins (behind β425) were exposed on the microtubule external side. Immunofluorescence microscopy revealed tyrosine phosphorylation of microtubules in plant cells, implying that β-tubulins

could be one of the targets for tyrosine kinases. The results suggest that prediction of epitope exposure on microtubules by means of homology modelling combined with site-directed antibodies can contribute to better understanding of the interactions of plant microtubules with associated proteins (**Blume et al., 2010**). This study was a joint project between our laboratory that focused on tubulin epitope mapping and Institute of Food Biotechnology and Genomics, National Academy of Sciences of Ukraine, that focused on homology modelling.

2. 6. New research tools and methods

Tubulin has been extensively used on daily basis in our laboratory. However, tubulin is a thermolabile protein and is usually stored at -80°C or in liquid nitrogen to preserve its conformation and polymerization properties. We developed a novel method for freeze-drying of assembly-competent tubulin in the presence of non-reducing sugar trehalose. Even after prolonged storage at ambient temperature, rehydrated tubulin is capable of binding anti-mitotic drugs and assembling to microtubules that bind microtubule-associated proteins in a usual way. Electron microscopy confirmed that rehydrated tubulin assembles into normal microtubules which are able to generate motility by interaction with the motor protein kinesin in a cell-free environment. Freeze-drying also preserved pre-formed microtubules. Rehydrated tubulin and microtubules can be used for preparation of diverse *in vitro* and *in vivo* assays and for preparation of bionanodevices (**Dráberová et al., 2010**). This study was performed in our laboratory, except for microtubule gliding assay that was performed at Leibnitz Institute for Age Research-Fritz Lipmann Institute, Jena. Technical details of the method were described in a subsequent publication from our laboratory (**Dráber et al., 2014**).

Sensitive, reproducible and rapid assays are necessary to monitor tubulin changes in cytosolic pools after treatment with anti-mitotic drugs, during the cell cycle, or activation and differentiation events. We developed new assays for α-tubulin quantification. The assays are based on sandwich ELISA, and the signal is amplified with biotinyl-tyramide or immuno-PCR (iPCR). The matching monoclonal antibody pair recognizes phylogenetically highly conserved epitopes localized outside the C-terminal isotype-defining region. This makes it possible to detect α-tubulin isotypes in different cell types of various species. Immuno-PCR detection showed enhanced sensitivity and wider dynamic range when compared to ELISA with biotinyl-tyramide detection. Our results on taxol-treated and activated BMMCs demonstrated that the assays allowed sensitive quantification of tubulin in complex biological fluids (**Dráberová et al., 2013**). ELISA was developed in our laboratory and immuno-PCR amplification in the Laboratory of Signal Transduction at IMG.

Microtubule-associated protein tau detected in cerebrospinal fluid (CSF) is an important biomarker of Alzheimer's disease. We have developed ELISA with biotinyl-tyramide amplification for quantification of this protein. The signal was also amplified with Nano-iPCR. In this case antibodies were connected with multiple DNA templates through gold nanoparticles. Nano-iPCR is simpler to perform than iPCR. Nano-iPCR was superior in sensitivity and detection range when compared with ELISA (**Stegurová et al., 2014**).

ELISA was developed in our laboratory and Nano-iPCR amplification in the Laboratory of Signal Transduction at IMG. Other partners provided clinical samples of CSFs.

Deeper insight into the molecular mechanisms leading to mast cell migration and chemotaxis is essential for understanding the mast cell function in health and disease. We have successfully used new μ-Slide Chemotaxis^{3D} chambers to study directed migration of non-adherent mast cells towards the chemoattractant. The mast cell movement was monitored by time-lapse microscopy followed by data analysis (**Bambousková et al., 2014**). This methodological study was performed in our laboratory (μ-Slide Chemotaxis, data analysis) and in the Laboratory of Signal Transduction at IMG (simple motility assay, chemotaxis to chemoattractants embedded in agarose).

Methods well established in our laboratory were also applied in the other projects at IMG. We performed heterokaryon assays to study specific mRNA export (**Lounková et al., 2014**), Laboratory of Viral and Cellular Genetics. We performed 2D-PAGE analysis of oocytes (**Chalupníková et al., 2014**), Laboratory of Epigenetic Regulations.

An integral part of our research is production of unique probes (recombinant proteins and monoclonal antibodies) recognizing microtubule components. For example, we prepared the first monoclonal antibodies specific for GCP2 (**Hořejší et al., 2012**). Based on business agreement, the hybridoma cell lines were transferred to a spin-off company of IMG, Exbio, a.s., for commercial use of the antibodies.

2.7. Review publications

Most publications of our laboratory prepared in the evaluated period were research papers. However, we also published several review articles. We published a review on microtubules (**Dráber and Dráberová, 2012**) and a review on cytoskeleton in mast cell signalling **(Dráber et al., 2012**). These reviews were produced exclusively in our laboratory. We also published a review on targeting βIII-tubulin in gliomas (**Katsetos et al., 2011**) and review on mitochondrial dysfunction in gliomas (**Katsetos et al., 2013**). In these reviews one co-author was from our laboratory. Finally, we published a review on tubulins as therapeutic targets in cancer. The clinical usefulness of many anti-cancer tubulin-binding agents has been held back as a result of tumour cell drug resistance. Elucidation of the three-dimensional structure of αβ-tubulin dimer has provided an opportunity for rational drug design aimed at generating compounds that will target tubulin in therapeutically more efficacious ways compared to presently available drugs. In this review we offered a critical appraisal of the current knowledge of tubulins in cancer and an update of new anti-neoplastic microtubule-targeted treatment strategies. Specifically, we examined, across disciplines, the cellular/molecular, biochemical, clinical/pathological, and pharmacological aspects of β-tubulin isotypes, posttranslational modifications of tubulin dimers, γ-tubulin and microtubule nucleation, and microtubule regulatory proteins. We also offered appraisal of the concept of hypersensitization to tubulin-binding agents as promising therapeutic strategies in taxane resistant epithelial cancers and in high-grade gliomas (**Katsetos and Dráber, 2012).** The authorship of

this review was equally shared between our laboratory and our colleagues from Drexel University College of Medicine.

2.8. References

(Authors and/or co-authors of the evaluated team are in bold; corresponding author from the evaluated team is marked by asterisk; titles of the papers published during the evaluation period are underlined)

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Research Report of the team in the period 2010–2014

Institute	Institute of Molecular Genetics of the CAS, v. v. i.

Scientific team	Laboratory of Epigenetic Regulations

2.1. Introduction

Our research combines developmental biology, RNA biology and bioinformatics. Our primary research interest aims at understanding the molecular mechanisms governing control of gene expression during mammalian oocyte-to-embryo transition (OET); our research is **curiosity-driven**, we are not strict about staying focused on a particular molecular mechanism or a model system – as long as we have enough resources, we enjoy collaborating and exploring any interesting problem related to what we do.

OET is a fascinating process, which has important ties to biotechnology (most importantly stem cell research, generation of genetically modified organisms, and animal cloning) as well as to clinical practices (understanding molecular causes of infertility & improvement of artificial reproduction techniques). The mammalian OET occurs as a highly orchestrated sequence of transitions. At the beginning, there is a highly specialized cell – the oocyte, which resumes and completes meiosis while being released from the ovary. During this time initiates maternal mRNA degradation, which can be seen as a mechanism gradually erasing the oocyte's cellular identity. During fertilization, a zygote is formed, which subsequently activates its genome in order to execute the genome-encoded developmental program. A successful zygotic genome activation (ZGA) is an essential event in the life of every sexually reproducing organism. Importantly, ZGA is closely associated with acquisition of pluripotency in embryonic cells, i.e. the ability of cells to differentiate into any body cell type.

What follows is an overview of our research in 2010-2014 organized into thematic blocks, starting with those related to OET.

2.2 Transcriptome dynamics and maternal mRNA metabolism during OET

A large part of OET takes place in the absence of transcription. Animal cloning by nuclear transfer demonstrates that gene expression reprogramming is executed by maternally deposited cytoplasmic factors – either proteins or mRNAs. Importantly, OET transition involves extensive post-transcriptional control of maternal mRNAs. This assures elimination of maternal mRNAs, which are no longer needed, while mRNAs are maintained and translated, whose products are needed for successful ZGA. Maternal mRNA degradation facilitates erasure of oocyte identity and sets up the stage for establishment of the zygotic program. Below is the list of our contributions to understanding maternal mRNA metabolism (excluding small RNAs, these are addressed separately in section 2.3).

In 2010, we showed that many RNA-binding proteins normally found in cytoplasmic foci called p-bodies have increased concentration in oocyte's cortex while the oocyte loses typical p-bodies at the onset of its growth. We proposed that this "subcortical domain" has a function in maternal mRNA storage and

we provided some evidence supporting our hypothesis (Flemr et al., 2010). During our work on the subcortical domain, we noticed that the abundance of the decapping complex is very low in the fully-grown ovarian oocyte while it dramatically increases in an ovulated, fertilization-ready, oocyte. Since the decapping complex is an important molecular mechanism involved in mRNA degradation, we investigated its control during OET in detail. We have found that the decapping complex is dormant, i.e. *Dcp1a* and *Dcp2* mRNAs encoding the complex are not translated until resumption of meiosis, which triggers synthesis of the decapping complex from deposited mRNAs, which in turn participates in the first wave of maternal mRNA degradation (Ma et al., 2013). This work on the dormancy of decapping was significant because it provided the first specific molecular mechanism how maternal mRNA degradation is activated. This work was done in collaboration with Richard M. Schultz laboratory, with whom we had collaborative grants supported by the Ministry of Education, Youth and Sports (MEYS) (Kontakt I in 2009-2012 and Kontakt II in 2013-2015). In the first paper, my lab produced most of the data presented in the paper. In the second paper, we contributed by experimental design, high-throughput data analysis, and writing up the story.

The latest contribution to post-transcriptional control during OET was our work on ELAVL2, which was identified in a bioinformatic screen as a candidate for selective post-transcriptional control. Functional analysis showed that ELAVL2 has properties of a translational repressor that is eliminated from the oocyte during acquisition of developmental competence. This work is significant because it for the first time reveals selective protein degradation during acquisition of developmental competence and points out to the rate of translation as one of the factors that might contribute to it (Chalupnikova et al., 2014). This was a complex work, on which we used technical expertise of people outside of the lab (2D gels & time-lapse microscopy of microinjected oocytes).

We currently focus on the molecular foundation of the selectivity of mRNA degradation. While some mRNAs are naturally unstable in the oocyte, others are relatively stable and their degradation occurs in waves triggered by three major developmental transitions: resumption of meiosis, fertilization, and ZGA. We aim to develop an integrated model based on the dynamics of mRNA degradation pathways, mRNA binding proteins, and combinatorial composition of 3'UTR motifs, which would explain the observed pattern of maternal mRNA dynamics. This work has mainly the form of collaborative bioinformatic data mining on high-throughput datasets. The key collaboration is with the Department of Bioinformatics, University of Zagreb headed by Kristian Vlahovicek and it is supported by a grant from ASCR. We also collaborate with Fugaku Aoki, who generated next-generation sequencing (NGS) datasets from oocytes and early embryos and we contribute to data mining.

Since 2013, we have also paid attention to ZGA and establishment of pluripotency. Along these lines, we established a qPCR array assay for monitoring OET and the pluripotent program in embryonic stem cells. This assay constituted our contribution to the analysis of the role of Nanog in pluripotency, which was published by our collaborators in Lisbon (Abranches et al., 2014). Finally, together with Kristian Vlahovicek, Fugaku Aoki, and Richard Schultz, we put together NGS analysis of the very first wave of ZGA in mouse zygotes and showed that it is highly promiscuous and produces non-functional RNAs. This work has been accepted for publication in EMBO J recently. My contribution was coordination of NGS data mining and I played a major role in writing up the article. We plan to continue data mining of high-throughput datasets and focus on several interesting observations we have made during our work on the EMBO J article.

2.3. Role of small RNAs during OET

Small RNAs acting as sequence-specific guides for repressive ribonucleoprotein complexes represent a unique layer of control of gene expression and retrotransposon activity. Our research focuses on the role of small RNAs (microRNAs (miRNAs), short interfering RNAs (siRNAs) and PIWI-associated RNAs (piRNAs)) in the mammalian female germline. Our primary research model are mouse oocytes, which offer a unique co-existence of all three classes of small RNAs, which exhibit interesting functional overlaps and unique adaptations.

2.3.1. miRNAs during OET – dead miRNAs in mouse oocytes

At the beginning of the reported period, we published that mouse oocytes do not have p-bodies, which are intimately linked with miRNA function (Flemr et al., 2010), and that miRNA activity is suppressed from oocyte growth to early zygotes (Ma et al., 2010). These two studies were done in collaboration with Richard Schultz's lab – he simply could do experiments we could not (e.g. microinjections) or could not afford (microarray profiling on Affymetrix expression arrays); we contributed with our expertise in bioinformatics, molecular biology, and miRNA analysis. As a spin-off of these results, we also published two reviews (Svoboda, 2010; Svoboda and Flemr, 2010) and a method paper (Flemr and Svoboda, 2011). Our expertise in p-bodies and miRNA analysis laid foundations for a collaborative research on the relationship between Lsm proteins and p-bodies (Novotny et al., 2012). The last contribution to miRNA biology in the female germline during the reported period was the functional analysis of LIN28 during OET (Flemr et al., 2014). Analysis of LIN28 actually started before the discovery of non-functional miRNAs reported in 2010. LIN28 is an inhibitor of expression of let-7 miRNA, which is associated with differentiation and somatic cells and is highly abundant in oocytes but not in early embryos. In mammals, there are two paralogs of LIN28, Lin28a and Lin28b, which were highly maternally expressed. Thus, we hypothesized that LIN28 helps reprogramming gene expression to the totipotent state by suppressing zygotic expression of miRNAs. Functional analysis involving a unique use of transgenic RNAi to knock-down two genes in an oocyte-specific manner revealed that indeed, LIN28 prevents let-7 expression in early embryos, but since miRNAs are inactive anyway, the LIN28 role is functionally insignificant in mice (Flemr et al., 2014). Since mouse oocytes represent a derived case when it comes to small RNA pathways, we speculate that the role of LIN28 might be more pronounced in other mammals.

2.3.2. RNAi in mouse oocytes – a dominant sibling of miRNA

RNAi in mouse oocytes was actually the first discovered mammalian small RNA pathway and I was one of the pioneers of RNAi in mammals, showing for the first time in 2000 that long dsRNA induces sequence-specific mRNA degradation in mammalian cells (one earlier paper showed gene repression at the level of phenotype or protein but not mRNA degradation, the hallmark of RNAi). In any case, our RNAi research evolved during the period 2010-2014 in three directions:

(i) **Development of RNAi tools for knocking-down gene expression** (mainly in oocytes). In this directions, in 2010-2014 we published experimental (Sarnova et al., 2010) and method papers (Chalupnikova et al., 2013; Nejepinska et al., 2012a; Stein et al., 2013) and reviews (Malik and Svoboda, 2012; Podolska and Svoboda, 2011). These are solid technical papers/reviews, which helped to feed indicators into annual reports in order to sustain our grant portfolios. They also helped to increase our

visibility and helped to improve paper writing skills. Other than that, I think that RNAi has been dethroned by CRISPR and TALEN technologies, which we intensely use now (Jankele and Svoboda, 2014).

(ii) **Effects of dsRNA in mammalian cells**. dsRNA is RNAi trigger but can also induce many other effects. We generated many interesting stories, but I'm most proud of two of them. The first one is a transgenic experiment where we produced a genetically modified mouse ubiquitously expressing dsRNA in its cells (Nejepinska et al., 2012b). This work challenged the general notion of dsRNA toxicity in somatic cells by demonstrating tolerance to expressed dsRNA in a transgenic mouse model. It also showed that RNAi is highly effective only in the oocyte. Most of the work was carried out in my laboratory, except for production of transgenic mice and microarray profiling (outsourced at FMI). This work demonstrated an aptitude to challenge a common belief by performing an experiment predicted to fail. The publishing process was uneasy, ending first with rejection of a revised manuscript in NAR, followed by a determined rebuttal and then acceptance of the rejected manuscript as is.

The second story I'm proud of is the analysis of transcriptomes of transiently transfected plasmids in mammalian cells (Nejepinska et al., 2012c). This is the most underestimated and one of the most interesting papers from my group because we reveal how messed up have transcriptomes become upon transient transfection and that innocent-looking commercial reporter plasmids may generate dsRNA and dramatically disturb expression of co-transfected plasmids. This paper was rejected in NAR and JBC on the basis "unclear physiological relevance", very technical focus, and limited target audience. However, I maintain that everyone working with transient transfection should see these results. After spending all those money on deep sequencing and ending up with PLOS ONE, we were not discouraged enough and we moved on to get an insight into dsRNA-induced repression, which apparently affected transiently transfected plasmids more than genome-encoded genes. The reason was that we might uncover a novel dsRNA-responding pathway and provide a mechanistic explanation for a volume of data accumulated since the 1980s, and we showed that plasmid expression is somehow suppressed by PKR, a well-known dsRNA-responding protein. So, after spending more time and a lot of funding, with a grant nearing to the end and knowing that there weren't many enthusiastic reviewers following our research, we decided to dump the not-completely-finished story into PLOS ONE (Nejepinska et al., 2014). The paper is still good though, because it shows, on a set of very systematically conducted experiments, that dsRNA expression during transfection mediates a robust, sequence-independent, and PKR-dependent repression of translation of all mRNAs expressed from a transiently-transfected plasmid but endogenous genes are resistant to this repression. I regret that we were not able to work out the story until the very end but, as an adaptation to salami-slicing reporting policy combined with expiring funding, this was just one of the many sacrifices to funding deities we had to make along the way to keeping the lab moving on and up. In any case, all the work described in (ii) opened the door to our later understanding why dsRNA does not trigger RNAi in somatic cells but does it very well in the mouse oocyte.

(iii) **Molecular mechanism of RNAi in mammalian cells**. We initiated collaboration with Thomas Ohrt from Petra Schwille's group as their fluorescence correlation and cross-correlation spectroscopy approaches seemed a perfect tool. Data from this collaboration were interesting as they, for the first time, showed behaviour of selected RNAi pathway factors in the nucleus and cytoplasm. Unfortunately, as Thomas graduated and moved on, we did not develop a full paper from the data and summarized them eventually as a review (Ohrt et al., 2012). Of note is that the results confirming our conclusions were subsequently published in 2014 in Cell Reports. But I simply didn't have the manpower and funding to explore this research area as we were working on the molecular mechanism of RNAi. This

study was first invited for reviews in Science, but after rejection and a major revision, which included producing a mouse knock-out model, was published in 2013 in Cell (Flemr et al., 2013). This study brought together the past 14 years of research on RNAi in mammals as it provided a mechanistic explanation for a number of unexplained observations and opened new research directions. This work included the first use of a pair of TAL effector nucleases to cut out a defined genomic sequence. The benchwork was done in my laboratory; bioinformatic analyses were a collaboration with K. Vlahovicek (U. of Zagreb). I designed research, analysed data, prepared the manuscript, and I am the corresponding author. A Cell paper conceived and produced in a Czech lab without western co-authors is extremely rare (there is only one other locally produced Cell paper – from J. Forejt's lab (IMG) published in 1992).

Our current research focuses on several endogenous RNAi-related questions: What is its precise biological role in mouse oocytes? How do small RNAs operate in oocytes of other mammals? Which effects have ectopically enhanced RNAi in somatic cells? Can ectopically enhanced RNAi change antiviral resistance in mammals?

2.4. Chemical biology of small RNAs

In this project, we decided to take advantage of our expertise in small RNAs and Petr Bartunek's lab (IMG) chemical biology and focused on identification of small compounds that affect RNA and miRNA pathway. Although technically relatively straightforward, this project was cursed since the beginning. It was included in rejected grant applications for Wellcome Trust, ERC starter and GACR grants in 2008-2009. A common objection of the reviewers was feasibility – they did not believe that we would manage developing optimized assays and performing high-throughput screening (HTS) with two PhD students. Therefore, I used the remaining money of my EMBO start-up grant and we optimized an HTS assay and performed the pilot screen on 10 000 compounds, and I submitted the updated application for GACR funding in the spring 2011. It was deliberately removed from the competition on so utterly stupid and unbelievable administrative grounds that it made me seriously considering resignation and taking a position of a PhD coordinator in Vienna. The next year I submitted an identical application without a supplement containing CVs of my collaborators from IOCB and MUNI and the project got funded. The momentum and enthusiasm was lost though because the priority in 2012 and 2013 had to be given to analysis of endogenous RNAi in mouse oocytes. In addition, the main person who developed all the assays and completed all the raw screens went on maternity leave in 2013 and goes the second one in 2015. The project is going as planned though. So far, we summarized experience with *in vitro* screening of dsRNA processing (Podolska et al., 2014). We completed all primary screens in 2012 (five assays, 20 000 compounds each) and finished validation screens in 2014. We are now moving on to the second phase, which is identification of lead compounds and testing their molecular effects. New research directions, which appeared recently, might steer the project more towards compounds activating RNAi. This would generate an unexpected but fantastic synergy with analysis whether RNAi could be turned into a functional antiviral response in mammals.

2.5. Roles of long non-coding RNAs during OET

Long non-coding RNAs (lncRNAs) are a heterogeneous group of genome-encoded RNAs, many of which have been shown to have important biological functions. We took advantage of the outstanding bioinformatic expertise of our collaborators from the Zagreb University and we generated a catalogue of lncRNAs expressed during OET from microarray and next-generation sequencing datasets. Remarkably, many of the identified lncRNAs are novel and have unique expression patterns. We currently work on functional analysis of selected candidates. This research is performed within the Marie Curie Initial Training network RNATRAIN.

2.6. Teaching and training – worth of noticing

Apart from conducting research in my lab, I am strongly engaged in educating and training the youngest generation of Czech scientists. I am constantly gaining experience and benchmarking PhD students during training outstanding European PhD students in Heidelberg during the **EMBO YIP PhD course**, a weekly course which I am co-organizing since 2008. I have also been teaching and training biomedical PhD students in Croatia and Czech Republic during EMBO Young Scientist Forums (2009&2010 – I organized the 2010 YSF). I also presented to MSc students in Palestine (2010) and PhD students in India (2013) during EMBO YIP workshops.

I am implementing my experience from Ivy League courses into my **Epigenetics** class, a semestral class taught in English, which uses as much as possible original research data. Also the take-home exam concept is something I think I am pioneering in the Czech Republic.

With Jiri Jonak (IMG), I am a co-organizer of the largest regular Czech biomedical PhD course **Advances in Molecular Biology and Genetics**. This is a two-week lecture series broadly covering molecular biology. Since I started co-organizing it in 2008, the course is in English, includes a career development day covering career-related topics, and hosts international speakers.
http://www.img.cas.cz/education/advances-in-molecular-biology-and-genetics/

I am the main organizer of a special biomedical edition of the course **Elements of Science** (Zaklady vedecke prace), a weekly career development course modelled exactly after the EMBO YIP PhD course I'm co-organizing in Heidelberg (organized 2012-2014).

With several colleagues, I am responsible for setting up PhD student matters at IMG, which includes optimization of the selection system for PhD candidates, organizing an annual **IMG Interview Day** for selecting PhD applicants (since 2008), and an **IMG Bootcamp**, a career development training weekend for new PhD students (since 2010).

I also take part in administrative bodies related to PhD studies, I'm a deputy head of the PhD board (oborova rada) for Cell and Developmental Biology, a member of an ASCR committee for collaboration with universities, and a member for Biomedical PhD studies panel (DSPB).

Hundreds of Czech PhD students went through my courses, where speakers were systematically stripped of academic titles and left with their names, so the students would be better exposed to peer-to-peer communication. In some respect, **this work might be far more important for ASCR and Czech science in general than anything else I do.**

Finally, there is one more project I consider a major achievement during 2010-2014. It was conceived at IMG; some fifteen people spent five months in 2010 developing it to the point of an application for a science training centre for kids for a call of the Ministry of Education – only to be shut down by some short-sighted colleagues ignoring the fact that sustained public support is the only way to achieve stable funding of our research. Anyway, **Bioskop**, (www.bioskop.cz) was finally realized in 2013 by our more enthusiastic colleagues at the Masaryk University. IMG is the owner of the domain bioskop.cz and the trademark and logo of Bioskop. I (P.Svoboda) am a member of the scientific board of Bioskop.

2.7. Publication output 2010-2014

During the evaluated period, my group produced 26 publications. Twenty-two of them are listed in the Web of Science Core Collection. Altogether, these publications were cited 237 times (without self-citations) in 198 articles (without self-citations) with an average citation per item = 12.36. Authors and/or co-authors of the evaluated team are in bold; corresponding author from the evaluated team is marked by asterisk

2.7.1 Original research papers 2010-2014

Flemr, M., Ma, J., Schultz, R.M., and **Svoboda, P.*** (2010). P-body loss is concomitant with formation of a messenger RNA storage domain in mouse oocytes. *Biol. Reprod.* 82, 1008-1017.

Sarnova, L., Malik, R., Sedlacek, R., and **Svoboda, P.*** (2010). Shortcomings of short hairpin RNA-based transgenic RNA interference in mouse oocytes. *J. Negat. Results Biomed.* 9, 8.

Ma, J., **Flemr, M.,** Stein, P., Berninger, P., **Malik, R.,** Zavolan, M., **Svoboda, P.*,** and Schultz, R.M. (2010). MicroRNA activity is suppressed in mouse oocytes. *Curr. Biol.* 20, 265-270.

Nejepinska, J., Malik, R., Filkowski, J., Flemr, M., Filipowicz, W., and **Svoboda, P.*** (2012). dsRNA expression in the mouse elicits RNAi in oocytes and low adenosine deamination in somatic cells. *Nucleic Acids Res.* 40, 399-413.

Nejepinska, J., Malik, R., Moravec, M., and Svoboda, P.* (2012). Deep sequencing reveals complex spurious transcription from transiently transfected plasmids. *PLoS One* 7, e43283.

Novotny, I., **Podolska, K.,** Blazikova, M., Valasek, L.S., **Svoboda, P.,** and Stanek, D. (2012). Nuclear LSm8 affects number of cytoplasmic processing bodies via controlling cellular distribution of Like-Sm proteins. *Mol. Biol. Cell* 23, 3776-3785.

Ma, J., **Flemr, M.,** Strnad, H., **Svoboda, P.*,** and Schultz, R.M. (2013). Maternally recruited DCP1A and DCP2 contribute to messenger RNA degradation during oocyte maturation and genome activation in mouse. *Biol. Reprod.* 88, 11.

Flemr, M., Malik, R., Franke, V., **Nejepinska, J.,** Sedlacek, R., Vlahovicek, K., and **Svoboda, P.*** (2013). A retrotransposon-driven dicer isoform directs endogenous small interfering RNA production in mouse oocytes. *Cell* 155, 807-816.

Nejepinska, J., Malik, R., Wagner, S., and **Svoboda, P.*** (2014). Reporters transiently transfected into mammalian cells are highly sensitive to translational repression induced by dsRNA expression. *PLoS One* 9, e87517.

Flemr, M., Moravec, M., Libova, V., Sedlacek, R., and **Svoboda, P.*** (2014). Lin28a is dormant, functional, and dispensable during mouse oocyte-to-embryo transition. *Biol. Reprod.* 90, 131.

Chalupnikova, K., Solc, P., Sulimenko, V., Sedlacek, R., and **Svoboda, P.*** (2014). An oocyte-specific ELAVL2 isoform is a translational repressor ablated from meiotically competent antral oocytes. *Cell Cycle* 13, 1187-1200.

Abranches, E., Guedes, A.M., **Moravec, M.,** Maamar, H., **Svoboda, P.,** Raj, A., and Henrique, D. (2014). Stochastic NANOG fluctuations allow mouse embryonic stem cells to explore pluripotency. *Development* 141, 2770-2779.

Podolska, K., Sedlak, D., Bartunek, P., and **Svoboda, P.*** (2014). Fluorescence-based high-throughput screening of dicer cleavage activity. *J. Biomol. Screen.* 19, 417-426.

2.7.2. Reviews and Methods papers

I use every opportunity to write reviews into impacted journals or chapters into books. This forces me to keep up with the latest literature, helps to improve my writing skills, and gives me some opportunity to see the data from different angles. Methods papers help to get oriented in the current tools and offer an opportunity to put together our technical expertise. During 2010-14, I published 6 reviews (incl. book chapers) and 7 methods papers on topics ranging from small RNAs in oocytes to the use of guided nucleases, which are briefly summarized bellow.

Reviews

Svoboda P.* (2014) Renaissance of mammalian endogenous RNAi, *FEBS Letters* 588: 2550-2556.

Ohrt, T., Muetze, J., **Svoboda, P.,** and Schwille, P. (2012) Intracellular Localization and Routing of miRNA and RNAi Pathway Components. *Curr Top Med Chem* 12(2): 79-88.

Svoboda, P.* (2010) Why mouse oocytes and early embryos ignore miRNAs? *RNA Biol* 7(5): 559-63.

Svoboda, P. and Flemr, M.* (2010) The role of miRNAs and endogenous siRNAs in maternal-to-zygotic reprogramming and the establishment of pluripotency. *EMBO Rep* 11(8): 590-7.

Book chapters

Nejepinska, J., Flemr, M., and Svoboda, P. (2012) The Canonical RNA Interference Pathway in Animals, in *Regulatory RNAs* (B. Mallick and Z. Ghosh, Editors) Springer: Heidelberg. 111-150.

Malik, R. and Svoboda, P. (2012) Nuclear RNA silencing and related phenomena in animals. in *Toxicology and Epigenetics* (S.C. Sahu, Editor). John Wiley & Sons

Methodological reviews

Jankele, R. and Svoboda, P.* (2014) TAL effectors: tools for DNA Targeting. *Brief Funct Genomics*. 13(5):409-19.

Malik, R. and Svoboda, P.* (2012) Transgenic RNAi in mouse oocytes: the first decade. *Anim Reprod Sci*. 134(1-2):64-8.

Flemr, M. and Svoboda, P.* (2011) Ribonucleoprotein localization in mouse oocytes. *Methods* 53(2): 136-41.

Podolska, K. and Svoboda, P.* (2011) Targeting genes in living mammals by RNA interference. *Brief Funct. Genomics* 10(4): 238-47

Protocols

Stein, P., **Svoboda, P.,** Schultz RM. (2013) RNAi-based methods for gene silencing in mouse oocytes. *Methods Mol Biol*. 957:135-51.

Chalupnikova, K., Nejepinska, J., Svoboda, P.* (2013) Production and application of long dsRNA in mammalian cells. *Methods Mol Biol.* 942:291-314.

Nejepinska, J., Flemr, M., and Svoboda, P.* (2012) Control of the interferon response in RNAi experiments. *Methods Mol Biol* 820: 133-61.

Varia

Svoboda, P. (2010) Journal club. A molecular biologist explores how new genomic tools can be applied to wild animals. *Nature* 465(7298): 529.

Research Report of the team in the period 2010–2014

Institute	Institute of Molecular Genetics of the CAS, v. v. i.

Scientific team	Laboratory of Transcriptional Regulation

2.1. Introduction

The main research interest of the Laboratory of Transcriptional Regulation is the study of development and evolution of development (evo-devo). Our focus is on the role of transcription factors and signalling cascades, especially on the role of the Wnt/β-catenin signalling pathway and transcription factors of the Pax gene family. We use a combination of gain-of-function (transgenic) and loss-of-function (conditional knock-outs) approaches using laboratory mouse as a model organism to study embryonic development. We utilize several model systems including fish, cephalochordate amphioxus, annelid worm platynereis and cnidarians to study various aspects of evo-devo, especially the evolution of eyes and gene regulatory networks.

2.2. Development

Lens formation in the mouse is critically dependent on proper development of the retinal neuroectoderm that is located close beneath the head surface ectoderm. We have shown that Pax6 directly controls expression of several Wnt inhibitors such as Sfrp1, Sfrp2, and Dkk1 in the presumptive lens. In accordance, the absence of Pax6 function leads to aberrant canonical Wnt activity in the presumptive lens that subsequently impairs lens development. Thus, Pax6 is required for down-regulation of canonical Wnt signalling in the presumptive lens ectoderm (Machon et al., 2010).

We investigated the effect of ectopic activation of Wnt/β-catenin signalling during lens fibre cell differentiation. Constitutive activation of Wnt/β-catenin signalling in lens fibre cells of αA-CLEF mice resulted in abnormal and delayed fibre cell differentiation. Moreover, adult αA-CLEF mice developed cataract, microphthalmia and manifested downregulated levels of γ-crystallins in the lenses. Our results indicate that precise regulation of the Wnt/β-catenin signalling activity during later stages of lens development is essential for proper lens fibre cell differentiation and lens transparency (Antosova et al., 2013).

During mouse eye development, all retinal cell types are generated from the population of retina-committed progenitors originating from the neuroepithelium of the optic vesicle. Conditional gene inactivation provides an efficient tool for studying the genetic basis of the developing retina; however, the number of retina-specific Cre lines is limited. We reported generation of the mRx-Cre BAC transgenic mouse line in which the expression of Cre recombinase is controlled by regulatory sequences of the mouse Rx gene, one of the earliest determinants of retinal development. When mRx-Cre transgenic mice were crossbred with the ROSA26R or ROSA26R-EYFP reporter lines, the Cre activity was observed in the optic sulcus from embryonic day 8.5 onwards and later in all progenitors residing in the

neuroepithelium of the optic cup. Our results suggest that mRx-Cre provides a unique tool for functional genetic studies in very early stages of retinal development. Moreover, since eye organogenesis is dependent on the inductive signals between the optic vesicle and head surface ectoderm, the inductive ability of the optic vesicle can be analysed using mRx-Cre transgenic mice (Klimova et al., 2013).

The physical contact of optic vesicle with head surface ectoderm is an initial event triggering eye morphogenesis. This interaction leads to lens specification followed by coordinated invagination of the lens placode and optic vesicle, resulting in formation of the lens, retina and retinal pigmented epithelium. Although the role of Pax6 in early lens development has been well documented, its role in optic vesicle neuroepithelium and early retinal progenitors is poorly understood. Here we show that conditional inactivation of Pax6 at distinct time points of mouse neuroretina development has a different impact on early eye morphogenesis. When Pax6 is eliminated in the retina at E10.5 using an mRx-Cre transgene, after a sufficient contact between the optic vesicle and surface ectoderm has occurred, the lens develops normally but the pool of retinal progenitor cells gradually fails to expand. Furthermore, a normal differentiation programme is not initiated, leading to almost complete disappearance of the retina after birth. By contrast, when Pax6 was inactivated at the onset of contact between the optic vesicle and surface ectoderm in Pax6(Sey/flox) embryos, expression of lens-specific genes was not initiated and neither the lens nor the retina formed. Our data show that Pax6 in the optic vesicle is important not only for proper retina development, but also for lens formation in a non-cell-autonomous manner (Klimova and Kozmik, 2014).

2. 3. Evolution of development (evo-devo)

Evolutionary developmental biology (evolution of development or informally, evo-devo) is a field of biology that compares the developmental processes of different organisms to determine the ancestral relationship between them, and to discover how developmental processes have evolved. Several model systems including fish, amphioxus, platynereis and cnidarians are used in the Laboratory of Transcriptional Regulation to study various aspects of evo-devo, especially the evolution of eyes and gene regulatory networks.

Formation of a dorsoventral axis is a key event in the early development of most animal embryos. It is well established that bone morphogenetic proteins (Bmps) and Wnts are key mediators of dorsoventral patterning in vertebrates. In the cephalochordate amphioxus, genes encoding Bmps and transcription factors downstream of Bmp signalling such as Vent are expressed in patterns reminiscent of those of their vertebrate orthologues. However, the key question is whether the conservation of expression patterns of network constituents implies conservation of functional network interactions, and if so, how an increased functional complexity can evolve. Using heterologous systems, namely by reporter gene assays in mammalian cell lines and by transgenesis in medaka fish, we compared the gene regulatory network implicated in dorsoventral patterning of the basal chordate amphioxus and vertebrates. We found that Bmp but not canonical Wnt signalling regulates promoters of genes encoding homeodomain proteins AmphiVent1 and AmphiVent2. Furthermore, AmphiVent1 and AmphiVent2 promoters appear to be correctly regulated in the context of a vertebrate embryo. Finally, we show that AmphiVent1 is able to directly repress promoters of AmphiGoosecoid and AmphiChordin genes. Repression of genes encoding dorsal-specific signalling molecule Chordin and transcription factor Goosecoid by Xenopus and zebrafish Vent genes represents a key regulatory interaction during vertebrate axis formation. Our data

indicate high evolutionary conservation of a core Bmp-triggered gene regulatory network for dorsoventral patterning in chordates and suggest that co-option of the canonical Wnt signalling pathway for dorsoventral patterning in vertebrates represents one of the innovations through which an increased morphological complexity of vertebrate embryo is achieved (Kozmikova et al., 2011). In chordates, early separation of cell fate domains occurs prior to the final specification of ectoderm to neural and non-neural as well as mesoderm to dorsal and ventral during development. Maintaining such division with the establishment of an exact border between the domains is required for the formation of highly differentiated structures such as neural tube and notochord. We hypothesized that the key condition for efficient cell fate separation in a chordate embryo is the presence of a positive feedback loop for Bmp signalling within the gene regulatory network (GRN), underlying early axial patterning. We therefore investigated the role of Bmp signalling in axial cell fate determination in amphioxus, the basal chordate possessing a centralized nervous system. Pharmacological inhibition of Bmp signalling induces dorsalization of amphioxus embryos and expansion of neural plate markers, which is consistent with an ancestral role of Bmp signalling in chordate axial patterning and neural plate formation. Furthermore, we provided evidence for the presence of the positive feedback loop within the Bmp signalling network of amphioxus. Using mRNA microinjections we found that, in contrast to vertebrate Vent genes, which promote the expression of Bmp4, amphioxus Vent1 is likely not responsible for activation of cephalochordate orthologue Bmp2/4. Cis-regulatory analysis of amphioxus Bmp2/4, Admp and Chordin promoters in medaka embryos revealed remarkable conservation of the gene regulatory information between vertebrates and basal chordates. Our data suggest that emergence of a positive feedback loop within the Bmp signalling network may represent a key molecular event in the evolutionary history of the chordate cell fate determination (Kozmikova et al., 2013).

The neural crest is unique to vertebrates and has allowed evolution of their complicated craniofacial structures. During vertebrate evolution, the acquisition of the neural crest must have been accompanied by the emergence of a new GRN. To investigate the role of protein evolution in the emergence of the neural crest GRN, we examined the neural crest cell (NCC) differentiation-inducing activity of chordate FoxD genes. Amphioxus and vertebrate (Xenopus) FoxD proteins both exhibited transcriptional repressor activity in Gal4 transactivation assays and bound to similar DNA sequences *in vitro*. However, whereas vertebrate FoxD3 genes induced differentiation of ectopic NCCs when overexpressed in chick neural tube, neither amphioxus FoxD nor any other vertebrate FoxD paralogues exhibited this activity. Experiments using chimeric proteins showed that the N-terminal portion of the vertebrate FoxD3 protein is critical to its NCC differentiation-inducing activity. Furthermore, replacement of the N-terminus of amphioxus FoxD with a 39-amino-acid segment from zebrafish FoxD3 conferred neural crest-inducing activity on amphioxus FoxD or zebrafish FoxD1. Therefore, fixation of this N-terminal amino acid sequence may have been crucial in the evolutionary recruitment of FoxD3 to the vertebrate neural crest GRN (Ono et al., 2014).

Pax genes encode highly conserved transcription factors vital for metazoan development. Pax transcripts, particularly those in Group II (Pax2/5/8), are extensively alternatively spliced. This study compares the transcriptional activation capacity and developmental stage-specific expression of major isoforms of Group II Pax proteins in amphioxus (*Branchiostoma floridae*) and in *Xenopus laevis*. The comparison reveals considerable divergence of splice forms between the lineages, with the *X. laevis* Group II Pax genes (Pax2, Pax5, and Pax8) possessing a greater repertoire of regulated and functionally distinct splice forms than the single amphioxus gene (Pax2/5/8). Surprisingly, some apparently

conserved splice forms are expressed at quite different levels during development in the two organisms and present different capacities to activate transcription. However, despite this divergence, the combinatorial transcriptional activation capacity of the isoforms present in early *X. laevis* and amphioxus development are broadly similar. This suggests that some of the conserved functional roles, implied by the expression of Group II Pax genes in homologous tissues of amphioxus and *X. laevis* embryos, may depend upon the combination of isoforms expressed in a particular tissue at a particular time in development. Thus, during early development, the evolutionary constraint on the net effect of several isoforms co-expressed in a given tissue may be more strict than that on specific isoforms. This flexibility may facilitate the appearance of new exons and splicing patterns in the vertebrate duplicates, leading to isoforms with subtly distinct functions critical to the subsequent development of vertebrate-specific cell types and structures (Short et al., 2012).

The origin of vertebrate eyes is still enigmatic. The "frontal eye" of amphioxus, our most primitive chordate relative, has long been recognized as a candidate precursor to the vertebrate eyes. However, the amphioxus frontal eye is composed of simple ciliated cells, unlike vertebrate rods and cones, which display more elaborate, surface-extended cilia. So far, the only evidence that the frontal eye might indeed be sensitive to light has been the presence of a ciliated putative sensory cell in the close vicinity of dark pigment cells. We set out to characterize the cell types of the amphioxus frontal eye molecularly, to test their possible relatedness to the cell types of vertebrate eyes. We have shown that the cells of the frontal eye specifically co-express a combination of transcription factors and opsins typical of the vertebrate eye photoreceptors and an inhibitory Gi-type alpha subunit of the G protein, indicating an off-responding phototransductory cascade. Furthermore, the pigmented cells match the retinal pigmented epithelium in melanin content and regulatory signature. Finally, we revealed axonal projections of the frontal eye that resemble the basic photosensory-motor circuit of the vertebrate forebrain. These results support homology of the amphioxus frontal eye and the vertebrate eyes and yield insights into their evolutionary origin (Vopalensky et al., 2012).

2.4. Search for Wnt inhibitors

Our interest in Wnt signalling resulted in an international collaboration with Prof. Stefan Krauss aimed to identify pharmacological inhibitors of canonical Wnt/β-catenin signalling. Inhibition of Wnt/β-catenin signalling therefore is an attractive strategy for anticancer drugs. We have identified a novel small molecule inhibitor of the b-catenin signalling pathway, JW55. In a dose-dependent manner, JW55 inhibited canonical Wnt signalling in colon carcinoma cells that contained mutations in either the APC (adenomatous polyposis coli) locus or in an allele of β-catenin. In addition, JW55 reduced XWnt8-induced axis duplication in Xenopus embryos and polyposis formation in conditional APC mutant mice (Waaler et al., 2012). Most colorectal cancers (CRC) are initiated by mutations of APC, leading to increased β-catenin-mediated signalling. However, continued requirement of Wnt/β-catenin signalling for tumour progression in the context of acquired KRAS and other mutations is less well-established. To attenuate Wnt/β-catenin signalling in tumours, we developed potent and specific small-molecule tankyrase inhibitors, G007-LK and G244-LM, that reduce Wnt/β-catenin signalling by preventing poly(ADP-ribosyl)ation-dependent AXIN degradation, thereby promoting β-catenin destabilization. We show that novel tankyrase inhibitors completely block ligand-driven Wnt/β-catenin signalling in cell culture and display approximately 50% inhibition of APC mutation-driven signalling in most CRC cell

lines. It was previously unknown whether the level of AXIN protein stabilization by tankyrase inhibition is sufficient to impact tumour growth in the absence of normal APC activity. Compound G007-LK displays favourable pharmacokinetic properties and inhibits *in vivo* tumour growth in a subset of APC-mutant CRC xenograft models. In the xenograft model most sensitive to tankyrase inhibitor, COLO-320DM, G007-LK inhibits cell-cycle progression, reduces colony formation, and induces differentiation, suggesting that β-catenin-dependent maintenance of an undifferentiated state may be blocked by tankyrase inhibition. The full potential of the anti-tumour activity of G007-LK may be limited by intestinal toxicity associated with inhibition of Wnt/β-catenin signalling and cell proliferation in intestinal crypts. These results establish proof-of-concept anti-tumour efficacy for tankyrase inhibitors in APC-mutant CRC models and uncover potential diagnostic and safety concerns to be overcome as tankyrase inhibitors are advanced into the clinic (Lau et al., 2013).

2.5. References

(Authors and/or co-authors of the evaluated team are in bold; corresponding author from the evaluated team is marked by asterisk; titles of the papers published during the evaluation period are underlined)

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Research Report of the team in the period 2010–2014

Institute	Institute of Molecular Genetics of the CAS, v. v. i.

Scientific team	Laboratory of Structural Biology

2.1. Introduction

The main interests of the Laboratory of Structural Biology are structural studies of various proteins of biological or medicinal interest. We use the knowledge of three-dimensional structure in understanding the protein function and in some projects also in modulating its function by design of specific inhibitors.

In our structure-based drug discovery project, we target enzymes from pathogenic organisms as well as human enzymes (e.g. human nucleotidases or cancer-specific carbonic anhydrase IX). The knowledge of 3D structure of proteins provides a platform for rational design of therapeutics. Crystal or solution structures of a target protein in complex with small molecules further increase the impact of structural biology in drug discovery. The main results achieved in the evaluated period 2010-2014 are described in Sections 2.2-2.4.

Our Laboratory also focuses on engineering recombinant antibody fragments of potential diagnostic use[1; 2; 3]. We employed several approaches aiming at practical use of antibodies and recombinant antibody fragments (Section 2.5).

In addition to own research projects, the Laboratory of Structural Biology also participates, by its structural biology expertise, in other projects. Relatively high involvement in collaborative projects is owing to the fact that our expertise is quite unique within our country and we are often approached by potential collaborators with interesting structural biology projects. The Laboratory is open to collaboration, because running several own and collaborative projects in parallel increases the chance to obtain positive results – especially in protein crystallography, where crystallization is very often a bottleneck (if not an obstacle) to successful conclusion of the project. Also, a balance between high-risk projects and safe projects ensures sustainable scientific output of the team, which is essential (not only) for successful grant applications.

2.2. Structure-based design of 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 carbonic anhydrases play important roles in various physiological and pathological processes (e.g. tumorigenicity, obesity, and epilepsy), and thus many CA isoenzymes represent established diagnostic and therapeutic targets. In particular, there is a significant interest in the development of selective inhibitors targeting carbonic anhydrase isoform IX (CAIX). CAIX is a tumour-associated transmembrane isoenzyme, overexpression of which is induced by hypoxia. CA IX is used as a tumour

marker and as a prognostic factor for several human cancers, and thus represents a valuable target for anti-tumour therapy. Design of a novel generation of selective inhibitors is the current challenge in the development of new therapeutic agents able to inhibit specific isoenzymes. The conventional carbonic anhydrase inhibitors contain a sulphonamide, sulphamate or sulphamide group connected to an organic moiety usually composed of a 5- or 6-membered aromatic ring or conjugated ring system. The novelty of our approach lies in utilization of substituted boron cluster compounds as active-site inhibitors of CA isoenzymes.

The idea to use boron clusters and the first compound we have designed originated from the analysis of X-ray structures of CAII in complex with organic sulphonamide-based inhibitors we determined previously[4;5]. Our analyses revealed that structurally related inhibitors can bind by two distinct binding modes and engage two opposite sides of the active site. Following this analysis, we hypothesized that the binding space within the enzyme active site cavity can be effectively filled with inhibitor with three-dimensional scaffold and hydrophobic nature, and we have designed a molecule containing the sulphamide group connected to carborane cluster optimally filling the active site cavity of CAII. Our compounds inhibited the enzyme activity with K_i value in submicromolar range and showed increased selectivity toward the tumour-associated isoform CAIX over widespread CAII. The crystal structure of this compound in complex with CAII confirmed the binding into the enzyme active site in the predicted pose and revealed key interactions responsible for binding of the inhibitors and enzyme inhibition and provided information that can be applied to the structure-based design of specific inhibitors.[6; 7].

2.3. Structure-based design of inhibitors of human nucleotidases

5’-Nucleotidases are ubiquitous enzymes that catalyse 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 analogues 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 analogues 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 analogues.

Two human 5’-nucleotidases, cytosolic 5'(3')-deoxyribonucleotidase (cdN) and mitochondrial 5'(3')-deoxyribonucleotidase (mdN), were subjects of our structural studies. We determined the crystal structures of both enzymes and attainment of an atomic resolution structure allowed us to use interatomic distances to assess the probable protonation state of the phosphate anion and side chains in the enzyme active site. A detailed comparison of the cdN and mdN active sites helped us in design of cdN-specific inhibitor.[8]

We have published a study on the 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. The crystal structures of mdN with two inhibitors revealed two different binding modes of these compounds in the mdN binding site[9].

2.4. Structural studies of HIV protease

HIV protease (HIV PR) plays an essential role in the viral replication cycle and this enzyme became one of the primary targets for drug design. The major problem that limits the therapeutic efficiency of inhibitors is drug resistance caused by extensive enzyme mutations.

Our laboratory participated in a project on HIV PR aimed to gain information on the molecular mechanisms of HIV resistance against protease inhibitors. We have focused on understanding the mechanisms of HIV PR resistance against protease inhibitors and novel mechanisms connected with insertions in HIV PR genes. The contribution of the laboratory was in structural analyses of complexes of mutated HIV PR species with clinically used protease inhibitors [10; 11; 12; 13].

2.5. Antibody fragments of potential diagnostic and therapeutic use

We have employed several approaches of engineering recombinant antibody fragments aiming at their practical use. For instance, we recently constructed single-chain variable fragment, scFv, comprising an auxiliary polypeptide segment which is rich in tyrosine. This protein shows a higher capacity to bind iodine radionuclide, as compared to the parental scFv[3]. We also participated in design of targeted polymers carrying toxic payloads or fluorescence tags. 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[14].

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Research Report of the team in the period 2010–2014

Institute	Institute of Molecular Genetics of the CAS, v. v. i.

Scientific team	Laboratory of Genomics and Bioinformatics

2.1. Introduction

Our team has two main research topics, which derive from our expertise in sequencing, transcriptome profiling, and bioinformatics and statistics. The first topic covers early evolution of eukaryotes, the evolutionary history of eukaryotic organelles, developmental processes in metazoans and evolution of endogenous herpesviruses in eukaryotic genomes. The second topic concerns transcriptomic analyses of the epithelium, with focus on intercellular interactions between the epithelial cells and fibroblasts, both in normal healing tissue and in cancer. We work on both topics with collaborators from Charles University in Prague and the Institute of Chemical Technology in Prague. In all below-mentioned activities, our team designed or co-designed the experiments, performed genomic and bioinformatic analyses, including nucleic acid isolation, library preparations, sequencing or microarray analyses, designed and maintained required databases, performed bioinformatic and statistic analyses, and interpreted the results or contributed to biological or clinical interpretation of the results.

2.2. Genomics of unicellular and multicellular eukaryotes

Unicellular eukaryotes (protists) with their phylogenetic branching close to the root of the evolutionary tree are the best candidates for genome studies of the common eukaryotic ancestor. Here, our main interests lie in the origin of two emblematic endosymbiotic organelles of the eukaryotic cell, the mitochondrion and the plastid. Using next-generation sequencing platforms we have characterized genomes and transcriptomes of several protist species, namely *Diplonema papillatum, Mastigamoeba balamuthi, Andalucia godoyi,* and *Malawimonas*. Adding genome sequences from diverse protists to currently available eukaryotic genomes enables us to deduce, with a much higher accuracy, details of many steps and processes of evolution of the eukaryotic cell.

In most eukaryotes, mitochondrion is the main organelle for formation of iron-sulphur clusters (FeSC). In *Archamoebae*, including *Mastigamoeba balamuthi*, the complex FeSC machinery was replaced by a nitrogen fixation system consisting of two components: NifS and NifU. *M. balamuthi* is rather unique as its FeSC formation is mediated through two machineries present in two cellular compartments. We showed that NifS and NifU are duplicated in the *M. balamuthi* genome. One pair of paralogs encodes proteins targeted to mitochondria, and the second pair encodes the cytosolic form of the machinery (**Nývltová et al., 2013**).

The most bizarre mitochondrial DNA (mtDNA) is that of the euglenozoan eukaryote *Diplonema papillatum*. The genome consists of numerous small circular chromosomes none of which appears to encode a complete gene. Here, we examined how many genes are encoded by *Diplonema* mtDNA and

whether all genes are fragmented and their transcripts trans-spliced. Our results opened new intriguing questions about the biochemistry and evolution of mitochondrial trans-splicing in *Diplonema* (**Vlček et al., 2011**).

Euglenids represent a group of protists that comprises species with diverse feeding modes. One distinct and diversified clade of euglenids is photoautotrophic, and its members bear green secondary plastids. We have described the plastid genome of the euglenid Eutreptiella, which we assembled from 454 sequencing events of Eutreptiella gDNA. Comparison of this genome and the only other available plastid genome of photosynthetic euglenid, *Euglena gracilis*, revealed that they contain a virtually identical set of 57 protein-coding genes, 24 genes fewer than the genome of *Pyramimonas parkeae*, the closest extant algal relative of the euglenid plastid. Searching within the transcriptomes of Euglena and Eutreptiella showed that six of the missing genes were transferred to the nucleus of the euglenid host while 18 have probably been completely lost. Euglena and Eutreptiella represent the deepest bifurcation in the photosynthetic clade, and therefore all these gene transfers and losses must have had happened before the last common ancestor of all known photosynthetic euglenids. After the split of Euglena and Eutreptiella, only one additional gene loss took place. The conservation of gene content in the two lineages of euglenids is in contrast to the variability of gene order and intron counts, which diversified dramatically. Our results show that the early secondary plastid of euglenids was much more susceptible to gene losses and endosymbiotic gene transfers than the established plastid, which is surprisingly resistant to changes in the gene content (**Hrdá et al., 2012**).

Organisms inhabiting low oxygen environments often contain mitochondrial derivatives known as hydrogenosomes, mitosomes or neutrally as mitochondrion-like organelles. Detailed investigation has shown unexpected evolutionary plasticity in the biochemistry and protein composition of these organelles in various protists. We have investigated the mitochondrion-like organelle in *Trimastix pyriformis*, a free-living member of one of the three lineages of anaerobic group Metamonada. Using 454 sequencing events we obtained 7,037 contigs from its transcriptome and on the basis of sequence homology and presence of N-terminal extensions we selected contigs coding for proteins that putatively function in the organelle. Together with the results of a previous transcriptome survey, the list now consists of 23 proteins – mostly enzymes involved in amino acid metabolism, transporters and maturases of proteins, and transporters of metabolites. We have no evidence of the production of ATP in the mitochondrion-like organelle of Trimastix, but we have obtained experimental evidence for the presence of enzymes of the glycine cleavage system (GCS), which is part of amino acid metabolism. Using homologous antibody we have shown that H-protein of GCS localizes into vesicles in the cells of Trimastix. When overexpressed in yeast, H- and P-protein of GCS and cpn60 were transported into the mitochondrion. In case of H-protein we have demonstrated that the first 16 amino acids are necessary for this transport. The glycine cleavage system is at the moment the only experimentally localized pathway in the mitochondrial derivative of *Trimastix pyriformis* (**Zubáčová et al., 2013**).

To understand the evolution of the developmental processes in eukaryotes, it is necessary to analyse the genomes and transcriptomes of diverse species. We have sequenced transcriptomes of two multicellular eukaryotes, both of phylum Radiata, sweet water medusa *Craspeducusta sowerbyi* and cubozoan *Tripedalia cystophora*. Having sequenced the transcriptomes, we characterized the expression of selected developmental genes (Pou and Six class homeoboxes) *in situ*. These homeobox genes are key members of the regulation cascades determining development of sensory organs, nervous system,

gonads and muscles. We have described their diversity and expression patterns in jellyfish, and highlighted their evolutionarily conserved functions (**Hroudová et al., 2012**).

2.3. **Transcriptomics of cancer-associated fibroblasts**

With our collaborators from the Anatomical Institute of Charles University in Prague, we have been working for an extended period of time on the properties of tumour microenvironment as a critical compartment influencing tumour progression and cancer prognosis. The research, which combines analyses of clinical samples, *in vitro* studies, and genomic and bioinformatic approaches, has resulted in several publications on the crosstalk between the most important players of the tumour microenvironment in carcinomas, the tumour epithelial cells and cancer-associated fibroblasts (CAF), and has yielded new insights into the so-called epithelial-mesenchymal interaction (EMI).

Specifically, we compared fibroblasts prepared from the stroma of squamous cell carcinoma and normal dermal fibroblasts and assessed their biological activity on normal keratinocytes by gene expression profiling on arrays and by immunocytochemistry of growth factors/cytokines. We determined and validated IGF2 and BMP4 as the factors responsible for the biological activity of stromal fibroblasts on normal epithelia. The results are published (**Strnad, Lacina et al., 2010**) and patent protected.

In completion, we studied the chemokine and cytokine expression in normal fibroblasts influenced by malignant epithelium of squamous cell carcinoma. Transcriptome profiling revealed strong up-regulation of interleukins IL6, IL8 and chemokine CXCL1 both *in situ* and *in vitro*. Experiments with addition of these factors to culture medium have confirmed that they reciprocally contributed to the maintenance of a low-differentiation phenotype of epithelial cells. Our results show the importance of epithelial-mesenchymal interaction in carcinoma and established a potential anticancer therapy. The results are published (**Kolář, Szabo et al., 2012**) and patent protected.

Next, we investigated the role of CAF in the formation of stem cell supporting properties of tumour stroma. We tested the influence of CAF from basal cell carcinoma on mouse 3T3 fibroblasts, focusing on expression of stem cell markers and plasticity *in vitro*. We demonstrated the biological activity of CAF by influencing the 3T3 fibroblasts to express markers such as Oct4, Nanog, and Sox2 and to show differentiation potential similar to mesenchymal stem cells. The role of growth factors such as IGF2, FGF7, LEP, and TGFβ has been established to participate in the activity (**Szabo et al., 2011**).

Frequently, CAF express smooth muscle actin (SMA), a marker of poor prognosis of cancer. We have studied the presence of SMA-positive CAF in relation to galectin-1. We applied microarray analyses and observed significant correlations of several transcripts with the protein level of galectin-1 in the CAF. The genes are related to known poor-prognosis factors, e.g., NF-κB up-regulation. Our results provide new insights into the significance of the presence of SMA-positive CAF in squamous cell carcinoma and were published in (**Valach et al., 2012**).

Recently, we have been studying the intercellular crosstalk in nodular melanoma, in completion to head and neck squamous cell carcinoma. We study the effect of melanoma cells (MC) on human primary keratinocytes (HPK). MC are important modulators of the tumour microenvironment and we compare their effect to the effect of non-malignant lowly differentiated cells that also originate from neural crest (NCSC). We have observed that MC and NCSC are able to increase expression of keratins 8, 14, 19, and

vimentin in the co-cultured HPK. This *in vitro* finding partly correlates with pseudoepitheliomatous hyperplasia observed in melanoma biopsies. We provide evidence of FGF-2, CXCL-1, IL-8, and VEGF-A participation in the activity of MC on keratinocytes and conclude that the MC are able to locally influence the differentiation pattern of keratinocytes both *in vivo* and *in vitro* (**Kodet et al., 2015**).

2.4. Genomics and metagenomics of soil bacteria

In our ongoing collaboration with the Institute of Chemical Technology Prague, we study soil bacteria with capacities to degrade industrial pollutants and the influence of diverse plants on their activity. We have identified bacteria associated with biodegradation of aromatic pollutants such as biphenyl, benzoate, and naphthalene in a long-term polychlorinated biphenyl- and polyaromatic hydrocarbon-contaminated soil. In order to avoid biases of culture-based approaches, stable isotope probing was applied in combination with sequence analysis of 16 S rRNA gene pyrotags amplified from (13)C-enriched DNA fractions. The metagenomes isolated after the incubation of soil samples with all three studied aromatics were largely dominated by Proteobacteria, namely sequences clustering with the genera Rhodanobacter, Burkholderia, Pandoraea, Dyella as well as some Rudaea- and Skermanella-related ones. Pseudomonads were mostly labelled by (13)C from naphthalene and benzoate. Our results also show that many biphenyl/benzoate-assimilating bacteria derive carbon also from naphthalene, pointing out broader biodegradation abilities of some soil microbiota. The results also demonstrate that, in addition to traditionally isolated genera of degradative bacteria, yet-to-be cultured bacteria are important players in bioremediation (**Uhlik, Wald et al., 2012; Uhlik, Musilová et al., 2012**).

Further, we have reported the complete genomic sequence of *Achromobacter xylosoxidans* strain A8. The bacterium was isolated from the soil contaminated with polychlorinated biphenyls and can use 2-chlorobenzoate and 2,5-dichlorobenzoate as sole sources of carbon and energy. This property makes it a good starting microorganism for derivation of a bioremediation system. The genome of *A. xylosoxidans* consists of a 7-Mb chromosome and two large plasmids (98 kb and 248 kb). Besides genes for the utilization of xenobiotic organic substrates, it contains genes associated with pathogenesis, toxin production, and resistance (**Strnad, Rídl et al., 2011**).

Next, we have reported complete genomic sequence of *Rhodobacter capsulatus* SB 1003. This bacterium belongs to the group of purple non-sulphur bacteria. Its genome consists of a 3.7-Mb chromosome and a 133-kb plasmid. The genome encodes genes for photosynthesis, nitrogen fixation, utilization of xenobiotic organic substrates, and synthesis of polyhydroxyalkanoates. These features made it a favourite research tool for studying these processes (**Strnad, Lapidus et al., 2010**).

2.5. Genomics of human endogeneous retroviruses

A significant fraction of mammalian genomes is composed of endogenous retroviral sequences (HERV). These elements are categorized to families, and members of some of these families (e.g. HERV-H, HERV-W and HERV-K) have been shown to be transcribed. These transcriptions are associated with several severe diseases such as mental disorders, AIDS, autoimmune diseases and cancer. We have discussed several bioinformatic strategies for genome-wide scan of HERV transcription using high-throughput RNA

sequencing on several platforms. We have shown that many more HERVs than previously described are transcribed to various levels (**Pačes et al., 2013**)..

Lentiviruses (LV), in contrast to other retroviral genera, only rarely form endogenous copies. We have searched for endogenous LV in vertebrate genomes *in silico* and discovered the first endogenous LV in the mammalian order *Dermoptera*, a taxon close to the *Primates*. The virus represents the oldest documented endogenization event and our discovery can lead to new insights into LV evolution. It is also the first reported endogenous LV in an Asian mammal, indicating a long-term presence of the LV family in Asia (**Hron et al., 2013**).

2.6. Bioinformatics support and methods development

As a laboratory specialized in genomics and bioinformatics, we have been continually developing new methods and pipelines, together with providing support to other research groups both intra- and extramurally. The resulting collaborative articles include, among others (**Kolář, Meier et al., 2012; Balounová et al., 2014; Arefin et al., 2014; Fafílek et al., 2013**).

2.7. References

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Research Report of the team in the period 2010–2014

Institute	Institute of Molecular Genetics of the CAS, v. v. i.

Scientific team	Laboratory of Molecular Virology

2.1. Introduction

During the period of evaluation, members of the group remained focused on their main long-term scientific orientation – the complex investigation of cancer. More specifically, the following topics were addressed: mechanisms and genes involved in experimentally induced malignant transformation of different cell types, selected biological properties of model cancer cells, interactions of cancer cells and a surrounding tissue (tumour microenvironment), and development and testing of anticancer compounds. In addition, some work was also devoted to identification of genes involved in the fate determination of neural crest and to studies of regulatory molecules participating in the differentiation of myogenic precursors. Both *in vitro* and *in vivo* approaches were exploited. The group continued its productive collaboration with colleagues from several academic and non-academic institutions. The results of the group are represented by scientific papers and summarized in the main body of the report, and by patents listed in the References section.

2.2. Initiation, promotion, and progression of experimental malignancies

The work by Pečenka and co-workers (**Pečenka et al., 2011**) represents the summary of our long-standing work on experimental chicken model of retroviral insertional mutagenesis.

The introductory part of the chapter summarizes published results of searching for cancer-related genes obtained using chicken models of insertional mutagenesis and presents new views of the mechanisms by which integrated proviruses deregulate host gene expression. The major part of the chapter is devoted to the most advanced model based on MAV-2 retrovirus that has been elaborated recently by the authors. So far, the model allowed identification of genes participating in the formation of nephroblastomas, lung and liver hemangiosarcomas, liver carcinomas and cholangiocarcinomas. Most of these tumour types were induced with the help of the new phenomenon discovered recently by the authors: non-tumour cells released into the blood circulation and passing further into tissues strongly promote formation of a tumour from latent malignant cells primed by MAV-2 insertional mutagenesis (**Pajer et al., 2009**).

The data described in the chapter are up-to-date and include the newest results of the authors that have not been published yet. The chapter shows that the chicken model of cancerogenesis is a prolific one, can advantageously complement mammalian models and contribute to our knowledge of genes, pathways and networks implicated in cancerogenesis, leading to data that could translate to human medicine (**Pečenka et al., 2011**).

An important collaborative project ("Cell transformation as a result of aberrant gene expression") with the laboratory of prof. Bose (University of Texas at Austin) was established during the postdoctoral

fellowship of Dr. Králová. Based on her work discovering an important role of AP-1 transcription factors in transformation induced by the v-rel oncogene (**Kralova et al., 1998),** further studies continued and defined distinct and complex roles of these transcription factors in Rel/NF-κB oncogenesis in detail (**Liss et al., 2010**). In addition to transcriptional regulation of AP-1 factors, their activation is also achieved through post-translational phosphorylation by the mitogen-activated protein (MAP) kinases. The expression of v-Rel results in strong and sustained activation of the ERK and JNK MAPK pathways, which was shown to be essential for this process (**Kralova et al., 2010**). The design of experiments and most of the immunodetection assays including siRNA experiments in these studies were performed by J. Kralova.

2.3. Tumour-tumour microenvironment interaction

Reciprocal interactions between a tumour and its microenvironment control both expansion and properties of tumour cells and the integrity and physiological functions of the tumour- surrounding microenvironment. In life-threatening stages of leukaemia, leukemic blast cells often penetrate the bone marrow-blood barrier and flood the periphery.

To understand the egress of leukemic blasts from the bone marrow, we analysed the aggressive and attenuated model myeloid leukaemias, which respectively develop and do not develop to the blast phase. Aggressive leukemic blasts, unlike attenuated leukemic cells, caused destruction of the BM microenvironment followed by the blast crisis. These aggressive blasts secreted high amounts of material – secretome. Significant parts of this secretome were represented by nucleosomes. The attenuated leukemic blasts secreted significantly less nucleosomes. *In vitro* experiments showed that the leukemic blast secretome caused death of different cell types when added to the cultivation media. We found that the cytotoxic agent of the secretome is represented by the fragmented DNA derived from the genome of leukemic cells and occurring in nucleosome-like complexes. This DNA enters nuclei of bone marrow or other cells and induces phosphorylation of histone H2A.X at Serine 139 and cell death, similarly as double-strand break-inducing agents. There is a correlation between large amounts of acquired DNA and death of recipient cells. Although the DNA from the attenuated blast cell secretome also entered nuclei of stromal cells, its lower amount was rather well tolerated by stromal cells. Importantly, the secretome DNA integrated into chromosomal DNA of recipient cells, likely by the DNA end joining repair mechanisms of cells. Primary human acute myeloid leukaemia cells also released fragmented DNA that penetrated the nuclei of other cells both *in vitro* and *in vivo*, suggesting that the observed mechanism is generally applicable. We suggest that DNA fragments released from leukemic and perhaps also other types of tumour cells can activate DNA repair mechanisms or death in recipient cells of a tumour microenvironment, depending on the amount of the acquired DNA. This can impair DNA stability and viability of tumour stromal cells, undermine homeostatic capacity of the tumour microenvironment, and facilitate tumour progression (**Dvořáková et al., 2013).**

Importantly, our data inspired other researchers (**Mittra et al., 2015),** who fully confirmed our pioneer observations by performing analysis of fragmented DNA and chromatin (DNAfs and Cfs) isolated from blood of cancer patients and healthy volunteers. These authors found that the DNA is readily taken up by a variety of cells in culture and localizes in their nuclei within a few minutes, causes DNA damage response, and integrates.

2.4. Metastasis- and fibrosis-related properties of tumour cells and myofibroblasts

The tumour microenvironment, in many aspects resembling the environment of healing wound, is created by a specific interaction between cancer cells and the surrounding tissue called tumour stroma. Very important cellular part of tumour stroma is represented by myofibroblasts, producing and remodelling extracellular matrix (ECM), and releasing a number of cytokines, thereby supporting cancer progression. Myofibroblasts are also the cause of fibrosis, another practically untreatable serious disease. While the causes of neoplastic cell transformation and the tumour formation have been at least partially elucidated, we have only a poor knowledge of the mechanisms behind the metastatic process. Efficient invasion of primary tumour cells into the surrounding tissue is a critical step in metastasis, and ECM is the first barrier interfering with the migration of primary tumour cells and metastasis. That is why we focused our research on the molecular mechanisms leading to dedifferentiation of the key tumour stroma cell type, myofibroblasts (2.4.1.) and mechanisms enabling cancer cells to efficiently invade tumour stroma and other tissues (2.4.2.).

Our research as a whole shows and confirms the relevance and matter of the chicken (Gallus gallus) model in search for generally valid molecular mechanisms operating in amniotes, especially in research of human untreatable diseases such as fibrosis and cancer.

2.4.1. Dedifferentiation of myofibroblasts

To study primary myofibroblast molecular and cellular biology, we have introduced a novel cell model based on primary chicken embryo dermal myofibroblasts (CEDM). The phenotypical homogeneity and temporal stability of these cells warrant their use as an experimental model in myofibroblast biology research **(**Kosla et al., 2013a**)**. Using this model, we showed the extensive evolutionary conservation of basic regulatory mechanisms involved in wound healing and fibrosis between two animal classes (birds, mammals) and revealed several genes whose dependence on TGFβ has not been known to date. Our data suggest possible existence of a specific and regulated process of myofibroblast dedifferentiation, characterized by a specific gene expression profile activated by decreased TGFβ signalling. Inhibition of TGFβ signalling alone does not lead to full dedifferentiation of CEDM cells **(**Kosla et al., 2013a**)**.

We have further shown that sustained activation of certain MAPK signalling pathway components (namely PDGFB, Ha-Ras(G12V) and transcriptional factor EGR4) lead to the complete loss of the myofibroblastic phenotype of CEDM cells. We identified a few gene products that might mediate the effects induced by EGR4. According to the present knowledge, EGR4 is the only part of the MAPK signalling not causing neoplastic transformation of cells. Among mediators of the EGR4 effect, transcriptional factor FOXG1 appears to hold an exceptional position because its sustained expression is able to dedifferentiate myofibroblasts as efficiently as expression of EGR4. Sustained expression of EGR4 and FOXG1 activates mechanisms that inhibit both the TGFβ and MAPK signalling. Activation of the tested components of the MAPK signalling pathway resulted in all cases in inhibition of the TGFβ signalling and perturbations of the MAPK signalling. That is why we simulated similar conditions by drugs. The particular combination of drugs inhibiting the TGFβ pathway and stimulating the MAPK pathway led to efficient loss of the myofibroblastic phenotype of CEDM cells *in vitro* **(**Kosla et al., 2013b**)**.

2.4.2. Invasiveness and metastasis of v-src-transformed fibrosarcoma cells

To study the genes regulating sarcoma metastasis, we used the unique chicken fibrosarcoma cell lines PR9692 and PR9692-E9, which we characterized. Upon inoculation into the pectoral muscle of experimental chicks, parental PR9692 cells give rise to rapidly growing sarcomas which efficiently metastasize into lungs, while PR9692-E9 cells (a subclone of PR9692 cells) form sarcomas that (in contrast to PR9692) never metastasize (**Cermak et al., 2010**).

Microarray analysis revealed that expression of some genes whose protein products are involved in processes associated with metastasis was decreased in PR9692-E9 cells. Such processes involve cell adhesion, motility and degradation of ECM, but also ECM production. There was the an obvious decrease of transcriptional factor EGR1 gene expression in non-metastasizing PR9692-E9 cells. Constitutive expression of EGR1 in PR9692-E9-EGR1 cells led to metastatic activity comparable to the activity displayed by PR9692 cells. Further analysis showed that PR9692-E9-EGR1 cells share with PR9692 cells not only the ability to metastasize, but also the polarized morphology with distinct stress fibres and long thin filopodia. Non-metastatic cells had typically rounded morphology with actin-rich membrane ruffles and fewer short thick filopodia. Time-lapse microscopy of particular cell lines revealed that part of the population of PR9692 and PR9692-E9-EGR1cells moved in a manner typical of mesenchymal cells. The motion of control cells PR9692-E9 was rather defective, with no obvious front-rear cellular polarity.

Gene expression analysis of PR9692-E9-EGR1 revealed that exogenous expression of EGR1 in non-metastatic cell line reconstituted expression of a number of genes to levels comparable to the parental PR9692 metastatic cells. These genes (reconstituted by EGR1) might play a role in a presumptive metastatic transcription programme. Among such genes, the MYL9 gene, encoding myosin light chain (MLC), was identified. MYL9 is the regulatory protein that is a direct target of phosphorylation by myosin light chain kinase (MLCK) and Rho-kinase (ROCK). This phosphorylation drives actin cytoskeleton contractions and is important for processes such as adhesion, establishment of polarity, and subsequent migration of the cells. Our analysis has thus shown that the metastatic PR9692 cells differ from non-metastatic cells by their ability to migrate and adhere, cell functions associated with actin cytoskeleton action (**Cermak et al., 2010**).

Further, we wanted to get a deeper insight into the invasive abilities of these model cell lines. The decreased expression level of MYL9 gene (chicken MLC2) in non-metastatic PR9692-E9 cell line suggested involvement of actomyosin contractility, controlled by regulatory proteins Rho/ROCK/MLC in the metastatic process. Recent publications show decisive roles of some regulatory components of Rho signalling in the invasive mode of migration in three-dimensional (3D) environment. PR9692 cells possess rounded morphology in 3D collagen, typical for cells using the amoeboid mode of migration. The amoeboid phenotype of PR9692 cells was confirmed by inhibition of their invasiveness by the ROCK inhibitor and low levels of metalloproteinases (MMP14 and MMP-2) produced by these cells. Following experiments confirmed the important role of Rho, ROCK, and MLC activities in the invasiveness of metastatic cells PR9692 in 3D collagen and in metastasis. Our results **(Kosla et al., 2013c)** show that activation of RhoA/ROCK/MLC signalling (by the expression of constitutively active RhoA) is sufficient to restore both invasiveness in 3D collagen and metastasis of normally non-metastatic PR9692-E9 cells. Parallel analysis of mammalian (rat), strongly metastatic sarcoma cell line A3, which primarily uses amoeboid cell invasion, confirmed the evolutionary conservation of regulatory mechanisms controlling

the amoeboid phenotype of sarcoma cells. Our work is the first study to show the effective amoeboid invasion and metastasis of cancer cells in a non-mammalian system, which further supports the general importance of the phenomena of the amoeboid invasion and also opens up possibilities for introducing novel interesting models into the field of different modes of invasiveness. Moreover, it revealed the important role of the Rho/ROCK/MLC signalling in sarcoma invasiveness and metastasis. Together with previous studies from the Mondello and Chiarugi labs, we believe our data provide the strongest evidence to date for the capability of amoeboid cancer cells to invade the tissue environment and effectively metastasize.

We also contributed to the design of a novel model for drug discovery of anti-invadopodial compounds (**Pulo Pobe et al., 2014**) based on large-scale screening of invadopodia formation in two-dimensional setting followed by validation of the results in complex 3D matrices such as dermis-based matrix. The ability of cancer cells to migrate and invade is often associated with the formation of invadopodia. The invadopodia are actin-based protrusions of tumour cells or transformed cells critically involved in protease secretion and targeting, and cytoskeletal rearrangements enabling cellular invasion. Anti-invadopodia therapy is a possible new strategy in anti-metastatic treatment.

We significantly participated in the analysis of the contribution of another transcriptional factor, homeodomain only protein X (HOPX), in metastasis formation and development **(Kovarova et al, 2013)** by preparation of DNA constructs, microarray and cell-cycle analyses, migration and invasive assays. HOPX-specific knockdown decreased HOPX expression in metastatic PR9692 and displayed reduced cell motility *in vitro*. Critically, HOPX knockdown decreased the *in vivo* metastatic capacity. Genomic analyses identified a cadre of genes affected by HOPX knockdown that intersected significantly with genes previously found to be differentially expressed in metastatic versus non-metastatic cells. Furthermore, 232 genes were found in both screens with at least a two-fold change in gene expression, and a number of high-confidence targets were validated for differential expression. Importantly, significant changes were demonstrated in the protein expression level of three metastatic associated genes (NCAM, FOXG1, and ITGA4), and knockdown of one of the identified HOPX-regulated metastatic genes, ITGA4, showed marked inhibition of cell motility and metastasis formation. Constitutive expression of HOPX in PR9692-E9-HOPX cells led to restoration of the metastatic activity of non-metastatic PR9692-E9 cells. These data demonstrate that HOPX is a metastasis-associated gene and that its knockdown decreases the metastatic activity of v-src-transformed cells through altered gene expression patterns.

2.5. Targeted intervention for cancer control

Specific elimination of cancer cells is a desirable goal that can be achieved by various strategies.

2.5.1. Targeted intervention for cancer control with synthetic ligands

In collaboration with The Institute of Chemical Technology (Prague) we explored several approaches utilizing novel, rationally designed chemical compounds and supramolecular complexes to target cancer cells.

i) **Photodynamic therapy agents.** Porphyrin derivatives with preferential accumulation in tumours were used as photosensitive drugs that after light exposure can mediate energy transfer and formation of

highly damaging reactive oxygen species. In the past, we demonstrated that glycol-substituted porphyrins represent such an example of efficient photosensitive compounds capable to induce apoptosis in various cancer cells (**Kralova et al., 2008**). The underlying molecular mechanisms of cell death were investigated for derivatives with very similar structure but different intracellular localization. Primary accumulation in lysosomes resulted in the activation the p38 MAP kinase cascade and induction of the mitochondrial apoptotic pathway, while localization in the endoplasmic reticulum induced dramatic changes in Ca2+ homeostasis, unfolded protein response, activation of calpains and stress caspases. These results showed that ROS generated in different subcellular compartments trigger diverse cell death pathways (**Moserova and Kralova, 2012).** Moreover, a new type of photosensitive compound combining two chromophoric systems, a porphyrin macrocycle and a polymethine moiety, was prepared and evaluated by **Bříza et al., 2012**. Here, we defined the photodynamic ability of this system and the prevailing photoinduced cell death was apoptosis.

ii) **New approach for versatile drug delivery and multimodal therapy.** The anticancer effect of photosensitive agents was further enhanced by joining them with cargo carriers accommodating an additional drug. For this purpose, porphyrin-cyclodextrin conjugates were prepared as multifunctional building blocks combining the capacity of cyclodextrin cavities to accommodate often poorly soluble drugs with the potency of porphyrin moiety to function as a tumour-targeting carrier. The mediated drug delivery and enhanced accumulation of porphyrin conjugates together with their photosensitive properties resulted in a synergistic effect of combined therapy, which was demonstrated for various cytostatic drugs by *in vitro* and *in vivo* methods (**Králová et al., 2010**). This therapeutic system can be further extended by incorporating therapeutic proteins. The validity of the concept was demonstrated by Zn–porphyrin–cyclodextrin conjugates and their supramolecular coordination complexes with therapeutic protein – immunoglobulin. In this way, the combination of photodynamic therapy, cell-targeted chemotherapy and immunotherapy brought an increase of anticancer efficacy in the mouse tumour model. Thus, the therapeutic efficacy and flexibility of this system indicates perspectives for future development (**Kejík et al., 2011; Kejík et al., 2012; Králová et al. 2014**). All *in vitro* cellular studies were performed by J. Králová.

iii) Nanoparticle tumour targeting. Another way to increase the therapeutic efficacy is by immobilization of porphyrin-brucine conjugates on gold nanoparticles. Their administration resulted in pronounced permeation and retention effect and eradication of experimental tumours in mice. The combined effect of photodynamic and photothermal therapy after irradiation was caused by the porphyrin conjugates and gold nanoparticles, respectively. These results suggested that gold nanoparticles can be used as an efficient vector for intra-tumour delivery of photosensitizers. In this study we participated mainly by performing cellular *in vitro* experiments (**Záruba et al., 2010; Králová et al., 2012**).

iv) **Ligands for selective recognition of tumour markers.** We also partly contributed to the study defining recognition properties of methinium salts for sulphated steroids by performing a pilot cytotoxicity study (**Kejík et al., 2015**), defining their uses as sensors for tumour markers (**patent CZ304948**) and helping to write a review focused on selective recognition of saccharide-type tumour marker with natural and synthetic ligands **(Kejík et al., 2010)**

v) **Fluorescent probes for imaging and intracellular targeting.** In a collaborative effort with the Institute of Chemical Technology, unique properties of new polymethine salts were studied and several

important observations were made. Firstly, the binding studies demonstrated that certain polymethine salts possess high affinity and selectivity for particular analytes, which can act as signalling molecules in cells as mentioned above. Moreover, we substantially contributed to the testing of biological relevance of these interactions by determining the anticancer effects for certain polymethine salts both *in vitro* and *in vivo* (**Bříza et al., 2015**).Secondly, due to their florescent properties, polymethine salts can be utilized as probes for cell imaging. Selective interaction of pentamethine salts with cardiolipin, which is an important component of the inner mitochondrial membrane, indicates their use as mitochondrial probes with good photostability, fluorescent properties, and low phototoxicity. (**Patent CZ304094B6; Rimpelová et al., 2013; Bříza et al., 2014; Bříza et al., 2015**). Notable features were discovered for the derivative with an expanded conjugated quinoxaline unit directly incorporated into a pentamethinium chain. This compound exhibited remarkable fluorescence intensity, selective mitochondrial localization, high cytotoxicity, and selectivity toward malignant cell lines resulting in pronounced *in vivo* suppression of tumour growth in mice (**Bříza et al., 2015**). The described qualities of polymethine derivatives indicate that particular polymethine salts have a potential to be used as a starting point for more advanced development of selective ligands for the inhibition of specific pathway(s) in cancer as well as tools for diagnostic applications detecting mitochondrial dysfunction.

2.5.2. Targeted intervention for cancer control with natural phospholipid derivatives

Dietary phospholipids (PLs) and their derivatives have proved active in the suppression of various health problems and conditions including cancer. In this work we compared the effect of PLs from hen egg yolk enriched with N-acyl ether-phosphatidylethanolamine (NAEPE) termed BAP+ preparation with PLs lacking NAEPE (BAP- preparation) on the growth of transformed cells *in vitro* and on the promotion and progression of experimental tumours *in vivo*. For the *in vivo* experiments we used the chicken model in which liver, lung, and kidney tumours arose via natural selection from single cells initiated by experimentally introduced somatic mutations caused by insertional mutagenesis. Mutagenized animals were fed BAP+ or BAP- diet in various regimens. We observed that BAP+ at low concentrations killed cells of various tumour cell lines in culture but did not compromise the viability of non-transformed cells. Oral administration of the BAP+ preparation efficiently reduced progression of all tumour types. However, it did not significantly reduce the number of already initiated tumours and their growth when BAP+ was discontinued. Our data suggest that NAEPE combined with hen egg PLs significantly interferes with tumour progression, possibly through the inhibition of tumour cell viability (**Karafiát et al., 2014**)

2.6. Fate determination in multipotent neural cells and differentiation of myogenic precursors

The neural crest (NC) is a transient dynamic structure of ectodermal origin found in early vertebrate embryos. The multipotential NC cells migrate along well-defined routes, differentiate to various cell types including melanocytes and participate in the formation of various permanent tissues. Abnormal development of NC cells causes several human diseases – neurocristopathies. As there is only limited information about the molecular mechanisms controlling early events in melanocyte specification and development, we exploited the AMV v-Myb transcriptional regulator, which directs differentiation of *in vitro* chicken NC cells to the melanocyte lineage. This activity is strictly dependent on v-Myb specific binding to the Myb recognition DNA element (MRE). The two tamoxifen-inducible v-myb alleles were constructed; one which recognizes MRE and one which does not. These were activated in *ex ovo* NC cells, and the expression profiles of resulting cells were analysed using Affymetrix microarrays and RT-

PCR. These approaches revealed up-regulation of the BMP antagonist gremlin 2 mRNA, and down-regulation of mRNAs encoding several epithelial genes including KRT19 as very early events following activation of melanocyte differentiation by v-Myb. The enforced v-myb expression in neural folds of chicken embryos resulted in detectable presence of gremlin 2 mRNA (**Starostová et al., 2014**).

Our previous data reported the role of c-Myb transcription factor in myogenesis using satellite cell-derived mouse myoblast cell line C2C12 (**Kašpar et al., 2005**). Next, we found c-Myb expression in i) activated satellite cells of regenerating muscle, ii) in activated satellite cells associated with isolated viable myofibers, iii) in descendants of activated satellite cells, proliferating myoblasts. However, c-myb was not expressed in differentiating myotubes originating from fusing myoblasts. The constitutive expression of c-Myb lacking the 3′ untranslated region (3′ UTR) strongly inhibited the ability of myoblasts to fuse. Our findings thus indicate that c-Myb is involved in the regulation of the differentiation programme of myogenic progenitor cells as its expression blocks myoblast fusion (**Kašpar et al., 2013**).

2.7. References

(Authors and/or co-authors of the evaluated team are in bold; corresponding author from the evaluated team is marked by asterisk; titles of the papers published during the evaluation period are underlined)

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Research Report of the team in the period 2010–2014

Institute	Institute of Molecular Genetics of the CAS, v. v. i.

Scientific team	Laboratory of Cancer Cell Biology

2.1. Introduction

Our research team was established in January 2013 and originally consisted of a junior group leader and two PhD students. Since then our team grew up and currently consists of the group leader, one research fellow, three postdocs, four PhD students and a master student.

The major scientific focus of our group are cellular responses to genotoxic stress and their consequences for cell division. We study the molecular mechanisms controlling the DNA damage response pathway (DDR) and cell cycle checkpoints. In particular, we focus on protein phosphatase Wip1 (encoded by *PPM1D* gene) that limits the amplitude and duration of DDR and thus allows checkpoint recovery. We try to understand how cells regulate Wip1 activity and how Wip1 impacts the individual components of DDR. Further, we study clinically relevant mutations in the *PPM1D* gene and their role in cancer development. As an independent research line, we are interested in the mechanisms that control DDR in context of the cell cycle. In that respect, we focus mainly on the polo-like kinases that regulate checkpoint recovery and suppress DNA repair during mitosis. Finally, we study how large protein complexes involved in DDR are assembled and what are the underlying molecular mechanisms. We believe that our work contributes to better understanding of general mechanisms controlling responses to genotoxic stress and that our findings will have important implications for understanding pathogenesis of cancer.

2.2. Regulation of DDR by protein phosphatases

This part represents a major scientific focus of our laboratory and consists of several smaller projects. First, we wish to determine what mechanisms regulate switching on and off the checkpoint in the G2 phase of the cell cycle. We have found that following induction of DNA double-strand breaks, checkpoint is initiated by ATM, whereas maintenance of the checkpoint is controlled by ATR. The activity of ATM is counteracted by the chromatin-bound Wip1 phosphatase, whereas the activity of ATR is counteracted by polo-like kinase 1 (Plk1). To follow the dynamics of DDR in the cells we have generated a FRET-based biosensor that specifically detects ATM activity after addition of radiomimetic drug neocarzinostatin. We have found that the ATM signal present in the foci close to the damage site is not sufficient to trigger the checkpoint, but rather a global spread of ATM on the chromatin controls the checkpoint. This ATM activity on chromatin needs to be suppressed by Wip1 to allow for recovery. We have identified several novel substrates that are phosphorylated by ATM and dephosphorylated by Wip1. We study these Wip1 substrates in normal diploid RPE cells either parental or knock-out for *PPMD* that we have generated

using CRISPR. We have now written a manuscript that summarizes our data and we expect to submit this work in the first half of 2015. This study was performed in collaboration with the Department of Cell and Molecular Biology at Karolinska Institute. Our team performed several key experiments in this study (including development of the FRET probe and generation of the *PPM1D* knock-out cells) and shares a corresponding author position.

Next, we work on determining the molecular mechanisms that control Wip1 function. We have found that upon stress, Wip1 is a substrate of MK2 kinase and we have identified several modified residues using MS. We are now testing what consequences these modification might have for the Wip1 function. Using domain mapping we search for the chromatin localization region in the sequence of Wip1. Localization of Wip1 to chromatin is evaluated by subcellular fractionation as well as by FRAP microscopy. We have prepared a set of various truncating mutants of Wip1 and using MS we now compare their ability to form protein-protein interactions. Preliminary data suggest that Wip1 lacking exon 6 is still able to localize to the chromatin fraction. Finally, we have initiated our work on performing non-biased screen for ubiquitinating enzymes responsible for the control of Wip1 stability. Currently, we are optimizing conditions for the high-throughput microscopy screening on Olympus ScanR station. These experiments will also continue in the next reporting period.

2.3. Role of PPM1D/Wip1 mutations in cancer

This part represents a second major research line of our team where we wish to address the oncogenic potential of *PPM1D* mutations. Besides the well-known gene amplification observed in multiple human cancer types, we have recently described truncating mutations in exon 6 of the *PPM1D* gene that result in expression of a C-terminally truncated Wip1 phosphatase (**Kleiblova et al., 2013**). We have found that these mutations significantly increased the protein stability of enzymatically active Wip1, underlying their gain-of-function character. Cells with truncated Wip1 failed to activate checkpoint following treatment with various DNA-damaging agents and replicated their DNA despite the presence of damaged DNA. Deficient checkpoint was caused by impaired activation of p53, which is a direct substrate of Wip1. Importantly, truncating mutations in *PPM1D* were found not only in cancer cell lines, but also in humans suffering from breast cancer. We have found that these mutations occurred not only in the tumours, but also in the peripheral blood of affected individuals, suggesting that they did not arise as typical somatic mutations but rather during the early stages of embryonic development. We have postulated that truncating *PPM1D* mutations represent a novel genetic defect predisposing to breast cancer development. Our data are fully consistent with another study published simultaneously in *Nature*, but our data provide more mechanistic insight into the possible pathogenic role of these mutations. The major part of the work was performed by members of our team (including first author and corresponding author). Analysis of clinical data and experiments involving live-cell imaging were performed in collaboration. This study also opened new avenues for further research and we continue working on this topic. In particular, we study the molecular mechanisms that control protein stability, localization and enzymatic activity of Wip1 (see above in 2.2.). To test the hypothesis that truncated Wip1 promotes cancer development we have generated a mouse model that mimics the *PPM1D* mutation found in humans. This model was generated using the TALEN technology in collaboration with the Laboratory of Transgenic Models of Diseases at our Institute. We have now validated this new model and initiated experiments using isolated mouse embryonic fibroblasts. We have also already initiated

crossing of our *PPM1D*^{TAL1} mice with various cancer-prone mouse models (including APC^{min} for colon cancer and MMTV-Erbb2 for breast cancer). We expect that *PPM1D*^{TAL1} mice will show earlier onset of tumours in sensitized genetic background. In addition, we performed qPCR analysis of *PPM1D* expression in selected tissues and found its high expression in intestinal stem cells and in the thymus. Therefore, we have started with analysing tumour formation in a model of ionizing radiation-induced thymic lymphoma. These long-term experiments will also continue throughout the next reporting period. We have performed and continuously work on screening for *PPM1D* mutations in other cancer types (including colon cancer, lymphoma, and melanoma). This work proceeds in collaboration with clinical laboratories at the Faculty of Medicine, Charles University in Prague and at Oslo University Hospital. Our team performs functional characterization of the identified mutations in cell-based experiments.

In parallel to experiments mentioned above, we try to determine the crystal structure of the catalytic domain of Wip1 phosphatase. In the last year, we optimized conditions for expression and two affinity-step purification of various forms of Wip1 from bacterial and insect cells. Currently we are running pre-crystallization screens aiming to identify appropriate crystallization conditions. This work proceeds in close collaboration with the Laboratory of Structural Biology at our Institute. Determining the structure of Wip1 would facilitate development of a specific inhibitor. We are also testing effects of the only commercially available Wip1 inhibitor on DDR and the sensitivity of cancer cells. So far, we have established that inhibition of Wip1 enhances DDR responses following various forms of DNA damage (including IR, necarcinostatin, etoposide and doxorubicine). We have also observed that cancer cells expressing truncated Wip1 are more sensitive to certain forms of chemotherapeutics delivered together with the Wip1 inhibitor. In contrast, the viability of cancer cells that have no alteration in the *PPM1D* gene and the viability of non-cancer cells is not affected by the Wip1 inhibitor. Our data suggest that Wip1 might be a suitable pharmacological target for certain types of tumours. We are currently testing this possibility using xenograft models. To this end, we have knocked out the *PPM1D* gene in HCT116 cells using CRISPR technology and we compare the tumour growth after injection to immunocompromised mice with that of the parental HCT116 cells that contain the truncating mutation in *PPM1D*. These experiments will also continue in the next reporting period.

2.4. Regulation of DDR in context of the cell cycle

Most published data on DDR describe the situation in interphase cells, when most of DNA repair occurs. In contrast, it remains poorly understood how cells respond to DNA damage during mitosis. We have tried to fill this gap and our research yielded three publications. First, we found that Wip1 phosphatase is phosphorylated by cyclin-dependent kinases and degraded by an APC^{cdc20}-dependent mechanism, thus allowing activation of ATM during mitosis (**Macurek et al., 2013**). Most of this work was accomplished before an official start of our group, and this publication is therefore affiliated to another research team at IMG. Second, we have described a novel phosphorylation of 53BP1 protein at Ser-1618 by Plk1 that results in suppression of DNA repair through non-homologous end joining pathway during mitosis (**Benada et al., 2015**). Our team accomplished all experiments in this study. This manuscript was accepted at the end of 2014 and published a few weeks after this reporting period. Third, we followed up on our earlier work and studied what mechanisms regulate activation of Plk1 in mitosis. We have challenged previous models published by others and found that Aurora-A and hBora continue to

activate Plk1 during mitosis (**Bruinsma et al., 2014**). Most of the work on this project was done in a collaborating laboratory. We performed experiments for detecting the kinase activity in mitotic cells and assisted with training of a PhD student from the collaborating team.

2.5. Role of R2TP complex in DDR

Several large multi-subunit protein complexes are involved in DDR and DNA repair. Folding of such complexes is facilitated by chaperones and the R2TP complex that contains a newly described phosphopeptide-binding protein PIH1. Part of our team (supervised by research fellow Zuzana Horejsi) studies what is the role of R2TP in response to genotoxic stress. Using phosphopeptide binding arrays combined with MS, we identified the putative interacting partners of PIH1. Among others, Mre11 (a part of the MRN complex) binds to R2TP through PIH1 in a phosphorylation-dependent manner. Currently we are addressing the functional relevance of this interaction. Besides this interaction, we have found that Mre11 is phosphorylated upon DNA damage by ATM, and this may change its ability to form some of the protein-protein interactions.

2.6. New research tools and methods

We have developed and validated several tools including:

- FRET-based biosensor that allows detection of ATM activity in living cells
- RPE-□PPM1D and HCT116-□PPM1D cell lines with knock-out of the *PPM1D* gene
- C57BL6-PPM1D^{TAL1} mouse model for study of oncogenic properties of truncated Wip1
- Two polyclonal antibodies against human Wip1 phosphatase

These research tools will be essential for our further work and will also be provided to the scientific community through collaborations.

2.7. Review publications

We have written a review article summarizing the knowledge about the function of R2TP complex in protein folding and in cellular response to stress. This review is now published in *Frontiers in Genetics*.

2.8. References

(Authors and/or co-authors of the evaluated team are in bold; corresponding author from the evaluated team is marked by asterisk; titles of the papers published during the evaluation period are underlined)

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Research Report of the team in the period 2010–2014

Institute	Institute of Molecular Genetics of the CAS, v. v. i.

Scientific team	Laboratory of Cell Differentiation

2.1. Introduction

The main interest of the laboratory is to study the molecular mechanism of cell fate determination. In the lab we have established in vitro systems to study self-renewal and differentiation of hematopoietic and neural stem cells. We use growth factors and small molecules as tools to manipulate these systems. More recently, we have initiated more systematic search for such tools using chemical biology/genetics approaches and established zebrafish as a new model in our laboratory.

2.2. Disp3 in neural stem cell differentiation

Disp3/Ptchd2 is a sterol-sensing domain-containing protein that is related to the family of Dispatched proteins. Expression profiling of Disp3 revealed predominant expression in the brain and retina. DISP3 was found to colocalize with cholesterol in the ER and upon overexpression induced lipid droplet formation (Zikova et al. 2009). We have identified two neural stem cell lines that highly express Disp3 (NS5, O4-ANS). Disp3 expression is positively regulated by T3 treatment and upon differentiation the level of Disp3 dramatically changes suggesting Disp3 may modulate self-renewal or differentiation. Brain tumours such as medulloblastoma are believed to arise from neural precursor cells. Analysis of a small number of primary human tumours revealed very high expression of Disp3, suggesting this protein is important for their pathogenesis. We have performed RNAi and overexpression studies and found out that Disp3 is able to modulate cell fate of neural stem cells and their progeny. In addition, we used a multipotent cerebellar progenitor cell line, C17.2, to investigate the impact of DISP3 on the proliferation and differentiation of neural precursors. We found that ectopically expressed DISP3 promotes cell proliferation and alters expression of genes that are involved in tumorigenesis. Finally, the differentiation profile of DISP3-expressing cells was altered, as evidenced by delayed expression of neural specific markers and a reduced capacity to undergo neural differentiation (**Zikova et al. 2014**). This work was performed in the Laboratory of Cell Differentiation with contribution of M. Kolar (bioinformatics analysis of the microarrays, IMG).

2.3. Dissection of haematopoiesis using vertebrate models

Differentiation of haematopoietic stem cells and progenitors into various lineages is controlled by a complex array of extrinsic and intrinsic factors. Although several important regulators have been identified, the exact molecular mechanism of cell commitment of the common megakaryocytic and erythroid progenitor is not fully understood. Recently, we have introduced the experimental model of

the chicken bi-potent thrombo-/erythropoietic progenitor (Bartunek et al. 2008) that was used for identifying the key regulators of cell fate determination by expression profiling using microarrays. The activity of identified candidates was validated using qPCR and 25 selected candidates were analysed in vivo using the zebrafish morpholino knockdown technology. We have validated at least 12 genes to be active in vivo (**Svoboda et al., in preparation**) and these candidates will be studied in future in human primary cells ($CD34^+$ cord-blood or bone-marrow derived cells).

We have extended our studies on vertebrate haematopoietic development to the zebrafish model and we have established ex vivo cultures of haematopoietic cells (Stachura et al. 2009). Recently, we have produced several recombinant zebrafish growth factors (Epo, Gcsfa/b, Tpo) that allow us to establish, for the first time, zebrafish haematopoietic clonal assays in semisolid media (**Stachura et al. 2011**). Granulocyte colony-stimulating factor (Gcsf) drives proliferation and differentiation of granulocytes, monocytes, and macrophages. Analysis of the zebrafish genome indicates the presence of two Gcsfs, likely resulting from a duplication event in teleost evolution. We show that in addition to supporting myeloid differentiation, zebrafish Gcsf is required for the specification and proliferation of hematopoietic stem and progenitor cells. These findings may inform how hematopoietic cytokines had evolved following the diversification of teleosts and mammals from a common ancestor (**Stachura et al. 2013**). Moreover, these tools enabled us to reveal the clonogenic and proliferation capacity of bi-potent thrombo/erythropoietic progenitors with respect to their mammalian haematopoietic counterparts. Despite obvious phenotypic differences between fish and mammalian thrombocytes and erythrocytes, our results strongly demonstrate the evolutionary conservation of basic processes and molecular mechanisms of erythro/thrombopoiesis in the vertebrates (**Svoboda et al. 2014**).

The zebrafish studies were performed jointly in the Laboratory of Cell Differentiation at IMG, AS CR (identification, cloning and expression of recombinant cytokines, in vitro and in vivo assays) and the Department of Cellular and Molecular Medicine, University of California, San Diego, La Jolla, CA, USA (in vivo assays). O. Svoboda and D. Stachura share co-first authorship on two publications. Molecular modelling was performed by J. Brynda (Laboratory of Structural Biology at IMG).

2. 4. Chemical biology

2.4.1. Signalling pathways in normal development and cancer

We have concentrated on the pathways that are involved in stem cell biology and often mutated in cancer such as Wnt, Shh, HIF, and TRAIL and identified several novel modulators (agonists/antagonists) of these pathways.

In high-throughput screening for novel agents/drugs that could sensitize TRAIL-resistant colorectal cancer cells to TRAIL-induced apoptosis, we found homoharringtonine (HHT), a cephalotaxus alkaloid and tested anti-leukaemia drug, to be a very effective, nanomolar enhancer of TRAIL-mediated apoptosis. Moreover, combined HHT + TRAIL therapy also led to strong suppression of HT-29 tumours implanted into immunodeficient mice. Thus, HHT represents a very efficient enhancer of TRAIL-induced apoptosis with potential application in TRAIL-based, anti-cancer combination therapy (**Beranova et al. 2013**).

In another study, we identified monensin, a polyether ionophore antibiotic, as a potent inhibitor of Wnt signalling. The Wnt signalling pathway is required during embryonic development and for the maintenance of homeostasis in adult tissues. However, aberrant activation of the pathway is implicated in a number of human disorders, including cancer of the gastrointestinal tract, breast, liver, melanoma, and hematologic malignancies. In multiple intestinal neoplasia mice, daily administration of monensin suppressed progression of the intestinal tumours without any sign of toxicity on normal mucosa. Our data suggest monensin as a prospective anticancer drug for therapy of neoplasia with deregulated Wnt signalling (**Tumova et a. 2014**).

Both publications were 50/50 collaborations with co-first and co-corresponding authors from the teams of L. Andera and V. Korinek (IMG).

2.4.2. Steroid receptor modulators

In the laboratory, we have a strong focus on nuclear receptors that are ligand-dependent transcription factors with very pleiotropic effects. Recently, we concentrated on the identification on novel oestrogen receptor modulators (**Sedlak et al. 2010, Eignerova et al. 2010, Sedlak et al. 2013** and patents US8334280B2, EP2274316B1, CZ300376) and profiling steroid activities for our collaborators (**Hessler et al. 2012, Rarova et a. 2012, Jurasek et al. 2013**)

2.4.3. Novel method development

As part of our chemical biology efforts we are constantly developing tools, methods and novel approaches that are being used in high-throughput screening campaigns. Some of them were already published and are exemplified by e.g. panels of steroid receptor luciferase reporter cell lines (**Sedlak et al. 2011**), fluorescence polarization assay to identify novel ligands of GPCII (**Alquicer et al. 2012**) and development of the fluorescence assay of Dicer activity suitable for uHTS (1536-well format) (**Podolska et al. 2014**).

The last two publications were 50/50 collaborations with co-first and co-corresponding authors from the teams of C. Barinka (IBT AS CR) and P. Svoboda (IMG).

We also contributed to the development of new antibiotics by introducing a fast ex vivo validation method for identification of cytoxic effects and potential therapeutic window using human primary cells (**Rejman et al. 2011** and patents EP2527351B1, CZ303569).

2.4.4. Cheminformatics

We are very active in developing informatics infrastructure to be able to professionally handle vast amounts of data generated at our facility. For the last couple of years we were developing LIMS and internal databases ChemGen DB and ScreenX. However, part of the code that might be useful for general use will be released as exemplified by InCHlib (Interactive Cluster Heatmap Library), a highly interactive and lightweight JavaScript library for cluster heatmap visualization and exploration. The cluster heatmap is one of the most popular visualizations of large chemical and biomedical data sets

originating, e.g., in high-throughput screening, genomics or transcriptomics experiments. (**Skuta et al. 2014** and http://www.openscreen.cz/software/inchlib). Other tools include algorhitms for chemical space exploration and identification of novel bioactive compounds in silico (**Hoksza et al. 2014**). The second publication represented collaboration with D. Hoksza (MFF UK), who developed algorithms behind the Molpher.

2.5. Research infrastucture

During the last couple of years we set up the state-of-the-art platform for chemical biology that enables us to perform the type of research previously inaccessible to academic scientists. The available technology and the level of automation and integration allow us to execute a broad portfolio of high-throughput miniaturized assays starting from kinetic biochemical assays to multiplexed high-content experiments (more information can be found at www.openscreen.cz. "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).

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 14 racks containing 700+ fish tanks. Currently we possess a collection of more than 30 transgenic and mutant zebrafish lines. This makes this unique setting very competitive in the Czech Republic, as well as in Europe.

2.6. References

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Research Report of the team in the period 2010–2014

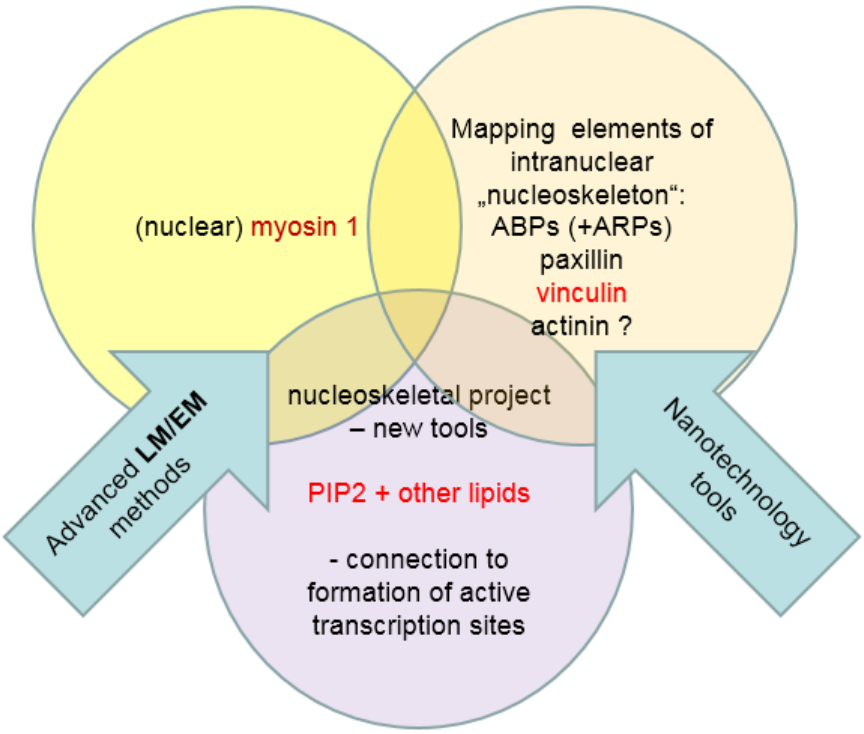
Institute	Institute of Molecular Genetics of the CAS, v. v. i.

Scientific team	Laboratory of Biology of the Cell Nucleus

2.1. Introduction – structure and aims of the lab

In diploid mammalian cells, some 6×10^9 base pairs of DNA fold as a nucleoprotein complex (i.e. chromatin) into higher-order arrays so as to fit in a nucleus measuring only 10 µm. The nucleus also contains machineries for transcription of genes and processing of RNA products, and for precise DNA replication, repair and recombination. Nuclear interior is therefore functionally highly compartmentalized, and the recent evidence points strongly to structure-related regulation of nuclear functions – however, the mechanisms forming the 3D-structure of the nucleus are still mostly obscure. We therefore employ a multi-disciplinary approach in order to study nuclear functions in relation to the higher-order nuclear structures. Our research concentrates on two long-term directions: (1) mechanisms of intranuclear structure formation and maintenance, and (2) formation of DNA transcription sites and modulation of their activity. These have been complemented by developing some novel imaging and nanotechnology tools.

The main areas of research during the period 2010–2014 are described in some detail below. They included work on functions of nuclear myosin I, actin, actin-related and actin-binding proteins, nuclear phosphoinosites, and signalling in cellular senescence.



Our research has always been international and we have maintained very useful collaborations especially with Dr. Masahiko Harata (Sendai), Dr. Monique Zetka (Toronto), Prof. Rolf Jessberger (Dresden), Prof. Susan Gasser (Zurich), and the German Mouse Clinic (Munich), most of them covered by collaborative grants.

In parallel to molecular/cell biology approaches, our laboratory also traditionally hosted the electron microscopy unit. We have accomplished a substantial volume of collaborative/service work in the field of cell ultrastructure, electron microscopic detection of various antigens, ultrastructural tomography, and cryo-electron microscopy.

During the period 2010–2014, a strong focusing of the lab had taken place; the signalling and genome stability topics were moved to a newly established Laboratory headed by prof. Jiří Bártek (a group led by Zdeněk Hodný), and the electron microscopy unit was set as an independent core facility at the beginning of 2015.

2.2. Methodological improvements and new research tools

We developed novel nanoparticles that improve immunoelectron detection methods by providing a wider scale of nanoparticles easily distinguishable in an electron microscope by their shapes. For the

first time in ultrastructural histochemistry, up to five molecular targets can now be identified simultaneously.

This was a truly collaborative effort in which each of the partner laboratories had its unique role, and the commercial partner guaranteed the future commercial use of the method. Our Laboratory was coordinating the entire project, developed the original idea, and performed all work on conjugating the nanoparticles with antibodies, and testing the conjugated nanoparticles on cell sections. The outcomes, besides the commercially available novel nanoparticle set, represented a few communications and a patent (Slouf et al., 2012; Philimonenko et al., 2014; Patent CZ 304250).

Electron microscopy is still a very valuable tool but often struggles with inadequately prepared samples. Based on the recent trends in sample preparation techniques going especially in the cryo direction, we have critically evaluated the existing methods, identified as the most critical issue extraction of proteins during the sample processing, and suggested some optimal procedures for obtaining the best results when working with sensitive antigens, especially in the cell nucleus. The recommendations were published in a series of articles and are now used by the scientific community (Sobol et al., 2010; 2011; 2012).

2.3. Signalling in cellular senescence

Cellular senescence guards against cancer and modulates aging; however, the underlying mechanisms remain poorly understood. Here, we showed that genotoxic drugs capable of inducing premature senescence in normal and cancer cells, such as 5-bromo-2'-deoxyuridine (BrdU), distamycin A (DMA), aphidicolin, and hydroxyurea persistently activate Janus kinase-signal transducer and activator of transcription (JAK/STAT) signalling and expression of interferon-stimulated genes (ISGs), such as MX1, OAS, ISG15, STAT1, PML, IRF1 and IRF7, in several human cancer cell lines. JAK1/STAT-activating ligands, interleukin 10 (IL10), IL20, IL24, interferon gamma (IFNgamma), IFNbeta and IL6, were also expressed by senescent cells, supporting autocrine/paracrine activation of JAK1/STAT. Furthermore, cytokine genes, including proinflammatory IL1, tumour necrosis factor and transforming growth factor families, were highly expressed. The strongest inducer of JAK/STAT signalling, cytokine production, and senescence was BrdU combined with DMA. RNA interference-mediated knockdown of JAK1 abolished expression of ISGs, but not DNA damage signalling, or senescence. Thus, although DNA damage signalling, p53 and RB activation, and the cytokine/chemokine secretory phenotype are apparently shared by all types of senescence, our data reveal so far unprecedented activation of the IFNbeta-STAT1-ISGs axis, and indicate a less prominent causative role of IL6-JAK/STAT signalling in genotoxic drug-induced senescence compared with reports on oncogene-induced or replicative senescence. These results highlight the shared and unique features of drug-induced cellular senescence, and implicate induction of cancer secretory phenotype in chemotherapy (Novakova et al., 2010).

2.4. Nuclear functions of myosin I

Different functions are spatially and temporarily organized in cell nuclei and many nuclear functions are connected with some kind of intranuclear movement and positioning. However, no nuclear molecular motor had been described until 1997, when in a collaborative effort we identified the 120 kDa nuclear protein as a new myosin IC isoform (NM1) that contains a unique 16 amino acid N-terminal extension. This had become the leading topic in our laboratory for some time, and we showed that the nuclear myosin and actin are required for transcription by RNA polymerases I and II both *in vitro* and *in vivo*, and reported that this myosin binds to TIFIA transcription factor of RNA polymerase I, which is connected with cellular metabolism (Philimonenko et al., 2004; Hofmann et al., 2004). These results allowed us to hypothesize that NM1 contributes to the movement of DNA relative to transcriptional complexes and/or it works as a conformational switch in the initiation/transcription complex. During the evaluated period, we concentrated on two aims: definition of NM1 binding properties and functional significance in DNA transcription, and definition of tissue-specific roles of NM1.

We investigated the mechanism of nuclear import of NM1 in detail. Using over-expressed GFP chimeras encoding truncated NM1 mutants, we identified a specific sequence that is necessary for its import to the nucleus. This novel nuclear localization sequence is placed within the

calmodulin-binding motif of NM1, and thus it is also present in Myo1c. We confirmed the presence of both isoforms in the nucleus by transfection of tagged NM1 and Myo1c constructs into cultured cells, and also by showing the presence of endogenous Myo1c in purified nuclei of cells derived from knock-out mice lacking NM1. Using pull-down and co-immunoprecipitation assays we identified importin beta, importin 5 and importin 7 as nuclear transport receptors that bind NM1. Since the NLS sequence of NM1 lies within the region that also binds calmodulin, we tested the influence of calmodulin on the localization of NM1. The

presence of elevated levels of calmodulin interfered with nuclear localization of tagged NM1.

Thus, we have shown that the novel specific NLS brings to the cell nucleus not only the "nuclear" isoform of myosin I (NM1 protein), but also its "cytoplasmic" isoform (Myo1c protein). This opens a new field for exploring functions of this molecular motor in nuclear processes, and for exploring the signals between the cytoplasm and the nucleus (Dzijak et al., 2012).

In order to trace the specific functions of the NM1 isoform, we generated mice lacking the NM1 start codon without affecting the cytoplasmic Myo1c protein. Mutant mice were analysed in a comprehensive phenotypic screen in cooperation with the German Mouse Clinic. We observed that Myo1c can replace NM1 in its nuclear functions. The amount of both proteins is nearly equal and NM1 knock-out does not cause any compensatory overexpression of Myo1c. We therefore suggest that both isoforms can substitute each other in nuclear processes – this opens a new view on the regulation of nuclear processes connected with nuclear myosin I (Venit et al., 2013).

2.5. Nuclear functions of actin and related proteins

Actin, actin-binding, actin-related proteins were the candidates to be found in myosin complexes in the nuclei. We have therefore previously performed a wide screen for the presence of these proteins in the cell nucleus (Dingová et al., 2009), summarized the findings and literature data in a review (Castano et al., 2010), and started deeper testing with a few hot candidates. We have worked on two most promising proteins: paxillin and vinculin, and we have now the manuscript and one research project submitted for evaluation. From the already published results, we concentrated on actin form in the cell nucleus, and together with our Japanese collaborators, on nuclear Arps.

Although actin monomers polymerize into filaments in the cytoplasm, the form of actin in the nucleus remains elusive. We searched for the form and function of beta-actin fused to the nuclear localization signal and to enhanced yellow fluorescent protein (EN-actin). Our results reveal that EN-actin is either dispersed in the nucleoplasm (homogenous EN-actin) or forms bundled filaments in the nucleus (EN-actin filaments). Among numerous actin-binding proteins tested, only cofilin is recruited to the EN-actin filaments. Overexpression of EN-actin causes increase in the nuclear levels of actin-related protein 3 (Arp3). Although Arp3, a member of actin nucleation complex Arp2/3, is responsible for EN-actin filament nucleation and bundling, the way cofilin affects nuclear EN-actin filaments dynamics is not clear. While cells with homogenous EN-actin maintain unaffected mitosis during which EN-actin re-localizes to the plasma membrane, generation of nuclear EN-actin filaments severely decreases cell proliferation and interferes with mitotic progress. The introduction of EN-actin manifests in two mitotic-inborn defects – formation of binucleic cells and generation of micronuclei – suggesting that cells suffer from aberrant cytokinesis and/or impaired chromosomal segregation. In interphase, nuclear EN-actin filaments pass through the chromatin region but do not co-localize with either chromatin remodelling complexes or RNA polymerases I and II. Surprisingly, the presence of EN-actin filaments was associated with increased overall transcription levels in the S-phase by a yet unknown mechanism. Taken together, EN-actin can form filaments in the nucleus that affect important cellular processes such as transcription and mitosis (Kalendová et al., 2014).

Nuclear actin and nuclear myosin I (NMI) are known important players in transcription of ribosomal genes, and transcription of rDNA takes place in a highly organized intranuclear compartment, the nucleolus. In the next study, we characterized the localization of these two proteins within the nucleolus of HeLa cells with high structural resolution by means of electron microscopy and gold-immunolabelling. We demonstrate that both actin and NMI are localized in specific compartments within the nucleolus, and the distribution of NMI is transcription-dependent. Moreover, a pool of NMI is present in the foci containing nascent rRNA transcripts. Actin, in turn, is present both in transcriptionally active and inactive regions of the nucleolus and co-localizes with RNA polymerase I and UBF. Our data support the involvement of actin and NMI in rDNA transcription and point out to other functions of these proteins in the nucleolus, such as rRNA maturation and maintenance of nucleolar architecture (Philimonenko et al., 2010).

The spatial organization of chromatin in the nucleus contributes to the genome function and is altered during the differentiation of normal and tumorigenic cells. Although nuclear actin-related proteins (Arps) have roles in local alteration of the chromatin structure, it is unclear whether they are involved in the spatial positioning of chromatin. In the interphase nucleus of vertebrate cells, the gene-dense and gene-poor chromosome territories (CTs) are located in the centre and periphery, respectively. We analysed chicken DT40 cells in which Arp6 had been knocked out conditionally and showed that the radial distribution of CTs was impaired in these knockout cells. Arp6 is an essential component of the SRCAP chromatin remodelling complex, which deposits the histone variant H2A.Z into chromatin. The redistribution of CTs was also observed in H2A.Z-deficient cells for gene-rich microchromosomes, but to a lesser extent for gene-poor macrochromosomes. These results indicate that Arp6 and H2A.Z contribute to the

radial distribution of CTs through different mechanisms. Microarray analysis suggested that the localization of chromatin to the nuclear periphery *per se* is insufficient for the repression of most genes (Maruyama et al., 2012). This was a collaborative effort, where we contributed all microscopy.

2.6. Deciphering nuclear roles of phosphatidylinositol 4,5-bisphosphate

Myosin I is generally known to be a regular binding partner of phosphatidylinositol 4,5-bisphosphate (PIP2) in the plasma membrane. We therefore asked whether a similar complexing takes place in the cell nucleus. This simple question evoked a long-term series of experiments and brought us in a mostly unexplored area of phosphoinosites in the cell nucleus – we have already published two papers in the evaluated period, and their topic has become a leading one, covered with a major grant, starting from 2015. The paper given below in fact represents the very first description of direct PIP2 involvement in the regulation of transcription.

When we investigated the role of PIP2 in RNA polymerase I (RNA Pol I) transcription using an *in vitro* transcription assay, we observed that the immunoblocking with anti-PIP2 antibody reduced the level of transcription by more than 80 %. The level of transcription was restored by the addition of exogenous PIP2 into the PIP2-depleted extract. We showed that PIP2 is present on the promoter along with transcription machinery [8] and there is a high level of PIP2 synthesis from a phosphatidylinositol precursor during on-going RNA Pol I transcription (unpublished results). We demonstrated that PIP2 interacts with the largest subunit of RNA Pol I (RPA116), UBF, and fibrillarin. UBF and fibrillarin are both essential components of rRNA biogenesis during RNA Pol I transcription initiation and rRNA early maturation steps, respectively. Super-resolution microscopy (SIM) confirmed PIP2 co-localization with UBF and fibrillarin in sub-nucleolar structures. Immunoelectron microscopy revealed that the interaction of PIP2 with UBF occurs in the inner space of fibrillar centres, where the inactive components of RNA Pol I transcriptional machinery are assembled, and in the dense fibrillar component, where rDNA transcription takes place. PIP2/fibrillarin clusters were detected only in the dense fibrillar component. In addition, PIP2 was present at the sites of nucleolar transcription *in situ* (labelled by Br-RNA). These data show the importance of PIP2 for rDNA transcription and possibly also for nucleolar compartmentalization (Yildirim et al., 2013).

In addition, we showed UBF complexes with phosphatidylinositol 4,5-bisphosphate in nucleolar organizer regions regardless of ongoing RNA polymerase I activity. We investigated transcription-related localization of PIP2 in regard to transcription and processing complexes of Pol I. To achieve this, we used either physiological inhibition of transcription during mitosis or inhibition by treatment of the cells with actinomycin D (AMD) or 5,6-dichloro-1-beta-d-ribofuranosyl-benzimidazole (DRB). We show that PIP2 is associated with Pol I subunits and UBF in a transcription-independent manner. On the other hand, PIP2/fibrillarin co-localization is dependent on the production of rRNA. These results indicate that PIP2 is required not only during rRNA production and biogenesis, as we have shown before, but also plays a structural role as an anchor for the Pol I pre-initiation complex during the cell cycle. We suggest that throughout mitosis, PIP2 together with UBF is involved in forming and maintaining the core platform of the rDNA helix structure. Thus we introduce PIP2 as a novel component of the NOR complex, which is further engaged in the renewed rRNA synthesis upon exit from mitosis (Sobol et al., 2013).

2.7. Other projects: Collaborative work and support to other labs

As mentioned previously, we provided collaborative or service support to many other projects, not started originally in our Laboratory – mostly with microscopy and data evaluation expertise. During the evaluation period, eight additional collaborative papers were published in the following areas: chromosomal dynamics (Philimonenko et al., 2010), neural stem cells (Kosi et al., 2014), anti-tumour agents acting on mitochondria (Rohlena et al., 2011; Dong et al., 2011), wound-healing gene therapy (Kolostova et al., 2012), biology of nuclear gamma-tubulin (Hořejší et al., 2012), autophagy physiology in bacteria (Hartlova et al., 2014), reproductive biology (Maňásková-Postlerová et al., 2011).

2.8. Other important research-related activities

We organized two international meetings: in 2013, the workshop “Intermediate filaments in health and disease” as a COST/SfH meeting with 140 participants (Roth et al., 2013), and in 2014, the world congress on microscopy “IMC2014” presided by P. Hozak with 3200 participants (www.imc2014.com).

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Research Report of the team in the period 2010–2014

Institute	Institute of Molecular Genetics of the CAS, v. v. i.

Scientific team	Laboratory of Genome Integrity

The Laboratory of Genome Integrity was established as a new laboratory at IMG in 2008. Our main research interests focus on the mechanisms of cellular DNA damage response (DDR) in relation to cancer and ageing. In 2010-2014, our aims were to unravel 1) mechanisms of cellular responses to persistent irreparable DNA damage lesions manifested as irreversible cell cycle arrest (cellular senescence) including a role of DNA damage-induced expression of secreted factors (cytokines) in autocrine/paracrine signalling, cancer microenvironment and cell reprogramming; 2) mechanisms of DNA damage signalling and repair including posttranslational modifications of key players involved in sensing and transmitting signals from DNA breaks to cellular effectors and cell cycle checkpoints, DNA repair and cell reprogramming; and DNA transactions mediated by RecQ DNA helicases, key players in the maintenance of genomic stability; 3) mechanisms of cell cycle control and cancer; 4) to develop new therapeutic strategies based on plasmonic photothermal properties of gold nanorods (cancer thermotherapy) or on approaches to overcome cancer cell plasticity linked with radioresistance and chemoresistance. In the evaluated period, we published 40 papers with IMG affiliation (cited > 400 times up to now: Scopus March 2015).

1. Cellular senescence

1.1. Senescence-associated secretory phenotype

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 foetal development, wound healing, tumorigenesis and ageing.

At the beginning of the evaluated period we published a study (Novakova et al., 2010) in which we showed for the first time that genotoxic stress induced by chemotherapeutic drugs activates a complex network of more than two dozens of various cytokine and chemokine species. These cytokines produced both in normal and tumour senescent cells activate corresponding signalling pathways and gene expression profiles in autocrine and paracrine ways and thus they can modulate tissue microenvironment. In follow-up studies, we have focused on the effects of this so-called senescence-associated secretory phenotype (SASP) in more detail. We studied the role of SASP in regulation of promyelocytic leukaemia protein (PML), which is a tumour suppressor upregulated in several forms of cellular senescence; however, the mechanism of its induction was at that time elusive. We showed (Hubackova et al., 2010) that genotoxic drugs that induce senescence, such as 5-bromo-2'deoxyuridine (BrdU), thymidine (TMD), distamycin A (DMA), aphidicolin (APH), etoposide (ET) and camptothecin (CPT) all evoke expansion of PML nuclear compartment and its association with persistent DNA lesions in

several human cancer cell lines and normal diploid fibroblasts. This phenomenon was accompanied by elevation of PML transcripts after treatment with BrdU, TMD, DMA and CPT. We found that JAK/STAT-mediated signalling is involved in regulation of the PML gene via Interferon Stimulated Response Element (ISRE) localized within the PML gene promoter. In summary, our data showed that upregulation of the PML tumour suppressor in cellular senescence triggered by diverse drugs including clinically used anti-cancer chemotherapeutics relies on stimulation of PML transcription by JAK/STAT-mediated signalling, possibly evoked by the autocrine/paracrine activities of senescence-associated cytokines.

Furthermore, we analysed the mechanism of PML expression in various cell types at unperturbed culture conditions (Hubackova et al., 2012b) as its cell type-specific regulation under non-stimulated conditions was poorly understood. We utilized three human cell types, BJ fibroblasts and HeLa and U2OS cell lines, each with a distinct PML expression pattern, to reveal that differences in the levels of activated STAT3 but not STAT1 correlate with PML mRNA and protein levels. We mapped the IL6/STAT3-responsive sequence to an ISRE(-595/-628) element of the PML promoter. Furthermore, the PI3K/Akt/NF-□B branch of IL6 signalling also showed cell-type dependence, being highest in BJ, intermediate in HeLa, and lowest in U2OS cells and correlated with IL6 secretion. Using RNAi-mediated knockdown of NEMO (NF-□B essential modulator), a key component of NF-□B activation, we found suppression of NF-□B targets LMP2 and IRF1 together with STAT3 and PML. When we analysed the role of both signalling pathways in PML gene expression, we found that combined knockdown of STAT3 and NEMO did not further promote PML suppression, and it could be bypassed by exogenous IL6, indicating the NF-□B pathway acts upstream of JAK-STAT3 through induction of IL6. In summary, our results indicate that the cell type-specific activity of IL6 signalling pathways governs PML expression under unperturbed growth conditions. As IL6 is induced in response to various viral and genotoxic stresses, this cytokine may regulate autocrine/paracrine induction of PML under these pathophysiological states as part of tissue adaptation to local stress.

In our follow-up study (Hubackova et al., 2012a), we identified IL1 and TGF□, two cytokine species secreted by senescent cells, that are responsible for development of secondary DNA damage and senescence in bystander cells. We found that this effect is caused by reactive oxygen species produced by NADPH oxidase Nox4, whose expression is controlled by IL1 and TGF□ signalling. These findings contribute to better understanding the link between persisting DNA damage and chronic inflammatory response and their participation in pathophysiology of several human diseases associated with cancer and aging.

In collaboration with the group of dr. Luciakova (Institute of Experimental Oncology, Slovak Academy of Sciences, Bratislava) we reported that adenine nucleotide translocase-2 (ANT2) is consistently down-regulated in all three major forms of cellular senescence: replicative, oncogene-induced and drug-induced, in both normal and cancerous human cells (Kretova et al., 2014). ANT2 is repressed by NF1/Smad transcription repressor complexes in non-proliferating cells and we identified formation of NF1/Smad transcription repressor complexes in etoposide-induced senescent cells. Mechanistically, binding of the NF1/Smad complexes to the NF1-dependent repressor elements in the ANT2 gene promoter repressed ANT2 expression. Etoposide-induced formation of these complexes and repression of ANT2 were co-incident with production and secretion of, and dependent on, TGFβ. siRNA-mediated knock-down of ANT2 in proliferating cells resulted in increased levels of reactive oxygen species (ROS) and activation of the DDR. Knock-down of ANT2, together with etoposide treatment, further intensified ROS production and DNA damage signalling, leading to enhanced apoptosis. Together, our data show

that TGF□-mediated suppression of ANT2 through NF1/Smad4 complexes contributes to oxidative stress and DNA damage during induction of cellular senescence.

In our last study so far on this topic (Hubackova et al., in press), we report that TNF□ and IFN□□ recently 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. Contrary to mouse tumour cells, where a concomitant presence of IFN□ and TNF□ was required, IFN□ alone (and after relatively short treatment) was sufficient to induce Nox4, Nox1 and DDR in human cells. The expression of Nox4 required unperturbed JAK/STAT signalling and, importantly, the effect was mediated by downstream activation of the TGF□/Smad pathway. Additionally, the expression of ANT2 was suppressed by IFN□ contributing to genotoxicity. In mouse TC-1 cells non-responding to IFN□/TNF□ by activation of DDR and senescence development, the inability to increase the expression of Nox4 was observed, supporting the role of ROS induced by NADPH oxidases in cytokine-induced senescence. Overall, our data reveal some unexpected differences between cytokine effects in mouse and human cells, and mechanistically implicate the TGF□/SMAD pathway, via induction of NADPH oxidases and suppression of ANT2, as key mediators of IFN□/TNF□-evoked genotoxicity.

1.2. Role of bacterial toxins in cellular senescence

Cytolethal distending toxins (CDTs) are proteins produced and secreted by facultative pathogenic strains of Gram-negative bacteria with potentially genotoxic effects. Mammalian cells exposed to CDTs undergo cell type-dependent cell-cycle arrest or apoptosis; however, the cell fate responses to such intoxication were mechanistically incompletely understood. We showed that both normal and cancer cells (BJ, IMR-90 and WI-38 fibroblasts, HeLa and U2-OS cell lines) that survive the acute phase of intoxication by *Haemophilus ducreyi* CDT possess the hallmarks of cellular senescence (Blazkova et al., 2010). This characteristic phenotype included persistently activated DNA damage signalling, enhanced senescence-associated □-galactosidase activity, expansion of PML nuclear compartment and induced expression of several cytokines (especially interleukins IL-6, IL-8 and IL-24), overall features shared by cells undergoing replicative or premature cellular senescence. Thus in analogy to oncogenic, oxidative and replicative stresses, bacterial intoxication represents another pathophysiological stimulus that induces premature senescence. Our study shows that activation of the two anticancer barriers, apoptosis and cellular senescence, together with evidence of chromosomal aberrations, support the emerging genotoxic and potentially oncogenic effects of this group of bacterial toxins.

1.3. Chromatin remodelling in cellular senescence

Human fibroblasts undergoing oncogene-induced or replicative senescence are known to form senescence-associated heterochromatin foci (SAHF), nuclear DNA domains enriched for histone modifications. We investigated the dependence of SAHF formation on the nature of senescence inducing stimulus and cell type context. We reported (Kosar et al., 2011) that human primary cells undergoing various forms of senescence show differential ability to form SAHF, and thus their formation is not a common feature of senescence. Furthermore, while the SAHF formation in cultured cells parallels expression of p16ink4a, SAHF are not observed in tissues, including premalignant lesions,

irrespective of enhanced p16ink4a and other features of senescence. This study documented the cell-type and insult-dependent formation of SAHF and its close link with the p16 tumour suppressor, and judged from the rapid and numerous citations, it is highly regarded by the scientific community.

1.4. Mechanism of oncogene-induced senescence

Both Myc and Ras oncogenes impact cellular metabolism, deregulate redox homeostasis and trigger DNA replication stress (RS) that compromises genomic integrity. However, how such oncogene-induced effects are evoked and temporally related, to what extent these kinetic parameters are shared by Myc and Ras, and how these cellular changes are linked with oncogene-induced cellular senescence in different cell context(s) remains poorly understood. We addressed the above-mentioned open questions by multifaceted comparative analyses of human cellular models with inducible expression of c-Myc and H-RasV12 (Ras), two commonly deregulated oncoproteins operating in a functionally connected signalling network (Maya-Mendoza et al., 2014). Our study of DNA replication parameters using the DNA fibre approach and time-course assessment of perturbations in glycolytic flux, oxygen consumption and production of reactive oxygen species (ROS) revealed the following results. First, overabundance of nuclear Myc triggered RS promptly, already after one day of Myc induction, causing slow replication fork progression and fork asymmetry, even before any metabolic changes occurred. In contrast, Ras overexpression initially induced a burst of cell proliferation and increased the speed of replication fork progression. However, after several days of induction Ras caused bioenergetic metabolic changes that correlated with slower DNA replication fork progression and the ensuing cell cycle arrest, gradually leading to senescence. Second, the observed oncogene-induced RS and metabolic alterations were cell-type/context dependent, as shown by comparative analyses of normal human BJ fibroblasts versus U2-OS sarcoma cells. Third, the energy metabolic reprogramming triggered by Ras was more robust compared to the impact of Myc. Fourth, the detected oncogene-induced oxidative stress was due to ROS (superoxide) of non-mitochondrial origin and mitochondrial OXPHOS was reduced (Crabtree effect). Overall, our study provides novel insights into oncogene-evoked metabolic reprogramming, replication and oxidative stress, with implications for mechanisms of tumorigenesis and potential targeting of oncogene addiction.

2. DNA damage signalling and repair

2.1. Role of RECQ5 in suppression of transcription-associated genomic stability

Replication-transcription encounters can cause chromosome breakage and genomic instability. Our studies (Kanagaraj et al., 2010; Paliwal et al., 2014) have shown that human DNA helicase RECQ5 binds to RNA-polymerase II during the elongation phase of transcription and suppresses transcription-associated genomic instability. More recently, we have found that RECQ5 also associates with RNA polymerase I during ribosomal DNA (rDNA) transcription and prevents amplification of DNA segments within the pre-rRNA coding region of rDNA arrays in cells exposed to replication stress. Moreover, we have found that RECQ5 depletion leads to replication fork stalling in both RNAPI- and RNAPII-transcribed genes. The blockage of transcription, but not of replication, immobilizes RECQ5 in DNA replication foci, suggesting that RECQ5 binds to transcription complexes at sites of concomitant transcription and

replication. Furthermore, we have found that RECQ5-depleted cells accumulate RAD51 foci that are formed in a BRCA1-dependent manner at sites of interference between replication and transcription and likely represent unresolved replication intermediates. Collectively, these results suggest a role for RECQ5 in resolution of conflicts between transcription and replication machineries.

2.2. Regulation of homologous recombination

Efficient repair of DNA double-strand breaks and interstrand cross-links requires the homologous recombination (HR) pathway, a potentially error-free process that utilizes a homologous sequence as a repair template. A key player in HR is RAD51, the eukaryotic orthologue of bacterial RecA protein. RAD51 can polymerize on DNA to form a nucleoprotein filament that facilitates both the search for the homologous DNA sequences and the subsequent DNA strand invasion required to initiate HR. Because of its pivotal role in HR, RAD51 is subject to numerous positive and negative regulatory influences. Using a combination of molecular genetic, biochemical, and single-molecule biophysical techniques, we have gained mechanistic insight into the mode of action of the FBH1 helicase as a regulator of RAD51-dependent HR in mammalian cells (Schwendener et al., 2010; Simandlova et al., 2013). We have shown that FBH1 binds directly to RAD51 and is able to disrupt RAD51 filaments on DNA through its ssDNA translocase function. Consistent with this, a mutant mouse embryonic stem cell line with a deletion in the FBH1 helicase domain fails to limit RAD51 chromatin association and shows hyper-recombination. Our data are consistent with FBH1 restraining RAD51 DNA binding under unperturbed growth conditions to prevent unwanted or unscheduled DNA recombination.

2.3. Molecular mechanism of ATR activation in response to DNA double-strand breaks

Ataxia telangiectasia and Rad3-related (ATR) protein kinase, a master regulator of DNA-damage response, is activated by RPA-coated single-stranded DNA (ssDNA) generated at stalled replication forks or DNA double-strand breaks (DSBs). We have identified the mismatch-binding protein MutSβ, a heterodimer of MSH2 and MSH3, as a key player in this process (Burdova et al., 2015). We have shown that MutSβ accumulates at sites of DSBs in a manner dependent on DNA-end resection and promotes DSB repair by homologous recombination. MSH2 and MSH3 form a complex with ATR and its regulatory partner ATRIP, and their depletion compromises the formation of ATRIP foci and phosphorylation of ATR substrates in cells responding to replication-associated DSBs. Purified MutSβ binds to hairpin loop structures persisting in RPA-ssDNA complexes and promotes ATRIP recruitment. Mutations in the mismatch-binding domain of MSH3 abolish the binding of MutSβ to DNA hairpin loops and its ability to promote ATR activation by ssDNA. These results suggest that hairpin loops might form in ssDNA generated at sites of DNA damage and trigger ATR activation in a process mediated by MutSβ.

2.4. Role of Rothmund-Thomson syndrome protein in maintenance of genomic stability

Inherited mutations in the RECQL4 gene cause Rothmund-Thomson syndrome that is associated with genomic instability and predisposition to osteosarcomas and skin cancer. Our goal is to define the functions of RECQL4 in the cell. We have found that RECQL4 is phosphorylated at serine 27 in response

to replication stress and plays a role in the restart of stalled replication forks (Singh et al., 2012). We have also discovered and characterized physical interaction between RECQL4 and the Bloom syndrome helicase (BLM). This study has shown that RECQL4 specifically stimulates the helicase activity of BLM on DNA fork substrates in vitro. Moreover, we found that the interaction between RECQL4 and BLM was enhanced during S-phase and after exposure of cells to ionizing radiation, which induces DSBs. The retention of RECQL4 at DSBs was shortened in BLM-deficient cells, and depletion of RECQL4 in BLM-deficient cells led to reduced proliferative capacity and increased frequency of sister chromatid exchanges. These results suggest that RECQL4 and BLM coordinate their activities to promote genomic stability.

2.5. Biochemistry of DNA-end resection

The 5'-3' resection of DNA ends is a prerequisite for repair of DSBs by homologous recombination, microhomology-mediated end joining and single-strand annealing. Recent studies in yeast have shown that following an initial DNA-end processing by the Mre11-Rad50-Xrs2 complex and Sae2, extension of resection tracts is mediated either by Exonuclease 1 or by combined activities of the RecQ-family DNA helicase Sgs1 and the helicase/endonuclease Dna2. Although human DNA2 was shown to cooperate with the BLM helicase to catalyse resection of DNA ends in vitro and in vivo, it remains a matter of debate whether another RecQ helicase, namely WRN, can substitute for BLM in DNA2-catalysed resection in mammalian cells. Using GFP-based reporter assays, we have shown that WRN and BLM act epistatically with DNA2 to promote long-range resection of endonuclease-induced DSBs in human cells (Sturzenegger et al., 2014). Our biochemical experiments have demonstrated that WRN and DNA2 interact physically and coordinate their enzymatic activities to mediate 5'-3' DNA-end resection in a reaction dependent on RPA. In addition, our experiments have revealed that BLM promotes DNA-end resection as part of the BLM-TOPOIIIa-RMI1-RMI2 complex, which is also required for resolution of recombination intermediates. This study provides new mechanistic insights into the process of DNA-end resection in mammalian cells.

2.6. The role of BRCA1 isoforms in DNA damage response

Alternative pre-mRNA splicing is a fundamental post-transcriptional regulatory mechanism. Cancer-specific misregulation of the splicing process may lead to formation of irregular alternative splicing variants (ASVs) with a potentially negative impact on cellular homeostasis. Alternative splicing of BRCA1 pre-mRNA can give rise to BRCA1 protein isoforms that possess dramatically altered biological activities compared with full-length wild-type BRCA1. During the screening of high-risk breast cancer (BC) families we ascertained numerous BRCA1 ASVs; however, their clinical significance for BC development is largely unknown. In this study (Sevcik et al., 2013), we examined the influence of the BRCA1Δ17-19 ASV, which lacks a portion of the BRCT domain, on DNA repair capacity using human MCF-7 BC cell clones with stably modified BRCA1 expression. Our results show that overexpression of BRCA1Δ17-19 impairs homologous recombination repair (sensitizes cells to mitomycin C), delays repair of ionizing radiation-induced DNA damage and dynamics of the ionizing radiation-induced foci (IRIF) formation, and also undermines the non-homologous end joining repair (NHEJ) activity. Mechanistically, BRCA1Δ17-19 cannot interact with the partner proteins Abraxas and CtIP, thus preventing interactions known to be

critical for processing of DNA lesions. We propose that the observed inability of BRCA1Δ17-19 to functionally replace wtBRCA1 in repair of DSBs reflects impaired capacity to form the BRCA1-A and -C repair complexes. Our findings indicate that expression of BRCA1Δ17-19 may negatively influence genome stability by reducing the DSBs repair velocity, thereby contributing to enhanced probability of cancer development in the affected families.

2.7. siRNA screens and the mechanism of nuclear import of 53BP1

53BP1 is a mediator of DDR and a tumour suppressor whose accumulation on damaged chromatin promotes DNA repair and enhances DDR signalling. In this study (Moudry et al., 2012b) we performed an siRNA-based functional high-content microscopy screen for modulators of cellular response to ionizing radiation and we show for the first time that a nuclear pore component NUP153 is a novel factor specifically required for 53BP1 nuclear import via importin-□ and as such is involved in maintenance of genome integrity.

2.8. Identification of ubiquitylation enzymes involved in DNA repair machinery

The cellular DDR machinery is orchestrated by signalling through protein modifications. Protein ubiquitylation regulates repair of DNA double-strand breaks (DSBs), toxic lesions caused by various metabolic as well as environmental insults such as ionizing radiation (IR). Whereas several components of the DSB-evoked ubiquitylation cascade have been identified, including RNF168 and BRCA1 ubiquitin ligases, whose genetic defects predispose to a syndrome mimicking ataxia-telangiectasia and cancer, respectively, the identity of the apical E1 enzyme involved in DDR has not been established. In this study (Moudry et al., 2012a), we identified ubiquitin-activating enzyme UBA1 as the E1 enzyme required for responses to IR and replication stress in human cells. We show that siRNA-mediated knockdown of UBA1, but not of another UBA family member UBA6, impaired formation of both ubiquitin conjugates at the sites of DNA damage and IR-induced foci (IRIF) by the downstream components of the DSB response pathway, 53BP1 and BRCA1. Furthermore, chemical inhibition of UBA1 prevented IRIF formation and severely impaired DSB repair and formation of 53BP1 bodies in G(1), a marker of response to replication stress. In contrast, the upstream steps of DSB response, such as phosphorylation of histone H2AX and recruitment of MDC1, remained unaffected by UBA1 depletion. Overall, our data establish UBA1 as the apical enzyme critical for ubiquitylation-dependent signalling of both DSBs and replication stress in human cells, with implications for maintenance of genomic integrity, disease pathogenesis and cancer treatment.

3. Cell cycle control and cancer

3.1. Heterochromatinization and DDR in germ cell tumours

Heterochromatinization has been implicated in fundamental biological and pathological processes including differentiation, senescence, ageing and tumorigenesis; however, little is known about its

regulation and roles in human cells and tissues in vivo. In this study (Bartkova et al., 2011), we showed distinct cell-type- and cancer-stage-associated patterns of key heterochromatin marks: histone H3 trimethylated at lysine 9 and heterochromatic adaptor proteins HP1α and HP1γ, compared with the γH2AX marker of endogenously activated DDR and proliferation markers in normal human foetal and adult testes, pre-invasive carcinoma in situ (CIS) lesions and a series of overt germ cell tumours, including seminomas, embryonal carcinomas and teratomas. Among striking findings were high levels of HP1γ in foetal gonocytes, CIS and seminomas; enhanced multimarker heterochromatinization without DDR activation in CIS; and enhanced HP1α in teratoma structures with epithelial and neuronal differentiation. Differential expression of the three heterochromatin markers suggested their partly non-overlapping roles, and separation of heterochromatinization from DDR activation highlighted distinct responses of germ cells vs. somatic tissues in early tumorigenesis. Conceptually interesting findings were that subsets of human cells in vivo proliferate despite enhanced heterochromatinization, and that cells can strongly express even multiple heterochromatin features in the absence of functional retinoblastoma protein and without DDR activation. Overall, these results provide novel insights into cell-related and tumour-related diversity of heterochromatin in human tissues in vivo, relevant for andrology and intrinsic anti-tumour defence roles attributed to activated DDR and cellular senescence.

3.2. Role of Wip1 phosphatase in DDR during mitosis

Cells are constantly challenged by DNA damage and protect their genome integrity by activation of an evolutionarily conserved DNA damage response pathway (DDR). A central core of DDR is composed of a spatiotemporally ordered net of post-translational modifications, among which protein phosphorylation plays a major role. Activation of checkpoint kinases ATM/ATR and Chk1/2 leads to a temporal arrest in cell cycle progression (checkpoint) and allows time for DNA repair. Following DNA repair, cells re-enter the cell cycle by checkpoint recovery. Wip1 phosphatase (also called PPM1D) dephosphorylates multiple proteins involved in DDR and is essential for timely termination of the DDR. We have investigated how Wip1 is regulated in the context of the cell cycle (Macurek et al., 2013). We found that Wip1 activity is downregulated by several mechanisms during mitosis. Wip1 protein abundance increases from G(1) phase to G(2) and declines in mitosis. Decreased abundance of Wip1 during mitosis is caused by proteasomal degradation. In addition, Wip1 is phosphorylated at multiple residues during mitosis, and this leads to inhibition of its enzymatic activity. Importantly, ectopic expression of Wip1 reduced γH2AX staining in mitotic cells and decreased the number of 53BP1 nuclear bodies in G(1) cells. We propose that the combined decrease and inhibition of Wip1 in mitosis decreases the threshold necessary for DDR activation and enables cells to react adequately even to modest levels of DNA damage encountered during unperturbed mitotic progression.

3.3. The temporal relationship among oncogene activation, DDR and induction of tumour suppressor ARF

Oncogenes trigger the DDR and induction of the ARF tumour suppressor (TS), both of which can activate p53 and provide intrinsic barriers to tumour progression. We analyse the timeframes and signal thresholds for ARF induction and DDR activation during tumorigenesis using mouse models and human samples of urinary bladder, colon, pancreatic and skin premalignant and malignant lesions (Evangelou et al., 2013). We have found that ARF expression occurred at a later stage of tumour progression than

activation of the DDR or p16INK4A, a TS gene overlapping with ARF. We propose that ARF provides a complementary and delayed barrier to tumour development, responding to more robust stimuli of escalating oncogenic overload.

3.4. The role of Wip1 mutations in cell cycle control during DDR

The DDR pathway and its core component p53 block cell cycle after genotoxic stress and represent an intrinsic barrier preventing tumour development. The phosphatase Wip1 inactivates p53 and promotes termination of the DDR. We identified novel gain-of-function mutations of Wip1 that result in expression of truncated Wip1 affecting the DDR pathway (Kleiblova et al., 2013). Mutations in Wip1 are present both in the tumours and in other tissues of breast and colorectal cancer patients, indicating that they arise early in development. We propose that mutations in Wip1 could predispose to cancer.

3.5. Activation of ATR kinase by mechanical stress

ATR controls chromosome integrity and chromatin dynamics. In collaboration with M. Foiani group (IFOM; Milan), we showed that ATR activity at the nuclear envelope responds to mechanical stress (Kumar et al., 2014). ATR associates with the nuclear envelope during S phase and prophase, and both osmotic stress and mechanical stretching relocalize ATR to nuclear membranes throughout the cell cycle. The ATR mediated mechanical response is reversible and is independent of DNA damage. We propose that mechanical forces derived from chromosome dynamics and torsional stress on nuclear membranes activate ATR to modulate nuclear envelope plasticity and chromatin association to the nuclear envelope. This novel checkpoint pathway that we reported in *Cell* opens numerous avenues for future studies in biomedicine.

3.6. Interplay between DAXX, p53, ATM kinase and Wip1 phosphatase

Death domain-associated protein 6 (DAXX) is a histone chaperone, putative regulator of apoptosis and transcription, and candidate modulator of p53-mediated gene expression following DNA damage. DAXX becomes phosphorylated upon DNA damage; however, regulation of this modification and its relationship to p53 remain unclear. We showed that in human cells exposed to ionizing radiation or genotoxic drugs etoposide and neocarzinostatin, DAXX became rapidly phosphorylated in an ATM kinase-dependent manner (Brazina et al., 2015). Our deletion and site-directed mutagenesis experiments identified serine 564 (S564) as the dominant ATM-targeted site of DAXX, and immunofluorescence experiments revealed localization of S564-phosphorylated DAXX to PML nuclear bodies. Furthermore, using a panel of human cell types, we identified the p53-regulated Wip1 protein phosphatase as a key negative regulator of DAXX phosphorylation at S564, both in vitro and in cells. Consistent with the emerging oncogenic role of Wip1, its DAXX-dephosphorylating impact was most apparent in cancer cell lines harbouring gain-of-function mutant and/or overexpressed Wip1. Unexpectedly, while Wip1 depletion increased DAXX phosphorylation both before and after DNA damage and increased p53 stability and transcriptional activity, knock-down of DAXX impacted neither p53 stabilization nor p53-mediated expression of Gadd45a, Noxa, Mdm2, p21, Puma, Sesn2, Tigar or

Wip1. Consistently, analyses of cells with genetic, TALEN-mediated DAXX deletion corroborated the notion that neither phosphorylated nor non-phosphorylated DAXX is required for p53-mediated gene expression upon DNA damage. Overall, we identify ATM kinase and Wip1 phosphatase as opposing regulators of DAXX-S564 phosphorylation, and propose that the role of DAXX phosphorylation and DAXX itself are independent of p53-mediated gene expression.

4. New therapeutic strategies

4.1. Development of new gold nanorods for cancer thermotherapy

Gold nanorods exhibit specific optophysical properties such as plasmonic photothemal effect, which can be utilized for eradication of tumour cells by lasers operating in the near infrared region of light. In collaboration with the group of Dr. J. Proska (CVUT/FJFI, Prague), we developed a new kind of gold nanorods with cationic surfactant suitable for photothermal therapy.

4.2. Utilising ERK/AKT inhibitors to suppress radioresistance and chemoresistance

We show that a type of genotoxic stress caused by radiotherapy or chemotherapy and used for clinical cancer treatment induces cellular reprogramming in stress-surviving epithelial tumour cells to cells with mesenchymal and stem cell traits and acquired resistance to subsequent treatment (Kyjacova et al., 2014). Examining the mechanism of this phenomenon we found that development of this phenotypic plasticity depends on active Erk/Akt signalling. Our results suggest the way to exploit the differential sensitivity of tumour cells to radiosensitizing drugs in overcoming radioresistance and chemoresistance.

5. Reviews

In the evaluated period we were invited to write several reviews and commentaries in the area of our expertise for journals such as Nature, Nature Genetics, Cancer Cell, Oncotarget, J. Chem. Med., etc., see (Andrs et al., 2014a; Andrs et al., 2014b; Bartek and Hodny, 2010; Bartek and Hodny, 2014a; Bartek and Hodny, 2014b; Bartek and Hodny, 2014c; Hodny et al., 2010a; Hodny et al., 2013; Hodny et al., 2010b).

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Research Report of the team in the period 2010–2014

Institute	Institute of Molecular Genetics of the CAS, v. v. i.

Scientific team	Laboratory of Transgenic Models of Diseases

The description (this form) requested by the Academy of Sciences is only partly relevant to this team, which ***not only comprises a regular scientific part but also service groups***, as it has an **exceptional role within IMG**, serving as an incubator where research projects and specialized research infrastructure (mainly transgenic technologies and functional genomics) have been developing, mainly (but not only) within the framework of the BIOCEV project.

The team has dynamically developed over the last four years, growing to 55 persons at the end of 2014. However, it must be stressed that the majority of the staff is not primarily involved in research as established in all other groups of IMG, but in the other activities important for the whole IMG.

As stated above, the team has a unique role in IMG, virtually serving as an incubator, which fosters both research and service (research infrastructure) activities. The team (actually rather an equivalent of a department with several semi-independent teams) has dynamically developed over the last years with the goal to give rise to new research infrastructure and research projects and teams. All the activities of the team successfully aimed to create several new research groups and research facilities forming the core of the BIOCEV division of the Institute, formally the research infrastructure "**Czech Centre for Phenogenomics" (CCP, http://www.phenogenomics.cz**). Although thematically distinct, all these projects and activities are based on the usage of mouse models as a tool to reveal gene functions in the complexity of the whole organism.

As the team developed into by far the largest group in IMG, its size, complexity, and dynamics also required professional management necessary for administration and organization of the BIOCEV division.

Regarding the output of the team, there are three basic categories: service activities (they will develop further), collaborative publications based on service and expertise of the CCP team, and own publications.

For this research report, altogether 55.6 FTE were considered in the team. The majority of them belong to the CCP infrastructure (whose aim is not primarily to produce its own publications). The staff primarily dedicated to research have only several postdocs/senior researchers, of whom one senior (M. Gregor) has established his own research group within the BIOCEV project since 1.1.2015. Another senior researcher (M. Balastik) has recently received an independent position as a group leader at the Institute of Physiology AS CR v. v. i., Prague although he is still also affiliated to our group.

2.2. Czech Centre for Phenogenomics

T**he Czech Centre for Phenogenomics (CCP)** provides expertise and services to the biomedical research community studying the function of genes in biological processes and/or human disorders *in vivo* using mouse or rat models. **CCP** is the **only site** in the Czech Republic with expertise and capacity for large-scale genetic engineering and for production of genetically modified mice and rats (from 2015), as well as for systematic and standardized comprehensive phenotyping. This know-how is combined with high breeding capacity under specific pathogen-free (SPF) conditions and cryo-archiving of animal models, ensuring access of Czech but also EU users to services and expertise at a level comparable with leading institutions in Europe. CCP services contributed substantially to Institute publications in high-impact journals (e.g. Flemr et al., Cell. 2013; 155, where CCP developed a mouse model to examine the role of a retrotransposon-driven dicer isoform).

Altogether, CCP covers a full spectrum of genetic engineering services, strain cryo-archiving services, advanced phenotyping and imaging services, as well as specific pathogen-free (SPF) animal housing and husbandry. CCP was subject to comprehensive evaluation within the national roadmap of large research infrastructures and received the grade A1 (best evaluation) prioritizing its funding from 2016 by MEYS, Czech Republic. **The focus of CCP** is to serve the biomedical research community in understanding gene functions through the following activities and services:

- developing technologies for gene targeting and genome editing
- utilizing gene targeting and editing strategies for generation of animal models
- characterizing the physiological impact of gene mutations in animal models
- providing comprehensive support for mouse strain cryo-archiving and cryo-recovery
- enabling timely import, export, and distribution of live animal strains and cryo-material
- providing extensive expertise, training, and support to customers and students

One of the key elements of CCP is the innovation potential in generation of new rodent models using programmable nucleases (TALENs and CRISPR/Cas-based approaches). Employing these new technologies, CCP has seen turnaround times reduced over 75 % from conventional methods. The efficiency of inducing the desired mutation has increased by a similar order of magnitude. Now mice harbouring targeted genetic mutations can be produced in as little as six weeks at a fraction of the cost using conventional methods. The use of these technologies has been an area of rapid growth within CCP, which at present is generating up to three mouse strains per week.

An expanded range of possibilities for the generation of mouse models now includes deletion mutations, introduction of point mutations, multiplexing mutant production, and various knock-in and fusion-protein mutant models. Whereas conventional gene targeting is restricted to the genetic strain of the employed embryonic stem cells, using programmable nuclease-based approaches avoids this inflexibility. Now mutations can be produced in a number of genetic backgrounds or species (e.g. from

wild mouse strains to spontaneously hypertensive rats). CCP is continually building upon our good track record for supporting the Czech research community. For example, we produced a mouse model to investigate the role of a retrotransposon-driven dicer isoform, which was then published in the prestigious journal *Cell* (***Flemr et al., 2013***). Such publications are testament to the importance of our research infrastructure for the Institute. CCP is also vitally important for securing Czech investments in basic research by providing long-term storage (cryo-archiving) and re-derivation services. Through these services, existing genetic mouse strains are insured against accidental loss and researchers are not unnecessarily burdened with the high costs of maintaining active breeding colonies. Worldwide distribution and import of animal resources are handled by our import and export service, with a significant volume of requests mediated through our integration in the EMMA network.

Phenotyping, i.e. comprehensive and standardized characterization of the impact of selected gene ablation or mutation on the main physiologic systems of the organism, is the next step of the established CCP production pipeline. In this regard, CCP builds a rat phenotyping pipeline to offer the research community more possibilities, especially in studies towards metabolic syndrome.

Finally, CCP also represents an educational hub where not only researchers but also undergraduate and postgraduate students as well as technical staff can obtain a broader range of skills and experience and access to state of the art resources.

2.3. Research topics

Besides the service-based activities, the team has also had its own research topics, historically dedicated mainly to understanding the protease function (this is going to be changed in 2015 and later – see below).

2.3.1. Proteases in physiology and disease

One research topic has been focused on proteases, particularly on matrix metalloproteinases (MMP), a disintegrin and metalloproteinase (ADAM), and kallikreins (Klk). MMP and Klk proteases are partly responsible for controlling extracellular matrix-cell interactions affecting cell differentiation, survival, migration, and other processes. ADAM proteinases (ADAM 10, ADAM17) release ligands and their receptors from the cell surface, thus guiding bioavailability of many important regulatory molecules. The balance among the proteases and their natural inhibitors determines whether biological processes are to be initiated or terminated. This balance is pivotal for tissue homeostasis and its disturbance may lead to development of various pathologies.

While all the metalloproteinase projects are about to be finalized in 2015, the project focused on kallikreins delivered promising results in 2014 and will be further carried on (see further description).

Metalloproteinases in physiology and pathology of liver and glucose metabolism

This topic was addressed using mouse models deficient for MMP-19, ADAM17, and ADAM 10.

The role of MMP-19 in the liver was described in ***Jirouskova et al. (2012).*** We showed that loss of MMP-19 was beneficial during liver injury, as plasma ALT and AST levels, deposition of fibrillar collagen, and phosphorylation of SMAD3, a TGF-ß1 signalling molecule, were all significantly lower in MMP19KO mice. The ameliorated course of the disease in MMP19KO mice likely results from a slower rate of basement membrane destruction and ECM remodelling, as the knockout mice maintained significantly higher levels of type IV collagen and lower expression and activation of MMP-2 after four weeks of CCl_4-intoxication. Accelerated liver regeneration in MMP19KO mice was associated with slightly higher IGF-1 mRNA expression, slightly increased phosphorylation of Akt kinase, decreased TGF-ß1 mRNA levels and significantly reduced SMAD3 phosphorylation. Primary hepatocytes isolated from MMP19KO mice showed impaired responsiveness towards TGF-ß1 stimulation, resulting in lower expression of Snail1 and vimentin mRNA. Thus, MMP-19 deficiency improves the development of hepatic fibrosis through the diminished replacement of physiological extracellular matrix with fibrotic deposits in the beginning of the injury, leading to subsequent changes in TGF-ß and IGF-1 signalling pathways.

Analysing measures of liver function in MMP19KO mice, we found reduced levels of cholesterol in the sera of MMP19KO mice and decided to evaluate this protective affect also in two models of biliary obstruction. We found that cholesterol and bile acids (especially cholic acid) are accumulated in the livers of MMP19KO mice. These findings correlated with elevated levels of sterolin-1 and -2 – members of the ABC-transporter family.

As preparation of ADAM10, ADAM17 and ADAM10/17-double-defficient mutants took a long time, we could only publish two small papers to date; however, several additional publications are currently being finalized (one is already submitted).

We showed that the liver-protective effect of ursodeoxycholic acid includes regulation of ADAM17 activity (Buryova et al., 2013). Ursodeoxycholic acid (UDCA) is used to treat primary biliary cirrhosis, intrahepatic cholestasis, and other cholestatic conditions. Although much has been learned about the molecular basis of the disease pathophysiology, our understanding of the effects of UDCA remains unclear. The beneficial effect of UDCA appears to be mediated in part by the inhibition of ADAM17 activation and, thus, the release of TNFα, a strong pro-inflammatory factor. The release of other ADAM17 substrates, TGFα and sMet, are also regulated in this way, pointing to a general impact. In parallel, UDCA upregulates TIMP-1 that, in turn, inhibits MMPs, which destroy the hepatic ECM.

The second paper describes ADAM10/17-dependent release of soluble c-Met correlating with hepatocellular damage (***Chalupsky et al., 2013***). The signalling pathway elicited by hepatocyte growth factor (HGF) and its receptor c-Met is indispensable for liver development and regeneration. We show that sMet is released by both ADAM17 and ADAM10, with ADAM17 appearing to be the major proteinase. Employing a model of 3,5-diethoxycarbonyl-1,4-dihydroxycollidine (DDC)-induced hepatobiliary obstruction we revealed that the serum levels of sMet correlated well with liver damage, consecutive regeneration, and with established markers of liver damage. sMet exhibited remarkably better correlation with liver damage and inflammation than did serum tumour necrosis factor α (TNF-α), whose shedding is also mediated by ADAM proteolytic activity. The differential serum levels of sMet

together with expression of c-Met/HGF might be a useful indicator not only for damage, but also for ongoing liver regeneration.

In addition, in a manuscript currently being prepared we describe complete protection of ADAM10-deficient mice against septic shock, providing attractive results for medicinal treatment and the impact of ADAM10/17 deficiency in liver function. Thus, the submitted manuscript reports on the role of ADAM10 in liver function and reveals a nice aging-related phenotype. Mice deficient for ADAM10 in hepatocytes, cholangiocytes and liver progenitor cells develop spontaneous liver necrosis and a ductular reaction in young animals at four weeks of age. In consequence, accumulation of LPCs through loss of ADAM10 enhances activation of HSCs and liver fibrosis formation.

Furthermore, we studied the role of MMP-19 in glucose metabolism via insulin release. Analysing the MMP-19-deficient mouse model, we demonstrated that MMP-19 regulates pancreatic islet beta cell function and insulin secretion through the fractalkine (FKN)/CX3CR1 system. Using glucose and insulin tolerance tests we showed that MMP-19 KO mice manifest impaired glucose tolerance with normal insulin sensitivity. Higher blood glucose levels were paralleled by impaired glucose-stimulated insulin secretion (GSIS). Static GSIS test using primary mouse islets confirmed reduced insulin secretion from islets isolated from MMP-19 KO mice when compared to their wild-type (WT) counterparts. Thus, the insulin secretory defect in MMP-19 KO islets is mediated by alterations in islet function independent of an extra-islet mechanism. Moreover, MMP-19 KO pancreatic islets exhibited reduced insulin secretion in response to arginine, further indicating that MMP-19 deficiency causes a β cell insulin secretory defect. Histological analysis of MMP-19 KO islets revealed increased β cell mass. Their ultrastructure was characterized by rupture of the membrane of the secretory granules, generally resembling necrotic pancreatic β cells after type 2 diabetes development. Altogether, these phenotypes recapitulated well the situation described for CX3CR1 KO mice. A recently published study on this model showed that the fractalkine/CX3CR1 system regulates β cell function and insulin secretion. (***manuscript in preparation***)

Metalloproteinases in physiology and pathology of intestine, inflammatory bowel disease and colon cancer

This topic was addressed using mouse models deficient for MMP-19, MMP-12, and MMP-18.

We demonstrated that MMP-19 is 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. Neutrophil recruitment is initially delayed; however, later on, *Mmp-19*$^{-/-}$ mice develop persistent inflammation. The dysregulated immune response in *Mmp-19*$^{-/-}$ mice was rescued by bone marrow transplantation from *Mmp-19*$^{+/+}$ mice. Moreover, we found that MMP-19 directly generates a soluble chemokine domain of CX3CL1 (fractalkine) and that *Mmp-19*$^{-/-}$ mice show diminished processing of CX3CL1. Altogether, MMP-19 mediates beneficial effects in intestinal inflammation and is an important factor in healing and homeostasis of the mucosa ***(Brauer et al., submitted).*** We have also comprehensively characterized its expression pattern in the intestine (***Cervinkova et al. 2014***).

The role of MMP-19 in colon cancer is completely unknown. We studied the consequences of functional ablation of this MMP on colon cancer. We focused our investigation on two kinds of cancer: colitis-

associated cancer (CAC) and an AOM-induced model of sporadic cancer (CRC). Our findings revealed that while MMP-19 deficiency in CAC leads to promotion of tumorigenesis, it appears that *MMP-19 plays a key protective role in colitis-associated cancer initiation or progression.* The tendency of MMP-19-deficient epithelia to form polyps and promote APCmin-driven tumorigenesis at sites of inflammation may be due to the previously reported compromised mucosal barrier, by severely retarded healing ability, and by aberrantly increased counts of Bmi1 positive crypt cells in the KO mice. In addition, both types of cancer can result from attenuated number of goblet cells in the deficient colon and by dysregulated Notch1 signalling. In summary, our data reveal multiple roles of MMP-19 in carcinogenesis. (***manuscript in preparation***)

2.3.2. Mouse models of human diseases

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 models, Diamond-Blackfan anaemia (DBA) and haemophilia A were selected to confirm gene-therapeutic possibilities. The selection of these diseases is based on the presence of relatively simple and well-characterized mutations, on the availability of human samples such as blood or bone marrow, on the possibility of their autologous transplantation, and on the availability of transgenic mouse models.

Thus, the main goal of this project is to introduce the TALEN application technology into the gene therapy field, by validating the specific repair of the gene function in mouse models and at the cellular level using cultures of cells isolated from patients. Particular goals of the project are: generation of new mouse models of the described diseases using the TALEN technology in order to better correspond to human mutations; correction of mutations using gene therapy; establishment of the set of specific TALE-nucleases for reparations of human mutations; and their application and validation using cells obtained from patients. In 2014, we succeeded in generating a new mouse model for DBA which recapitulates the phenotype of human patients; the mouse models die within the first two months after birth. Currently, we are preparing the manuscript that describes this model.

2.3.3. Ubiquitylation-mediated processes in health and disease

Using mutant mouse models we are addressing the role of several ubiquitin ligases whose function and role have not been described. A major focus of these studies is to understand the role of ubiquitylation in regulating the intestinal barrier function and to characterize links with human inflammatory bowel disease. This topic will be the major focus of our group as the field of Ub-ligases and deubiquitinases is of key importance for cellular processes and many of the genes in the Ub-ligase and deubiquitinase families (altogether around 900 members) have not been investigated so far, or investigated only poorly. In 2014, we already created the first, largely conditional, U3-ligase-deficient mouse models (among them Btbd3, Trim15, Rnf121, Wdsub1, Mex3b, and others) and each of the new postdocs works and studies one of them. Although this topic is new for the department, we have already obtained interesting results: for instance, RNF121 is indispensable for embryonic development as homozygous mice do not survive *in utero*.

2.3.4. Stem cell pluripotency and early embryonic development

Recently, the major effort has been dedicated to the Fam208a (D14Abb1e) gene and heterochromatic gene silencing. FAM208A (D14Abb1e) was identified in an ENU mutagenesis screen for genes that alter expression of a variegating reporter transgene in mice. Classified as a new suppressor of variegation, its mutation not only leads to deficient transgene silencing, but also to a failure of embryonic gastrulation. Using unique mouse models we are currently addressing the molecular mechanisms by which Fam208a influences embryonic development, stem cell pluripotency, and embryonic robustness to environmental stressors and teratogens.

2.4. DEVELOPMENT OF RESEARCH TOOLS

This area interlinks CCP and the other IMG and BIOCEV 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 last two years we have massively invested our effort into new transgenic technologies, especially those which include 'programmable nucleases' as they create a new base for efficient generation of animal models and open new possibilities for the development of human disease treatments.

These programmable nucleases, especially **TALE nucleases and the CRISPR/Cas9 system have revolutionized production of the genetically modified animals.** These technologies allow easy programming for targeting a specific locus in the genome through introduction of targeted DNA double-strand breaks. Repair of this DNA break by non-homologous end joining (NHEJ) leads to modifications of the targeted locus and creation of insertions or deletions (indels), which often lead to frame-shifts mutations. Since such frame-shifts often result in non-functional genes/proteins, TALENs or CRISPR/Cas9 can be used for generation of knock-out animals. However, these nucleases are not limited to production of knock-outs. Targeting two sites simultaneously also allows specific deletions within the genome. Also, once a DNA double strand-break is introduced, it is possible to introduce repair constructs (double-stranded DNA constructs or single-stranded oligonucleotides) via homologous recombination. With these new tools, modifications of any kind (including mutation of a few base-pairs, tagging of proteins, or creating conditional knock-outs) are possible in a short period of time.

In CCP, we have not only established the TALEN and CRISPR/Cas9 technologies, but also developed several of our own tools which make this technology more effective. We have set up a small team (one head, two technicians) as part of TAM that produces these tools and provides consultancy to end-users. We accomplished more than 50 projects using this technology during the last year, so far resulting in two publications: the first was a cooperation, for which we generated the transgenic models (***Flemr et al., 2013***) and the second was a technology-development publication (***Kasparek et al., 2014***). We have also developed additional tools and currently are preparing two additional technology publications focusing on programmable nucleases. Although all completed projects for transgenic model generation should eventually result in publications, this is expected on average to lag by 1-2 years.

We have started to improve and develop new methods also in the currently established phenotyping units. For example, we have already developed a new technology for bile acids analysis, which is very useful for biochemical and metabolic screenings.

Altogether, our own research and participation in research projects led by PIs outside of CCP is a main driving force for the development of our centre.

Publications (2010-2014):

(Authors and/or co-authors of the evaluated team are in bold; corresponding author from the evaluated team is marked by asterisk)

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