SYMPOSIUM 1: FUNCTIONAL GENOMICS, PROTEOMICS AND BIOINFORMATICS

1.1. Epigenetics: DNA Methylation and Far Beyond

IL 1.1–1
The role of MeCP2 in the brain

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The DNA of every cell in the body carries a pattern of chemical modifications due to the methylation of cytosine in the dinucleotide sequence 5′CG. It is thought that these chemical marks help to define the pattern of gene expression that is appropriate for each cell type. The nuclear protein MeCP2 was originally discovered because of its ability to specifically bind to methylated CG sites, but not to CGs lacking the methyl moiety. Because of its DNA binding preference, it was hypothesised that MeCP2 interprets the DNA methylation signal. A considerable body of evidence indicates that DNA methylation causes gene silencing and, in line with this view, early evidence established that MeCP2 can attract molecular machinery that contains enzymes capable of altering chromatin structure, for example by removing acetyl groups from histone tails. A plausible hypothesis is therefore that MeCP2 binds to methylated DNA and recruits deacetylase activity so that the chromatin environment becomes incompatible with efficient transcription. This scenario predicts that the absence of MeCP2, as in neurons of patients with the autism spectrum disorder Rett Syndrome, will cause inappropriate gene expression due to relaxed repression. Decisive evidence supporting this prediction has proven elusive so far and other potential functions for MeCP2 have been proposed – for example that it is an activator of transcription or a regulator of messenger RNA splicing. The functional significance of MeCP2 will be assessed in the light of studies of the structure and dynamics of the interaction between MeCP2 and methylated DNA at the molecular level. At the level of brain physiology, the unexpected reversibility of Rett Syndrome-like symptoms in *Mecp2*-null mice has important implications, as it demonstrates that MeCP2 can assume its normal functions in a brain that developed and acquired severe neurological symptoms in the complete absence of MeCP2. These results challenge the long-held view that Rett Syndrome is a 'neurodevelopmental disorder'. Taken together, the molecular and neurobiological information implicate MeCP2 as a protein that is essential for the tight maintenance and stability of gene expression programs in mature nerve cells.

IL 1.1–2
Asymmetric cell division through epigenetic differentiation of sister chromatids and their selective segregation in mitosis

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Our studies with the model system of fission yeast have discovered two new principles of biology. First, developmental asymmetry of sister cells simply results from the inheritance of older 'Watson' versus older 'Crick' chain-containing chromatids at the *mat1* locus where through epigenetic means nonequivalent sister chromatids are generated by chromosome replication. Second, epigenetic states controlling gene repression are inherited in mitosis and meiosis as remarkably stable conventional Mendelian markers (1). We propose that likewise asymmetric cell divisions in higher eukaryotes might result by further postulating biased segregation of differentiated sister chromatids of both copies of a specific chromosome to daughter cells (2,3). Can we explain hitherto unexplained developmental traits/disorders in humans and vertebrates by invoking such principles? The causes of schizophrenia and bipolar human psychiatric disorders are unknown. A novel somatic cell genetics, *SSIS* (*S*omatic *S*trand-specific *I*mprinting and *S*elective strand segregation) model, postulated biased segregation of differentiated older 'Watson' versus 'Crick' DNA chains of a chromosome to specific daughter cells. Such an oriented asymmetric cell division in embryogenesis may constitute the mechanism for development of healthy, functionally nonequivalent brain hemispheres in humans. For evidence, genetic translocations of the relevant chromosome might therefore cause disease by disrupting the chromosome-specific biased chromatid segregation process. This way the epialleles of a hypothetical gene controlling brain laterality development in the translocation-containing chromosome will be randomly distributed to sister cells. Accordingly, the model predicts that symmetrical brain hemispheres might develop in 50% of translocation carriers. Thus, the observation of only 50% of chromosome 1/6/9;11 translocation carriers that do develop disease is in accord with the model (4). Likewise, the SSIS model is also advanced for visceral laterality development in mice.

References:

1. Klar AJS. Lessons learned from studies of fission yeast mating-type switching and silencing. *Annual Review of Genetics* 2007; 41: 213–36.
2. Armakolas A and Klar AJS. Cell type regulates selective segregation of mouse chromosome 7 DNA strands in mitosis. *Science* 2006; 311: 1146–1149.
3. Armakolas A and Klar AJS. Left-right dynein motor implicated in selective chromatid segregation in mouse cells. *Science* 2007; 315:100–1.
4. Klar AJS. A genetic mechanism implicates chromosome 11 in schizophrenia and bipolar diseases. *Genetics* 2004; 167: 1833–1840.

IL 1.1–3
Epigenomic programs and reprogramming in mammals

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Epigenetic programs play an essential role for the establishment of pluri-and totipotency of cells in the early embryo. In mammals histone modificatons and DNA-methylation patterns are rapidly changed on the parental chromosomes merged from from the egg

and the sperm. This reprogramming begins rapidly after fertilization and predominantly affects the paternal (sperm) chromosomes in the first cell cycle. The reprogramming is apparently crucial to reset the chromatin for developmentally regulated genetic programs. Comparative immunofluorescence based analysis of chromatin changes reveals a striking conservation of the dynamic and specificty of such epigenetic reprogramming events in early mammalian embryos. Of particular interest are enzymatic mechanisms which trigger a rapid replication independent elimination of DNA-methylation from the paternal chromosomes in the zygote. Such 'active' demethylation mechanisms occur in a time window of about 4–7 hours postfertilization. We investigated the potential involvement of DNA-repair mechanisms in this DNA-demethylation process and identified a striking accumulation of strand breaks and repair markers around the early phases of zygotic development. We furthermore observe that potential repair activities can be separated from DNA-replication processes. We conclude that epigenetic reprogramming is indeed partially linked to DNA repair processes. I will discus the potential mechanisms of such DNA-demethylation processes and some of their general implications for genetic and epigenetic variation.

IL 1.1–4
Molecular coupling of X-inactivation regulation and pluripotency

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The integration of X-chromosome inactivation into the developmental process is a crucial aspect of this paradigm of epigenetic regulation. During early female mice development, X-inactivation reprogramming occurs in pluripotent cells of the inner cell mass of the blastocyst, when imprinted X-inactivation is replaced by random inactivation, via a transient stage characterized by the presence of two active X-chromosomes. Reactivation of the inactive X also occurs in pluripotent primordial germ cells and is also observed *in-vitro*, during the reprogramming of female somatic cells mediated by nuclear cloning, by fusion with embryonic stem (ES) cells, and during the generation of induced pluripotent stem (iPS) cells. Reprogramming of X-inactivation is therefore associated with the acquisition of pluripotency*in-vivo* and *in-vitro*. Using ES cells, we have demonstrated that the coupling of X-inactivation reprogramming with pluripotency depends on the functional interaction of the master genes controlling pluripotency with key players in the X-inactivation process such as its molecular trigger, the non-coding *Xist* RNA, and its antisens *cis*-repressor *Tsix*. Nanog, Oct4 and Sox2 (the triumvirate of factors underlying pluripotency) all cooperate to repress *Xist* in undifferentiated ES cells. Additionally, Rex1 (a well-known marker of pluripotent cells), Klf4 and c-Myc (which in conjunction with Oct4 and Sox2 are required to generate iPS cells) are involved in conferring maximal transcriptional activity to *Tsix* in undifferentiated ES cells. Our results provide a molecular framework linking X-inactivation reprogramming to the control of pluripotency, and shed light on how pluripotency and genome reprogramming factors reset established epigenetic states.

OP 1.1-1
Recognition of monomethylated histone peptides by the malignant brain tumor repeats of human SCML2

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SCML2 (Sex Comb on Midleg-like 2) is a constituent of the Polycomb repressive complex 1, a large multiprotein assembly involved in the long term silencing of gene expression required to maintain cell identity. SCML2 contains two N-terminal 100-residue malignant brain tumor (MBT) repeats, a protein module adopting a beta-barrel core similar to that of chromatin-binding domains like chromo- and Tudor domains. All are members of the Royal superfamily of effector modules able to "read" different types of histone post-translational modifications. We have used NMR spectroscopy to investigate the binding specificity of the MBT repeats of human SCML2. Our data show that they preferentially recognize histone peptides monomethylated at lysine residues, with no apparent sequence specificity, and also free monomethylated lysine. Patterns of chemical shift changes are very similar for all the monomethylated lysine-containing peptides and for the monomethylated lysine residue, mapping a cluster of residues at one end of the beta-barrel of the second repeat. The crystal structure of the complex between the protein and monomethylated lysine shows that the modified amino acid is buried deep into a conserved aromatic pocket formed by two phenylalanine and one tryptophan residues. A salt bridge between the monomethylammonium moiety and the carboxylate group of a conserved aspartate residue further provides specificity for the lowest lysine methylation state. This work is a good example of synergy between NMR and X-ray crystallography.

1.2. Evolution of Polyploid Genomes

IL 1.2-1
Epigenetic variation and inheritance

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Our group has been studying the regulation and function of cytosine methylation by combining different genetic approaches in Arabidopsis. One arm of this analysis has been characterization of variation in DNA methylation found in natural accessions. This approach has the advantage of allowing us to assess and dissect the contributions from both genetic and epigenetic variation. In some cases, we find that epigenetic variation and inheritance plays a major role in shaping extant variation in DNA methylation. For example, differences in DNA methylation of the *Sadhu* class of transposable elements among different natural accessions co-segregates in inter-strain crosses with the elements themselves. This *cis*-regulation is consistent with epigenetic inheritance of parental DNA methylation levels. However, this epigenetic inheritance can also be modulated by *trans*-acting genetic variation. In some cases, trans-acting genetic variation plays a dominant role. For example, we have identified one natural accession that has reduced centromere DNA methylation caused by deletion in a gene encoding an SRA domain methylcytosine-binding protein, VIM1 (variant in methylation 1). *VIM1* and a subset of its paralogs function together to maintain CpG methylation and transcriptional silencing throughout the Arabidopsis genome.

IL 1.2–2
Plant chromosomes at interphase - paired? cohesed? dynamic?

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Eukaryotic chromosomes occupy distinct territories within interphase nuclei. The arrangement of chromosome territories (and of specific chromatin domains therein) is likely to be important in key events that occur within cell nuclei such as replication, transcription, repair and recombination processes. Our knowledge about interphase chromatin arrangement, mainly based on results obtained by means of various *in situ* labelling approaches, is still meagre. Nevertheless, it is emerging that phylogenetic affiliation of a species, cell cycle and differentiation status, as well as environmental influences may have an impact on, and may cause alterations of, interphase nuclear architecture. Most data regarding interphase structural organization in plants have been obtained for Brassicaceae (*Arabidopsis thaliana* and related species) and for cereal species. I will survey the present knowledge about interphase arrangement of Brassicaceae chromosomes concerning the relative positioning of chromosome territories, somatic pairing of homologues, and sister chromatid alignment in meristematic and differentiated tissues. Furthermore I will discuss the morphological constraints and epigenetic impacts on the nuclear architecture and the evolutionary stability of chromosome arrangement patterns as well as alterations of nuclear architecture during transcription and repair, in mutants with increased recombination activity, and in lines carrying transgenic tandem repeat arrays.

IL 1.2–3
Genetics and epigenetics in diploid and tetraploid Arabidopsis

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Approximately three decades ago, a small genome and low genetic redundancy were major arguments for the choice of the small weed *Arabidopsis thaliana* as a model organism for molecular biology of higher plants. Nevertheless, genome analysis has revealed remnants from probably three ancient polyploidization events. Fertile polyploid Arabidopsis is easy to generate from recent diploid accessions. Furthermore, a substantial portion of cells undergo endoreplicationeven in diploid plants, reaching high levels of ploidy. Therefore, the plentiful resources of genetic and genomic Arabidopsis information have been helpful to study the consequences of auto- and allopolyploidization. These changes are suspected to be important driving forces for plant evolution, since many higher plants and most crop plants are polyploid. Like in many other species, polyploidization in Arabidopsis is associated with changes in the sequence and/or the chromatin configuration of nuclear DNA. Multiplications of chromosome numbers can thereby contribute to heritable, genetic and epigenetic diversity. We will report on the formation and stability of epialleles at transgenic and endogenous sequences and the role of chromatin-modifying factors, based on analysis with molecular, genetic and cytological approaches. The work in the lab is supported by grants from the Austrian Science Fund (FWF), the EU Network of Excellence 'Epigenome' and the GEN-AU program of the Austrian Ministry for Science and Research.

IL 1.2–4
Mechanisms of gene expression rewiring in hybrids and polyploids

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Genome merging, in interspecific hybrids and allopolyploids, is associated with novel patterns of gene expression. We have analyzed the genetic and epigenetic basis for this rewiring in two model systems, namely a yeast hybrid between *Saccharomyces cerevisiae* and *S. paradoxus*, and a synthetic wheat hybrid and allopolyploid analogous to bread wheat. In yeast, we have analyzed how hybrid-specific gene expression patterns are generated from the divergence in regulatory components between the parental species. Between the species, we have distinguished changes in regulatory sequences of the gene itself (cis) from changes in upstream factors (trans). Expression divergence was mostly due to changes in cis. Changes in trans were condition-specific and reflected mostly differences in environmental sensing. In the hybrid, over-dominance in gene expression occurred through novel cis-trans interactions or, more often, through modified trans regulation associated with environmental sensing. We will discuss the phenotypic impact of hybrid-specific expression patterns. In wheat we have previously shown rapid genetic and epigenetic alterations in genes or transposons at the onset of hybridization

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and/or in nascent allopolyploids. As small RNAs are candidates for affecting these events, we have analyzed the changes in small RNAs (Micro and siRNAs) populations in hybrids and allopolyploids and their connection with gene and transposon expression. We show that small RNA populations are altered in hybrids and polyploids with the strongest changes occurring upon polyploidization. Overall, in the first generation of the polyploid, there was a massive suppression of siRNAs that corresponds to repeats and transposons. This is consistent with the observed transcriptional activation of transposons upon polyploidization and supports the role of siRNAs in heterochromatinization and repression of transposons. These works emphasize how different levels of regulation, namely genetic, epigenetic and environmental, can bring about hybrid-specific expression patterns in lower and higher eukaryotes.

OP 1.2–1
Transcription through transgene is the most frequent cause of positive position effects in Drosophila melanogaster

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This work is dedicated to study position effects in Drosophila using a mini-white gene as a model system. As a result of insertion of P-element vectors containing a mini-white gene without enhancers into random chromosomal sites, flies with different eye color phenotypes appear. Such effects are usually explained by the influence of enhancer/silencer elements located around the insertion site of the mini-white transposon. As a consequence, insulators/MAR elements were broadly used to protect a transgene expression from position effects. Alternatively we supposed and showed that in many cases transcription through the transgene is responsible for high levels of its expression in most of chromosomal sites and be the cause of positive position effects. Moreover the white promoter was decayed by efficient transcription initiated from an upstream promoter. These results suggest that enhancer–promoter interactions are more specific and that incorrect stimulation of a promoter by a wrong enhancer is a relatively rare event. It seems likely that the initiation of white translation is able to induce from internal regions of transcripts. Thus, in the absence of this property, transcription through a transgene might lead to reducing of its expression. Our results also showed that transcriptional terminators but not a strongest Drosophila gypsy insulator, are efficient in protecting gene expression from transcription-mediated position effects. Therefore, combining an insulator and a terminator is the best way to make transgene expression independent from position effects.

1.3. Bioinformatics: from Comparisons to Functional Predictions

IL 1.3–1
The evolution of enzyme mechanisms and functional diversity

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Enzyme activity is essential for almost all aspects of life. With completely sequenced genomes, the full complement of enzymes in an organism can be defined, and 3D structures have been determined for many enzyme families. Traditionally each enzyme has been studied individually, but as more enzymes are characterised it is now timely to revisit the molecular basis of catalysis, by comparing different enzymes and their mechanisms, and to consider how complex pathways and networks may have evolved.

IL 1.3–2
UniProtKB/Swiss-Prot: from sequences to functions

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The UniProtKB/Swiss-Prot knowledgebase [1] strives to provide its users a corpus of manually annotated protein entries. Swiss-Prot is far from being a mere repository of sequence. Since its creation in 1986, its mission has always been to provide its users, an up-to-date description of what is known about a particular protein. Today, genomic sequences are very easily obtained and from them it is relatively trivial to predict the corresponding protein-coding regions. But there is still no shortcut to allow the high-throughput elucidation of the function of all of these predicted proteins. It is therefore important to capture in a knowledgebase such as Swiss-Prot experimentally-derived information that will permit to infer the function of related proteins in an increasingly widening variety of organisms. We therefore concentrate our annotation efforts on a palette of model organisms that are the target of characterization studies. For these organisms that range from bacteria (*E. coli*), fungi (*S. cerevisiae*), plants (*A. thaliana*) to mammals (human and mouse) we try to be as complete as possible and provide as much information as we can that helps travelling the path that leads from sequence to function.

Reference:

1. Nucleic Acids Res. 37:D169-D174(2009); DOI = 10.1093/nar/gkn664.

IL 1.3–3
Computational approaches to unveiling ancient genome duplications

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Recent analyses of eukaryotic genome sequences have revealed that gene duplication, by which identical copies of genes are created within a single genome by unequal crossing over, reverse transcription, or the duplication of entire genomes, has been rampant. The creation of extra genes by such duplication events has now been generally accepted as crucial for evolution and of major importance for adaptive radiations of species and the general increase of genetic and biological complexity. We have developed software to identify remnants of large-scale gene duplication events and more recently, we have also developed mathematical models that simulate the birth and death of genes based on observed age distributions of duplicated genes, considering both small and large scale duplication events. Applying our model to the model plant Arabidopsis shows that much of the genetic material in extant plants, i.e., about 60% has been created by several genome duplication events. More importantly, it seems that a major fraction of that material could have been retained only because it was created through large-scale gene duplication events. In particular transcription factors, signal transducers, and regulatory genes in general seem to have been retained subsequent to large-scale gene duplication events. Since the divergence of (duplicated) regulatory genes is being considered necessary to bring about phenotypic variation and increase in biological complexity, it is indeed tempting to conclude that such large scale gene duplication events have indeed been of major importance for evolution.

IL 1.3–4
The evolutionary design of proteins

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Natural proteins display structural and functional features that seem beautifully matched for their biological role. They fold spontaneously into well-defined three-dimensional structures, and can display complex biochemical properties such as signal transmission, efficient catalysis of chemical reactions, specificity in molecular recognition, and allosteric conformational change. These properties are known to arise from the cooperative action of amino acid residues, but the pattern of residue cooperativity in the tertiary structure is generally unknown. To address this, we have been developing an approach (the statistical coupling analysis or SCA) for estimating the evolutionary constraints between sites on proteins through statistical analysis of large and diverse multiple sequence alignments[1,2]. This analysis indicates a novel decomposition of proteins into sparse groups of co-evolving amino acids that we term 'protein sectors'[9]. The sectors are statistically quasi-independent and comprise physically connected networks in the tertiary structure. Experiments in several protein systems demonstrate the functional importance of the sectors[1,3,4,7,8] and recently, the SCA information was shown to the necessary and sufficient to design functional artificial members of two protein families in the absence of any structural or chemical information. These results support the hypothesis that the SCA captures the basic architecture of functional interactions in proteins. We are now working on understanding the physical mechanisms underlying statistical coupling, and perhaps more importantly, trying to understand any principles of why the SCA pattern might represent the natural design of proteins that emerge through the evolutionary process.

References:

1. Lockless, Ranganathan R. *Science* 1999; **286**: 295–9.
2. Suel *et al*., *Nature Struct*. *Biol*. 2003; **10**: 59–69.
3. Hatley, *et al*., *PNAS* 2003; **100**: 14445–14450.
4. Shulman et al., *Cell* 2004; **116**: 417–429.
5. Socolich et al., Nature 2005; **437**: 512–518.
6. Russ et al., *Nature* 2005 **437**: 579–583.
7. Mishra et al., *Cell* 2007; **131**: 80–92.
8. Lee et al., *Science* 2008; **322**: 438–442.
9. Halabi et al., 2009. manuscript submitted.

OP 1.3–1
Promoter mapping: in silico, *in vitro* and *in vivo*

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Nowadays a large number of promoter-search protocols for both eukaryotic and prokaryotic genomes have been designed. Being based on different platforms, they take into account practically all known features of promoter DNA and are attuned for accurate and efficient recognition of known promoters. However, highly sensitive promoter finders if used for genome scanning tend to generate a large amount of false positives, resembling promoters by formal criteria but functionally inactive. In this study we evaluate conformity of *in silico*, *in vitro* and *in vivo* data for the set of unexpected promoters predicted by pattern-recognition software PlatProm within coding sequences and intergenic regions. RNA polymerase binding capacity *in vitro* was verified for 32 out of 34 tested promoters, indicating high capacity of PlatProm to recognize promoter region. The coefficient of correlation between PlatProm scores and percentage of DNA-bound enzyme appeared to be rather high (0.63) assuming ability of the program to predict enzyme binding efficiency. However, only 23 tested promoter regions were captured by RNAPol *in vivo* showing hybridization signals with microarray probes in ChIP-on-chip assays. Correlation coefficient between PlatProm scores and efficiency of RNAPol binding according to ChIP-on-chip data was also low (0.23), reflecting yet unpredictable structural state of promoters within nucleoide. Since the final goal of genome analysis is to reconstruct regulatory events taking place on bacterial chromosome novel approaches are required to account this natural environment by promoter finders of new generation.

OP 1.3-2
Protein-protein interaction network analysis of exosomal proteome

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Exosomes are membrane vesicles secreted from endosomal membrane compartment by various cell types such as hematopoietic, epithelial, and tumor cells. Actively growing tumor cells shed exosomes, and the rate of shedding increases in malignant tumors. Although recent progress in this area has revealed that exosomes play multiple roles in intercellular communication including immune modulation and signal transduction, the precise sorting mechanism into exosomes and their complex biological roles are still unclear. Here, we organized a detailed proteinprotein interaction map of this extracellular organelle using comprehensive proteomic analysis and bioinformatics approach. This network showed the overall architecture of the exosomes and essential hub proteins such as 14-3-3 proteins, CSNK2A1 and SRC. Also, we revealed that exosome proteins are sorted together by protein-protein interactions and organized by functional modules tightly associated with cell structure and motility, intracellular protein traffic, protein targeting and localization. Our results highlights that the physically interacting proteins are sorted together into exosomes and form modules with functional relevance, which are associated with exosome biogenesis and functions. Taken together with previously reported results, our observations suggest that exosomes may act as communicasomes, i.e. extracellular organelles that play diverse roles in intercellular communication.

SYMPOSIUM 2: PROTEIN STRUCTURE AND INTERACTIONS

2.1. Protein Folding

IL 2.1–1
Quantifying interactions and energy landscapes of membrane proteins by single-molecule force spectroscopy and microscopy

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Molecular interactions drive all processes in life. They determine the molecular crosstalk and build the basic language of biological processes. By developing a combined approach of atomic force microscopy and single-molecule force spectroscopy (SMFS) we image individual membrane proteins and locate their molecular interactions at submolecular resolution. The approach observes how molecular interactions fold a polypeptide into the functional protein, stabilize the structure, or lead to protein misfolding. It also measures protein–protein interactions, interactions switching on and off ion channels, ligand- or inhibitor-binding, the functional states of receptors, and the supramolecular assembly of molecular machines as functional units. Dynamic SMFS (DFS) obtains insights into the mechanical rigidity, transition state, lifetime, and free energy stabilizing the structural regions of a membrane protein. Using DFS we reveal mechanistic insights how molecular interactions modulates these energetic parameters to precisely tune the function of a membrane protein.

IL 2.1–2
Towards physico-chemical understanding of fibril formation of the Alzheimer disease-associated amyloid beta-peptide

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Protein aggregation can result in a major disturbance of cellular processes, and is associated with several human diseases. The amyloid b peptide (Ab) seems to play an important role in the pathogenesis of Alzheimer's disease (AD). Ab is produced from a precursor protein, APP, by specific proteases and is kept at a constant concentration in healthy individuals. The main proteolytic products have 40 and 42 residues, respectively, and the 42 residue peptide is most aggregation prone and of higher significance for disease development. Onset of AD correlates with an imbalance in the ratio of the 42 versus 40 products or increased total concentration. The fibrillar form of Ab has a characteristic stacking of b strands perpendicular to the long axis of the fiber. The molecular events behind the process leading from native to fibrillar states remain elusive, but accumulated data from many studies suggest that it involves a number of intermediate oligomeric states of different association numbers and structures. Pre-fibrillar oligomers seem to be critical components for development of disease symptoms. Important questions regard molecular properties of Ab peptide and its environment which prevent or promote aggregation and amyloid fibril formation. To address these questions we have developed a recombinant expression system with a facile and scalable purification protocol for Ab(M1-40) and Ab(M1-42), which relies on inexpensive tools [Walsh et al., 2009]. This allows us to produce large quantities of highly pure monomeric peptide to enable large scale systematic studies. We have also made an effort to eliminate as many sources of experimental error as possible and can now acquire highly reproducible kinetic data on Ab fibrillation. We will report here the results of large scale systematic studies of the fibrillation kinetics of Ab and its dependence of physic-chemical factors such as peptide concentration, pH, temperature, ionic strength, salt type and concentration, as well as the results from studies of the effects of various kinds of biological macromolecules and surfaces including phospholipid membranes of different compositions.

Reference:

Walsh DM, Thulin E, Minuogue A, Gustafsson T, Pang E, Teplow DB, Linse S. *FEBS J.* 2009; **276**, 1266–1281.

IL 2.1–3
Molecular interactions/electron transfer protein complexes using Docking algorithms, spectroscopy (NMR) and site direct mutagenesis

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Chemera 3.0 is a molecular modeling software package that includes BiGGER (Bimolecular complex Generation with Global Evaluation and Ranking), a protein docking algorithm. We will focus on new features of Chemera 3.0, specially constrained docking, to the search for protein–protein complex consistent with the ambiguity of some experimental data. We take advantage of sets of experimental data obtained by NMR, site-directed mutagenesis, or other techniques. Other features of Chemera 3.0 include filtering the docking models according to different interaction scores, importing and creating new scores. Chemera 3.0 also interfaces directly with web services for domain identification, secondary structure assignment or sequence conservation, simplifying the analysis of the partners and complexes, and includes tools for the computation and display of electrostatic fields, protonation, accessible and contact surface, and other molecular properties. Protein–protein complexes formed by short live electron transfer proteins will be presented covering a wide range of examples: di-heme peroxidase, N2O and nitrite reductases, hydrogenase and aldehyde oxido reductase in interaction with specific redox partners.

Acknowledgements: Nuno Palma and Isabel Moura for several inputs and the financial support of the Fundação Ciência e Tecnologia - MCTES.

References to algorithm:

Palma PN, Krippahl L, Wampler JE, Moura JJG. BiGGER: A new (soft) docking algorithm for predicting protein interactions. Proteins: Structure, Function, and Genetics 2000; 39, 372–84.

Krippahl L, Moura JJG, Palma PN. Modeling protein complexes with BiGGER. Proteins: Structure, Function, and Genetics 2003; 52, 19–23.

IL 2.1–4
Towards quantitative predictions in cell biology using chemical properties of proteins

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It has recently been suggested that proteins in the cell are close to their solubility limits, and that the even minor alteration in their levels might results in misfolding diseases. This concept is intriguing because the abundance of proteins is closely regulated by complex cellular processes, while their solubility is primarily determined by the chemical characters of their amino acid sequences. I will discuss how the presence of a link between abundance and solubility of proteins offers the opportunity to make quantitative predictions in cell biology based on the chemical properties of proteins.

IL 2.1–5
Equilibrium H/D-exchange patterns are insensitive to reversal of the protein-folding pathway

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An increasing number of proteins are found to contain multiple folding nuclei, which allow their structures to be formed by several competing pathways. One example is the ribosomal protein S6 that comprises two folding nuclei, s1 and s2, defining two competing pathways in the folding energy landscape: s1–s2 and s2–s1. The balance between the two pathways, and thus the folding order, is easily controlled by circular permutation. In this study we demonstrate that the equilibrium H/D-exchange pattern of S6 remains the same regardless of how the folding sequence is routed: the dynamic character of the different parts of a protein is independent of their folding order.

OP 2.1–1
Cracking the lectin code : in silico modeling and structure-functional study of principles driving sugar preference in PA-IIL family

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Introduction: *Pseudomonas aeruginosa* is an opportunistic human pathogen, a bacterium capable of attacking individuals with lowered immunity barriers. It is e.g. responsible for lethal complications in patients with cystic fibrosis. The PA-IIL lectin (a C-type fucose-preferring lectin with sugar binding mediated by two calcium ions), produced by the bacterium plays a crucial role in the host-pathogen interaction. Similar lectin sequences were found in other bacteria, displaying distinct differences in preference despite only small differences in structure of binding site. *In vitro* and *in silico* mutants were constructed in order to analyze the principles driving the sugar preference.

Methods: Molecular docking was performed using the AUTODOCK and DOCK software. The AMBER package was used for molecular dynamics simulations. Isothermal titration calorimetry was used to determine the thermodynamics of binding behavior of the mutants, verifying the method for extrapolative application on protein design.

Results: The experiments showed the importance of the specificity-binding loop in the binding site. The strongly-directing effect of the aminoacid 22 is further reinforced by presence of longer charged residue in position 24. Docking experiments combined with subsequent molecular dynamics were performed to help with the structural reasoning and exploring induced-fit changes.

Conclusions: Molecular modeling greatly helps in elucidating the structural principles driving the sugar preference. The binding preferences of the PA-IIL family lectins and their mutants can be customized by mutations, and the knowledge obtained from this study can be applied in designing potential inhibitors of the host-pathogen interaction.

Supported by LC06030, MSM0021622413, GA303/09/1168

2.2. Bioactive Peptides

IL 2.2–1
Identification and characterization of novel anti-infectious peptides from the male genital tract

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Antimicrobial resistance has become aggravated over the last 20 years. During this period pharmaceutical industries have focused on making incremental improvements on long-established antibiotics and, to an extent, sidelined the search for new drugs to overcome pharmaco-resistance strategies currently employed by pathogens. As a consequence bacterial infections are, to date, the most morbid and resistant among infectious diseases. This is, in particular, true for: diarrheic or respiratory infections, meningitis, sexually transmitted diseases and nosocomial infections. The time has come to discover innovative molecules for anti-infection therapies. Among new molecules with potential interest are antimicrobial peptides, an important component of the natural defenses of most living organisms. These are welcomed as serious candidates considering: their rapid microbicidal action, their broad spectrum of activity (bacteria, fungi, parasites, enveloped virus) and their original mechanism of action; the latter being difficult to evade by the resistance strategies employed by bacteria. Over the past decade, more than 700 microbicidal peptides have been inferred from various species including vertebrates. In the latter it is known that organs of the male genital tract express a potent and sophisticated anti-infectious defense system based partly on antimicrobial peptides. It follows that major reproductive organs such as the testis and epididymis are an ideal source for novel, highly specific microbicidal peptides. Using state-of-the-art proteomics and innovative syntactical biocomputing approaches, we identified numerous peptides with antimicrobial properties. This establishes the male genital tract as a veritable gold-mine for new anti-infectious agents to be exploited for future medicine.

IL 2.2–2
Comparative neuropeptidomics: the singular contribution of amphibians to the discovery of mammalian neuropeptides

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The concentration of many neuropeptides in the brain of ectothermic vertebrates is several orders of magnitude higher than in the brains of mammals. This singular situation has allowed us to isolate a number of regulatory peptides from the brain of the European green frog, *Rana esculenta*. A peptidomic approach has led to the characterization of many biologically active peptides that are orthologous to mammalian neuroendocrine peptides including two GnRH variants, CRH, PACAP, NPY, two tachykinins, alpha-MSH, gamma-MSH, CGRP, CNP, GRP and ODN. More importantly, this project has led to the discovery of several novel neuroendocrine peptides that were first isolated from frog brain tissue and have subsequently been identified in mammals. Notably, we have characterized (i) the somatostatin-14 (S14) isoform [Pro2, Met13]S14 together with authentic S14, thereby providing the first evidence for the occurrence of two somatostatin variants in the brain of a single species; (ii) the first tetrapod urotensin II, thus demonstrating that this peptide was not only the appendage of the fish caudal neurosecretory organ; (iii) secretoneurin, a peptide derived from the post-translational processing of secretogranin II; and (iv) 26RFa, a novel member of the Arg-Phe-NH_2 family of regulatory peptides. Orthologs of all these frog neuropeptides have now been identified in man and have been shown to exert important regulatory effects in mammals.

Acknowledgements: Supported by INSERM (U413), IFRMP 23, the Platform for Cell Imaging (PRIMACEN) and the Conseil Régional de Haute-Normandie.

IL 2.2–3
Novel toxins from snake venoms

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Snake venoms are complex cocktails of pharmacologically active proteins and polypeptides. Studies on these proteins have led to (i) our understanding of mechanisms of toxicity of snake venom poisoning; (ii) development of research tools which help in deciphering various physiological processes; (iii) sharpening of skills in protein chemistry and molecular biology; (iv) understanding of mechanisms of the origin and evolution of this unique set of proteins expressed in a highly specialized venom gland; and (v) identification of pharmacological prototypes that could be developed as therapeutic agents. We have been interested in the structure-function relationships and the mechanism of action of snake venom proteins. In the recent years, we have purified and characterized a number of proteins with interesting pharmacological properties. Some of them are new members of the well-characterized toxin families, whereas others belong to new families of proteins, hitherto not described in snake venoms. Here I will present our findings on some of these new snake venom proteins. These studies may provide new impetus to search for novel proteins in snake venoms.

IL 2.2–4
Evolution and development of peptides with special activities such as on ion-channels and receptors

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Venoms from animals such as from cone snails, spiders, scorpions or snakes are a unique cocktail of often more than 100 different peptides acting specifically on a variety of exogenous targets, e.g., ion-channels and receptors. A large proportion of venom peptides adopt specific folds which are characterized by conserved cysteine patterns. Hypervariability in amino acid sequences occurs between the cysteins leading to numerous peptide isoforms. In effect, peptides with the same structural signature, the cysteine patterns, exhibit different functional properties. The genes encoding venom peptides have been found to

undergo an abnormally high rate of mutations which may allow a rapid adaptation to changes in availability of prey, in predatory pressure or to other environmental challenges. The mechanisms and the evolutionary impacts underlying these high mutation rates are unknown. Whether special selection pressures or simply random expression of genes induced by exogenous stress factors are involved, is still a matter of speculation. The high specificity of most peptides for a particular ion-channel or receptor type may indicate a strong coevolutionary adaptation to these targets, eventually also triggering changes in the target′s structure to avoid envenoming. Exploring the 'venome', the sum of all natural venomous peptides and proteins of an animal, provides a unique opportunity to study peptide evolution in general as well as the genetic mechanisms that lead to the development of the huge variety of these compounds.

OP 2.2–1
Selecting peptides for breast cancer treatment

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The monoclonal antibody trastuzumab has a tyrosine kinase receptor HER2 as a target and it is currently in use as a gold standard treatment in breast cancer patients who presents overexpression of this receptor. However, there are some reports of resistance to this treatment and it can develop a high rate of cardiac failure, despite the high cost. As an alternative to trastuzumab we have selected specific peptides to HER2 using a phage display technology. A cyclic 7 aminoacids random peptide library had been panned using an external domain of recombinant HER2. Specific peptides were dislodged and selected using trastuzumab. After each round of binding assays, peptides were selected, sequenced and analyzed by ClustalW program. These peptides were assayed using different breast cancer cell lines in comparison with trastuzumab. It was observed that one of the selected peptides (CXBBXXXXC), where C represents cysteine, X non charged and B positive charged aminoacids, had shown inhibitory effect in MTT proliferation assay. Cell cycle analysis demonstrated a cell death rate (sub-G_0/G_1 region) of 79% with positive 1 phage treatment, compared with 64% in the trastuzumab treated group. Annexin V and Propidium iodide assay confirmed cell death and suggest late apoptosis/necrosis as the main mechanism of death mediated by this peptide. Confocal microscopy confirmed co-localization of HER2 and selected peptides. The data suggest a potential use of this peptide as an alternative anti-tumor therapy for breast cancer. Supported by CNPq, FAPESP, CAPES, and NEPAS.

2.3. Protein Engineering and Directed Evolution

IL 2.3–1
Protein evolution - a reconstructive approach

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In spite the robustness and perfection of their mechanism of action, proteins possess a remarkable ability to rapidly change and adopt new functions. I will describe experimental work aimed at reproducing the evolution of new proteins in the laboratory, and unraveling the evolvability traits of proteins. Specifically, I will describe how the functional promiscuity of proteins, their conformational plasticity, and their modularity of fold, accelerate their rate of evolution. I will address the issue of neutral (or actually, seemingly neutral) mutations, and neutral networks, as facilitators of protein evolution. Finally, I will address mechanisms for buffering and compensating the deleterious effects of mutations – these relate primarily to loss of protein stability, and include compensatory stabilizing mutations and chaperones.

IL 2.3–2
From random mutagenesis to focused directed evolution: examples for altered enantioselectivity and broadened substrate range

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An impressive number of applications has been developed in the past decades for the use of enzymes in organic synthesis [1]. Whereas initially, commercial enzyme preparations have been used 'straight from the bottle', the current trend is to tailor-design the biocatalyst using methods of protein engineering such as rational design and directed (molecular) evolution [2]. One of the most important, but at the same time most challenging, properties of biocatalysts is their stereoselectivity. Examples will be shown, in which high-throughput screening (HTS) systems for hydrolases [3] were successfully developed and applied to substantially improve the enantioselectivity of esterases for the synthesis of an important secondary alcohol serving as building block [4] as well as for tertiary alcohols [5]. Here, the combination of rational protein design [6] and focused directed evolution [7] allowed to increase or invert selectivity. In addition, the use of Baeyer–Villiger monooxygenases (BMVO) will be presented, which allow the highly selective kinetic resolution of aliphatic ketones [8]. Finally, catalytic promiscuity [9] will be introduced and first results will be given.

References:

1. Bornscheuer UT, Kazlauskas RJ. Hydrolases in Organic Synthesis. 2nd edn. Wiley-VCH, Weinheim. Buchholz K; Kasche V, Bornscheuer UT. *Biocatalysts and Enzyme Technology*, 2005, 2006; Wiley-VCH, Weinheim.
2. Lutz S, Bornscheuer UT. (eds) Protein Engineering Handbook. 2008; Wiley-VCH, Weinheim.
3. *Angew. Chem. Int. Ed.* 2001, **40**, 4201–4204; *Nature Prot.* 2006, 1, 2340–2343; *Angew. Chem. Int. Ed.* 2003, **42**, 1418–1420.
4. ChemBioChem. 2006, 7, 805–809.
5. *Angew. Chem. Int. Ed.* 2002; **41**, 3211–3213; ChemBioChem. 2003, **4**, 485–493; Tetrah.: Asymmetry 2002; **13**, 2693–2696; *J. Org. Chem.* 2005; **70**, 3737–3040; *J. Org. Chem.* 2005; **70**, 8730–8733; *Org. Biomol. Chem.* 2007, **5**, 3310–3313.
6. *Prot. Eng. Des. Sel.* 2007; **20**, 125–131; *Adv. Synth. Cat.* 2007, **349**, 1393–1398.
7. *Angew. Chem. Int. Ed.* 2008; **47**, 1508–1511.
8. Appl. Microb. Biotechnol. 2007; **73**, 1065–1072; Appl. Microb. Biotechnol. 2007; 75, 1095–1101; Angew. Chem. Int. Ed. 2006; **42**, 7004–7006; Appl. Microb. Biotechnol. 2007, **77**, 1251–1260; Appl. Microb. Biotechnol. 2008; **81**, 465–472.
9. *Angew. Chem. Int. Ed.* 2004; **43**, 6032–6040.

IL 2.3–3
Teaching enzymes to catalyze new reactions

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Changing the catalytic activity of enzymes provides insight into how nature evolves new enzymes and also creates unnatural catalysts to solve synthetic problems. We have explored three ways to change the catalytic activity of enzymes: replace the active site metal, replace amino acids residue to enhance an existing minor catalytic activity, or replace amino acids residue to create a completely new catalytic activity. An example of metal replacement is replacing the active site zinc in carbonic anhydrase with manganese to create a stereoselective oxidation catalyst. An example of mutagenesis to enhance catalytic activity is a Leu29Pro mutation in Pseudomonas esterase to enhance perhydrolysis over hydrolysis. The resulting variant is an efficient catalyst for synthesis of peracetic acid. An example of a completely new catalytic activity are multiple mutations in an esterase to create an oxyntrilase.

IL 2.3–4
Novel enzymes and designer microorganisms for industrial application: tapping functional sequence space from nature and beyond

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Industrial 'white' biotechnology is regarded as a central feature of the sustainable economic future of modern industrialized societies. Highly effective enzymes and 'designer bugs' promise improvement for existing process or could enable novel product ideas. For any industrial application, enzymes need to function sufficiently well according to several application-specific performance parameters. Instead of designing a process to fit a mediocre enzyme, it is conceivable that a comprehensive access to the microbial diversity might be used to find a suitable natural enzyme(s) that optimally fits process requirements [1,2,3]. In view of multi-parameter process requirements current technologies and screening strategies for the development of optimised biocatalysts from microbial biodiversity as well as from 'metagenome' libraries will be presented. To tap into the next generation biocatalysis using engineered 'designer' microorganisms for multi-step bioconversions, it is necessary to move into the construction of artificial operons and the heterologous expression of modified biosynthetic pathways.

References:

1. Eck J, Gabor E, Liebeton K, Meurer G, Niehaus F (2009) From Prospecting to Product – Industrial Metagenomics is

coming of age. In: Protein Engineering Handbook (Lutz S & Bornscheuer U, eds.) Wiley-VCH Weinheim, pp. 295–323.
2. Gabor E, Liebeton K, Niehaus F, Eck J and Lorenz P. Updating the metagenomics toolbox. *Biotechnol. J.* 2007; **2**: 201–206.
3. Lorenz P and Eck J. Metagenomics and industrial applications. *Nat. Rev. Microbiol.* 2005; **3**: 510–516

OP 2.3–1
Cold-adaptation of a hyperthermophilic group II chaperonin

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Group II chaperonins exist in the archaeal and eukaryotic cytosol, and mediate protein folding in an ATP-dependent manner. We have been studying the reaction mechanism of group II chaperonin using hyperthermophilic archaum, *Thermococcus* sp. strain KS-1 chaperonin (T. KS-1) [Iizuka *et al.*, JBC (2003, 2004, and 2005), and Kanzaki *et al.*, JBC (2008)]. However, high thermophilicity of T. KS-1 chaperonin caused difficulty in utilization of various analytical methods. To resolve this difficulty, we tried to make T. KS-1 group II chaperonin mutants which function at relatively moderate temperatures. Comparison of amino acid sequences among 26 thermophilic and 17 mesophilic chaperonins has shown that three amino acid replacements are likely to be responsible for the difference of their optimal temperatures. Then, we compared three single mutant and three double mutant chaperonins as candidates for cold-adapted mutants. Consequently, K323R single mutant improved folding activity at lower temperature, 50 °C and 40 °C. Small angle X-ray scattering (SAXS) demonstrated that this improvement of folding activity at lower temperatures is ascribable to the conformational change ability at lower temperature. In group II chaperonins, an ATP dependent signal for the conformational change transmits from the ATP binding site, which is located at the bottom of a chaperonin subunit, to the helical protrusion, which is located at the tip of a chaperonin subunit. Our study suggests that the amino acid residues, such as K323R, located in the conformational change pathway from ATP binding site to helical protrusion are important for the functions such as the ATP dependent conformational change ability and folding activity.

2.4. Proteolysis on and within the Biological Membrane

IL 2.4–1
Rhomboid proteases and growth factor signaling

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The intramembrane proteases are an unexpected class of proteases that have been discovered over the last 10 years. They have the property of cleaving proteins within their transmembrane domains. This is a surprising reaction since proteolysis is a hydrolytic reaction that requires water; the hydrophobic lipid bilayer of biological membranes is therefore an unexpected environment for protease active sites. The rhomboids are a recently discovered and widely conserved family of intramembrane serine proteases. They were first identified as the primary activators of EGF receptor signalling in Drosophila but recent work has implicated them in a wide variety of other functions from bacteria to humans. Despite this, little is known about most of their functions. A major focus for us has been determining the biological function of this diverse family of proteases in organisms beyond Drosophila. Significantly, many of the rhomboid functions discovered to date have potential medical relevance, including in areas as diverse as cancer, mitochondrial diseases and parasitic infection. Bacterial rhomboids may also be suitable therapeutic targets. I will discuss our recent work investigating the function of rhomboids in growth factor signalling. I will also discuss our approaches to substrate identification – the bottleneck of much protease research.

IL 2.4–2
Proteinase dysbalance in disease: the ACE and NEP gene families

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Therapeutic targets in many diseases. Homologous proteinases can often serve counter-regulatory roles in metabolism. Examples will be taken from several disease paradigms: prostate cancer, neurodegeneration and cardiovascular regulation. The closely related proteinases neprilysin (NEP) and endothelin converting enzyme-1 (ECE-1) play a counter-balancing role in the metabolism of the mitogenic peptide, endothelin-1, which contributes to the development of androgen-insensitive prostate cancer. In contrast, both NEP and ECE play a role in the clearance of the amyloid beta-peptide in brain and are hence potential therapeutic targets in Alzheimer's disease. Therapeutic strategies based on modulating these proteinases through epigenetic and other approaches will be described. The third exemplar involves the newly appreciated complexity of the renin-angiotensin system as a regulator of the cardiovascular system. The application of functional genomics approaches to the discovery of angiotensin-converting enzyme-2 (ACE2) as a counterbalance to the well known hypertensive target ACE will be highlighted and their differential cellular targeting and enzymology addressed. Finally, the serendipitous discovery of ACE2 as the SARS virus receptor illustrates the surprises always in store from nature.

Acknowledgements: Supported by the U.K. Medical Research Council, M.R.C., Yorkshire cancer Research and British Heart Foundation.

IL 2.4–3
Intramembrane Proteolysis by GxGD Proteases

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Alzheimer's disease is the most frequent neurodegenerative disorder and is pathologically characterized by the invariant deposition of amlyloid plaques. The amyloid cascade hypothesis describes a series of cumulative events, which are initiated by Amyloid ß-peptide and finally lead to synapse and neuronal loss. Obviously, the proteases involved in Amyloid ß-peptide generation are targets for therapeutic treatment strategies. For the development of a safe therapeutic intervention, it is however, absolutely required to understand the precise physiological functions and the cellular mechanisms involved in substrate recognition, selection and cleavage. Moreover, homologous proteases, whose physiological function could be affected by inhibitors need to be discovered and assays must be developed allowing to determine the cross-reactive potential of such inhibitors. I will focus on the intramembrane cleavage of the ß-amyloid precursor protein, which is performed by the γ-secretase complex. In parallel the cellular and biochemical properties of similar proteases of the same family of GxGD-type aspartyl proteases (the signal peptide peptidases and their homologues) will be described. A common multiple intramembrane cleavage mechanisms performed by these proteases will be shown and evidence will be presented that Alzheimer's disease associated mutations lead to a partial loss of intramembrane proteolysis.

IL 2.4–4
Beyond proteolysis: glutamate carboxypeptidase II as a neuropeptidase and prostate specific membrane antigen

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Glutamate carboxypeptidase II (GCPII) is a membrane-bound metallopeptidase expressed in a number of tissues such as jejunum, kidney, prostate and brain. The brain form of GCPII is expressed in astrocytes and cleaves N-acetyl-aspartyl glutamate, an abundant neurotransmitter, to yield free glutamate. GCPII thus represents an important target for the treatment of neuronal damage caused by excess glutamate. The enzyme is also expressed in the prostate where it is known as prostate-specific membrane antigen (PSMA) since it is upregulated in prostate cancer. Using specific monoclonal antibodies, we show that GCPII is also expressed in other normal and malignant tissues and in the neovasculature of number of different human tumors. Several GCPII homologs have been described and partially characterized that might compensate for the activity of GCPII in knock-out animals. We cloned, expressed and characterized two of them – GCPIII and NaaladaseL. We determined the 3-D structure of free GCPII, GCPIII and their complexes with inhibitors and substrate analogs by protein X-ray crystallography. Based on an extensive site-directed mutagenesis studies, we are able to identify the key amino acid residues critical for the activity and substrate

recognition. The series of 3-D structures explains the substrate preferences of the enzymes, led to the hypothesis of an induced-fit mechanism of substrate recognition and suggest a plausible mechanism of action of GCPII. Taken together, these structure-activity data and might lead to the design of novel, potent GCPII inhibitors.

IL 2.4–5
Regulation of extracellular proteolysis by CUB-domain containing proteins

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The CUB domain, a discrete protein module originally defined by expression in complement ***C***1r/s, ***U***egf and ***B***one morphogenetic protein-1, has been shown recently to to underlie the enhancer function of procollagen proteinase enhancer for procollagen proteinase/BMP-1-mediated proteolytic degradation. This module is also expressed in systemically-represented serum attractin, a protein initially characterised as having a dipeptidyl peptidase IV (DPPIV) activity. Despite recent reports demonstrating that there is no DPPIV activity intrinsic to attractin, it is clear that attractin enhances the activity of minimal amounts of serum DPPIV leading to an activity out of proportion to its apparent representation. We can localise a significant component of this functionality to the attractin CUB domain. Data suggests that this could result from modulation of substrate presentation, increasing catalytic activity and specificity. We suggest that this enhancer function may be a unique means of downregulating the systemic activity of DPPIV substrates relatively restricting their activity to locally-active sites, a property critical for proper functionality of chemokine and neuropeptide substrates of DPPIV.

OP 2.4–1
Rhomboid intramembrane proteases: from substrate specificity and mechanism to biological function

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Intramembrane proteolysis is a novel regulatory mechanism in many biological processes. Intramembrane proteases comprise four evolutionarily unrelated enzyme families of different catalytic types. Despite their wide conservation, in most cases their natural substrates and biological functions are not known and their mechanism of action is unclear. Rhomboid proteases, currently the best understood family, are conserved across the tree of life and are key players in several biological processes, including EGF receptor signaling in flies, mitochondrial fission in yeast, apicomplexan parasite invasion, and protein secretion in some bacteria. We nevertheless lack knowledge of their biological functions beyond these few examples. A bottleneck in revealing their function is our lack of methods for substrate identification. One such method could be prediction of relevant candidate substrates based on sufficiently well understood specificity of the enzyme and bioinformatic analysis of the given proteome. Like other intramembrane proteases, rhomboids were thought to recognize a region of helical instability in the transmembrane domain of their substrates, which is not strictly dependent on amino acid sequence. However, substrate predictions based on that model have been poor. Here we demonstrate, contrary to expectation, that rhomboids do recognize a specific sequence in their substrates. We define a recognition motif that is present in several model substrates and required by evolutionarily distant rhomboids and show that our model has predictive power. Our work demonstrates that intramembrane proteases can be sequence-specific. We will discuss the significance of our findings in relation to rhomboid mechanism, the discovery of rhomboid substrates and inhibitors.

SYMPOSIUM 3: METABOLITES IN INTERACTIONS

3.1. Biotransformation of Carcinogens

IL 3.1–1
Effect of benzo[a]pyrene metabolism on cells and vice versa

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Although discovered and identified more than 75 years ago, the environmental carcinogen benzo[a]pyrene (BaP) is still widely studied and has become a standard test agent for exploring the metabolic capacity of biological systems and the responses of cells or tissues *in vitro* or *vivo* to external genotoxic insult. We have investigated the carcinogenic/genotoxic properties of benzo[a]pyrene in cell culture and in whole animals, using DNA adduct formation, gene expression and cell cycle distribution as biomarkers of its effects. In all these studies, the principal pathway of activation demonstrated is via formation of the 7,8-diol 10,11-epoxide of BaP (BPDE) to form an adduct with the N^2 position of guanine in DNA. In cells in culture this pathway is mediated by cytochrome P450, but it now appears that *in vivo* P450 metabolism acts primarily to detoxify BaP. Gene expression changes in vitro can be categorised as either resulting from induction of the Ah receptor, or from causation of DNA damage. In cells that are p53 competent BP causes accumulation of p53, evident at the protein level but not at the mRNA level. DNA adduct formation by BaP, but not by BPDE, appears to be p53 dependent, suggesting that loss of p53 affects metabolic activation. Meanwhile *in vivo* studies show that BaP forms DNA adducts with equal measure in both target and non-target tissues, while gene expression changes are organ specific. Further insight into the complexities of interactions of BaP with mammalian cells may shed further light on mechanisms of carcinogenicity.

IL 3.1–2
The Hepatic Cytochrome P450 reductase null (HRN) mouse model as a tool to study xenobiotic metabolism

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Hepatic cytochrome P450 (CYP) enzymes play a pivotal role in the metabolism of many drugs and carcinogens. Much of the work carried out on the role of hepatic CYPs in xenobiotic metabolism has been done *in vitro*. However, additional factors such as route of administration, absorption, renal clearance and extra-hepatic CYP expression, make it difficult to extrapolate from *in-vitro* data to *in-vivo* pharmacokinetics. Moreover, functional redundancy inevitably found in the large CYP family of isoenzymes make it difficult to determine the role of CYPs in metabolism as a whole. To overcome these limitations a mouse line, HRN (Hepatic Cytochrome P450 Reductase Null), has been developed in which cytochrome P450 oxidoreductase (POR), the unique electron donor to CYPs is deleted specifically in the liver, resulting in the loss of essentially all hepatic P450 function. We used the HRN model to evaluate the role of hepatic versus extrahepatic metabolism and disposition of different drugs (e.g. ellipticine) and environmental carcinogens (e.g. benzo[a]pyrene [BaP], aristolochic acid, 3-nitrobenzanthrone [3-NBA]). In one set of experiments, HRN and wild-type (WT) mice were treated i.p. with 2 mg/kg body weight (bw) 3-aminobenzanthrone (3-ABA), the metabolite of the air pollutant 3-NBA, for 24 hours. DNA binding by 3-ABA measured by 32P-postlabelling was reduced by 80% in the livers of HRN relative to WT mice, confirming previous results indicating that CYP1A1 and -1A2 are mainly responsible for the metabolic activation of 3-ABA. In contrast, no difference in DNA binding was observed after treatment with 3-NBA indicating that 3-NBA is predominantly activated by cytosolic nitroreductases rather than microsomal POR. In another experiment HRN and WT mice were treated i.p. with 125 mg/kg bw of BaP for 24 hours. DNA adduct levels were around 10-fold higher in livers of HRN than in WT mice. In contrast, when hepatic microsomal fractions from HRN and WT mice were incubated with DNA and BaP, DNA adduct formation was 7-fold higher in WT than in HRN fractions and most of the hepatic microsomal activation of BaP *in vitro* was attributable to CYP1A enzyme activity. These data reveal an apparent paradox, whereby CYP enzyme activity appears to be more important for detoxification of BaP *in vivo*, despite being essential for its metabolic activation *in vitro*.

IL 3.1–3
Aristolochic acid, an old drug, is a human carcinogen

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The use of traditional herbal medicines is increasing worldwide. The old herbal drug aristolochic acid (AA), derived from Aristolochia species has been associated with the development of a novel nephropathy, designated as aristolochic acid nephropathy (AAN), and human urothelial cancer. The major components of the plant extract are nitrophenanthrene carboxylic acids, which are genotoxic mutagens after metabolic activation. The major activation pathway involves reduction of the nitro group primarily catalysed by NAD(P)H:quinone oxidoreductase to an electrophilic cyclic N-acylnitreniumion with delocalised charge that reacts preferentially with purine bases to form covalent DNA adducts. These aristolochic acid specific DNA adducts have been identified and detected in experimental animals exposed to aristolochic acid or botanical products containing aristolochic acid, and in renal tissues from AAN patients. In rodent tumors the major adduct formed by AA has been associated with the activation of ras oncogenes through a specific A:T to T:A transversion mutation in codon 61. A:T to T:A transversions were also the predominant mutation type found in human p53 knock-in mouse fibroblasts treated with AA. In humans A:T to T:A transversions in the p53 gene have been identified in several patients suffering from Balkan endemic nephropathy and in an urothelial tumor from an AAN patient along with AA-specific DNA adducts. This concordance of specific mutations in patient tumours and AA-exposed cells supports the argument that AA was responsible for the high risk for cancer in individuals who ingested material from Aristolochia plants – in the form of weight-loss pills in Belgium, or from cereal fields in the Balkans where Aristolochia plants grow as weeds. IARC has classified AA as carcinogenic to humans (Group 1) and has urged a ban of all botanical products known or suspected to contain AA from the market worldwide.

IL 3.1–4
Biotransformation of the plant alkaloid ellipticine dictates its genotoxic and pharmacological effects

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Ellipticine is an antineoplastic agent, which should be considered a drug, whose pharmacological efficiency and/or genotoxic side effects are dictated by its cytochrome P450 (CYP) and/or peroxidase-mediated activation in target tissues. Namely, among the multiple modes of ellipticine antitumor action, formation of covalent DNA adducts by ellipticine mediated by its oxidation with CYPs and peroxidases was found to be the predominant mechanism of cytotoxicity to human breast adenocarcinoma MCF-7 cells and several leukemia and neuroblastoma cells. Such DNA adducts were found also *in vivo*, in target and non-target tissues of rats and mice exposed to ellipticine. We report the molecular mechanism of ellipticine oxidation by CYPs and peroxidases and identify CYPs responsible for ellipticine metabolic activation and detoxication. Whereas 9-hydroxy- and 7-hydroxyellipticine formed by CYPs and the major product of ellipticine oxidation by peroxidases, the ellipticine dimer, are the detoxication metabolites, two carbenium ions, ellipticine-13-ylium and ellipticine-12-ylium, derived from other two metabolites, 13-hydroxy- and 12-hydroxyellipticine, generate two major deoxyguanosine adducts in DNA seen *in vivo* in rats and mice treated with ellipticine. Ellipticine is also a strong inducer of CYP1A enzymes, modulating levels of detoxication and activation metabolites and thus its own genotoxic and pharmacological efficiencies. Furthermore, cytochrome b_5 dictates the pattern of ellipticine metabolites generated by CYP3A4 and mainly by CYP1A1 and 1A2 enzymes, increasing formation of ellipticine metabolites forming DNA adducts. The study forms the basis to further predict the susceptibility of human cancers to ellipticine.
Acknowledgements: Supported by GACR (303/09/0472, 203/09/0812) and Czech Ministry of Education (MSM0021620808, 1M0505).

IL 3.1–5
The fate of drugs conjugated to albumin: Metabolism and effects on cellular targets

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We are developing drugs covalently linked to human serum albumin (HSA), because of the following properties of HSA: most abundant protein in serum, transport of lipophilic molecules, stability against organic solvents which allows chemical modifications, non-immunogenic to rats or mice, accumulation in tumours and inflammation. Compounds were linked either as carbamides, via triazinyl chloride, glucuronosides, or by peptide spacers and their uptake into and effects upon cells, tumours or inflammation were followed. From our early *in vivo* studies it was clear that a molar loading ratio of 1.4 is not immunogenic and results in a conjugate with a half life of 12 days in humans, similar to that reported for 'native' HSA. Aminofluoresceine-HSA (AFL-HSA) is taken up by endocytosis into lysosomes of human epithelial tumour cells, but also of fibroblasts. It accumulates in glioblastoma and has been successfully used as intra-operative diagnostic agent in the fluorescence guided surgery of glioma. Methotrexate linked to HSA (MTX-HSA) was successfully tested in clinical Phase I and II studies, with the result of a much reduced toxicity and a very long half-life compared to MTX. Due to its cellular uptake by endocytosis MTX-HSA was able to overcome transport resistance to MTX. Intracellular liberation of the drug was reflected by S-phase arrest of cells and inhibition of thymidylate synthase. To improve efficacy of cleavage of the drug from HSA, MTX was linked by a g-polyglutamylate (PG) linker to HSA. This physiological compound is cleaved by lysosomal glutamylhydrolase to short chain glutamates. The enzyme is secreted into media by human melanoma cells and MTXPG4 was determined to be the substrate most efficiently cleaved to MTX (MTXPG1). In a human lung adenocarcinoma cell line MTXPG4-HSA was, however, much less active than MTX-HSA, but was potent against fibroblasts. *In vivo* MTXPG4-HSA was active against mouse Lewis lung carcinoma at 150 fold lower doses than MTX, but also much more toxic. This is contrary to the lower toxicity seen by conjugating aminopterin or MTX directly to HSA. The data show that the choice of linkers between drug and protein may have a drastic effect on the drug's pharmacologic potency. This might also hold true for drugs linked to therapeutic proteins like monoclonal antibodies.

OP 3.1–1
The mechanism of formation of (deoxy)guanosine adducts derived from peroxidase-catalyzed oxidation of the carcinogenic non-aminoazo dye 1-phenylazo-2-hydroxynaphthalene (Sudan I)

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Sudan I is a liver and urinary bladder carcinogen for rodents and a potent contact allergen and sensitizer for humans. Sudan I metabolic activation in urinary bladder is attributed to its peroxidase-mediated oxidation. We identified the structures of two peroxidase-mediated Sudan I metabolites and those of adducts of (deoxy)guanosine that are formed during Sudan I oxidation. Peroxidase oxidizes Sudan I to radical species, reacting with another Sudan I molecule to form a dimer, where the oxygen 2 radical of Sudan I reacted with carbon 1 in the second molecule. In the presence of (deoxy)guanosine, the carbon 4 radical of Sudan I can attack the exocyclic amino group of guanine, forming the 4-[(deoxy)guanosin-N^2-yl]Sudan I adduct. The Sudan I dimer is unstable and decomposes spontaneously to the second oxidation product. This compound consists of 4-oxo-Sudan I skeleton connected *via* oxygen of its 2-hydroxyl group and nitrogen of its azo group with carbon 1 of 2-oxonaphthalene. If (deoxy)guanosine is present during the formation of this metabolite, an adduct, in which this metabolite is bound to the exocyclic amino group of guanine, is generated. This adduct is again unstable and decomposes to the same adduct that is formed by the direct reaction of radical, 4-[(deoxy)guanosin-N^2-yl]Sudan I. The results presented here are the first structural characterization of Sudan I-(deoxy)guanosine adducts formed during oxidation of this carcinogen by peroxidase.
Acknowledgements: Supported by GACR (303/09/0472, 203/09/0812), Czech Ministry of Education (MSM 0021620808) and the Academy of Sciences of the Czech Republic (Z4 055 0506, KJB 400 550 903).

3.2. Heme Biosynthesis, Utilization and Degradation

IL 3.2–1

Metabolic basis of protoporphyrin accumulation in Erythropoietic Protoporphyria: Ferrochelatase deficiency versus Ala Synthase 2 gain of function

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Accumulation of protoporphyrin IX in human erythrocytes and other tissues causes Erythropoietic Protoporphyria (EPP) (MIM 177000) an inherited disease which leads to life-long photosensitivity and, in about 2% of patients, severe liver dysfunction. EPP is usually caused by partial deficiency in mitochondrial ferrochelatase (FECH) (EC 4.99.1.1), the terminal enzyme of heme biosynthesis. Most patients have autosomal dominant EPP (dEPP) in which clinical expression normally requires co-inheritance of a *FECH* mutation that abolishes or markedly reduces FECH activity *trans* to a hypomorphic *FECH IVS3-48C* allele carried by about 11% of western Europeans. A limited number of patient display recessive EPP (rEPP) with a marked ferrochelatase deficiency and *FECH* mutations on both alleles usually with compound heterozygosity. However, mutational analysis fails to detect *FECH* mutations in about 7% of EPP families of which about 3% are homozygous for the wild type *FECH IVS3-48T* allele, suggesting possible involvement of another locus. Each of the seven inherited porphyrias results from a partial deficiency of an enzyme of heme biosynthesis. Mutations that cause porphyria have been identified in all the genes of the heme biosynthetic pathway except *ALAS1* and *ALAS2* that encode the ubiquitously expressed (ALAS1) and erythroid-specific (ALAS2) isoforms of mitochondrial 5-aminolevulinate synthase (ALAS) (EC 2.3.1.37). ALAS2 is essential for haemoglobin formation by erythroid cells. All reported mutations in *ALAS2*, which encodes the rate-regulating enzyme of erythroid heme biosynthesis, cause X-linked sideroblastic anemia. We described eight families with deletions in *ALAS2*, either c.1706-1709delAGTG (p.E569GfsX24) or c.1699-1700delAT (p.M567EfsX2), resulting in frameshifts that lead to replacement or deletion of the 19–20 C-terminal residues of the enzyme. Prokaryotic expression studies show that both mutations markedly increase ALAS2 activity. These gain of function mutations cause a previously unrecognised form of porphyria, X-linked dominant protoporphyria, characterised biochemically by a high proportion of zinc-protoporphyrin in erythrocytes, in which mismatching of protoporphyrin production to the heme requirement of differentiating erythroid cells leads to overproduction of protoporphyrin in amounts sufficient to cause photosensitivity and liver disease.

IL 3.2–2

Lipid mediator signaling: structural and evolutionary insights

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Lipid mediators constitute a broad spectrum of molecules, including vasoactive substances in mammals and volatile organic compounds that confer characteristic flavors to fruits and vegetables. Plant oxylipins (such as jasmonates) and animal prostaglandins are short-lived but potent peroxide-derived lipid mediators that share strikingly similar biological activities, including metabolic regulation, reproduction, and host defense. Their biosynthesis involves extraordinary rearrangements of labile organic peroxides by a novel group of heme thiolate enzymes belonging to the cytochrome P450 superfamily. Despite three decades of intense research, it has been difficult to gain molecular insights into how some of these molecules are produced. Equally unclear is the evolutionary origin of the enzymes that synthesize these diverse group of signaling molecules. In this talk, I will offer an atomic description of the enzymes involved in oxylipin biosynthesis. I will also elaborate on how our structural efforts led to the discovery of new oxylipin signaling pathways in bacteria and marine invertebrates (Lee, Nioche, Hamberg, Raman 2008 *Nature* **455**: 363–368]. This work is funded by the Pew Charitable Trusts, The Robert A. Welch Foundation, and the National Institutes of Health.

IL 3.2–3

Structural insight of heme oxygenase catalysis

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Heme oxygenase (HO), a central enzyme in heme catabolism, converts heme to biliverdin, CO, and a free Fe ion through three monooxygenase reactions using seven electrons donated through NADPH-cytochromeP450 reductase [1]. Electronic states, reactivities, and the crystal structures of eight key intermediates have been determined with a help of cryo-reduction to trap unstable intermediates. HO forms an enzyme-substrate complex with heme, where heme serves both as the substrate and the active center. After reduction to the ferrous form, O_2 binds to the ferrous heme iron with an acute FeOO angle of ~ 110°, placing its terminal oxygen atom close to the α-meso-carbon. An extended distal pocket hydrogen bonding network functions as a conduit for transferring protons required for the formation of hydroperoxo, generated by one-electron reduction of the oxy form, and also for the activation of hydroperoxo, leading to the α-meso-hydroxylation. Hydroperoxo cannot be formed upon loss of the nearby H_2O, indicating a crucial role of this nearby H_2O molecule in HO catalysis. Ferrous verdoheme formation proceeds by a reaction of the ferrous porphyrin neutral radical of ferric α-meso-hydroxyheme with O_2 and one electron. Conversion of verdoheme to biliverdin [2] is realized through ferric hydroperoxo in a manner very similar to that for the hydroxylation of the heme α-meso-carbon, the first HO catalysis step.

References:

1. M. Unno, T. Matsui, M. Ikeda-Saito *Nat. Prod. Rep.* 2007; **12**, 553–570.
2. T. Matsui, K. Omori, H. Jin, M. Ikeda-Saito, M. *J. Am. Chem. Soc.* 2008; **130**, 4220–4221.

IL 3.2–4

Macromolecular complexes involving cytochrome P450 enzymes

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Cytochrome P450s comprise a superfamily of oxidative hemoproteins, capable of catalyzing an extensive array of chemical

transformations, with biological roles as varied as natural product biosynthesis and xenobiotic metabolism. One important and increasingly better characterized subsection of P450s include those which, instead of binding free ligands, accept their substrates bound to a carrier protein (CP). Such P450s have mostly been identified in antibiotic biosynthesis pathways and have been found to catalyze several different types of oxidative reactions with substrates bound to peptidyl carrier proteins (PCPs). These reactions include the hydroxylation of aliphatic or aromatic amino acid C-H bonds, found in the biosynthesis of the antibiotics novobiocin, coumermycin A, and nikkomycin, and the oxidative phenolic coupling of heptapeptides, found in the biosynthesis of vancomycin-type antibiotics. The latter reactions are catalyzed by the Oxy-proteins. Their mode of action will be reviewed, insights on P450 carrier protein systems will be exemplified using the model system $P450_{BioI}$.

OP 3.2–1
Efficient utilization of iron for hemoglobin synthesis requires endosomal transport of transferrin to mitochondria

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Developing red blood cells (RBC) are the most avid consumers of iron (Fe) in the organism and synthesize heme at a breakneck rate. Delivery of iron to erythroid cells occurs following the binding of Fe_2-transferrin (Tf) to its cognate receptors on the cell membrane. The Tf-receptor complexes are then internalized via endocytosis, and iron is released from Tf by a process involving endosomal acidification. Iron, following its reduction to $Fe_2{}^+$ by Steap3, is then transported across the endosomal membrane by the divalent metal transporter, DMT1. However, the post-endosomal path of Fe in the developing RBC remains elusive or is, at best, controversial. It has been commonly accepted that a low molecular weight intermediate chaperones Fe in transit from endosomes to mitochondria and other sites of utilization; however, this much sought iron binding intermediate has never been identified. We have formulated a hypothesis that in erythroid cells a transient mitochondrion–endosome interaction is involved in Fe translocation to its final destination. This hypothesis is based on our earlier finding that in Hb-synthesizing cells Fe acquired from Tf continues to flow into mitochondria even when the synthesis of protoporphyrin IX is suppressed. In this study we have collected strong experimental evidence supporting this hypothesis: We have shown that Fe, delivered to mitochondria via the Tf pathway, is unavailable to cytoplasmic chelators. Moreover, we demonstrated that Tf-containing endosomes move and contact mitochondria in erythroid cells, that vesicular movement is required for iron delivery to mitochondria and that 'free' cytoplasmic iron is not efficiently used for heme biosynthesis. Furthermore, performing flow cytometry on cell lysates from reticulocytes incubated with two different fluorescent markers for endosomes and mitochondria, we identified three distinct populations: endosomes, mitochondria, and a population of particles labeled with both fluorescent markers. The size of the double-labelled population increases with the incubation time and plateaus in 30 minutes. Re-incubation of the reticulocytes with unlabelled Fe2-Tf leads to a time-dependent decrease, and ultimate disappearance, of the double-labelled population, indicating a reversible nature of organel interactions. Hence, the 'chaperone'-like function of endosomes may be one of the mechanisms that keeps the concentrations of reactive $Fe_2{}^+$ at extremely low levels in oxygen-rich cytosol of erythroblasts.

3.3. Turnover and Recognition of Carbohydrates

IL 3.3–1
Interactions between bacterial lectins and host carbohydrates

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Recent interest in bacterial lectins demonstrated their role in host recognition, biofilm formation, tissue adhesion and virulence. *Pseudomonas aeruginosa* and *Burkholderia cenocepacia* are opportunistic pathogens responsible for lung infections. Both bacteria contain several calcium-dependant lectins that demonstrate high affinity for diverse oligosaccharides that are present on human tissues. We used combined titration microcalorimetry, x-ray crystallography and molecular modeling approaches to decipher the thermodynamical and structural basis for high affinity binding of bacterial lectins to host carbohydrates [1]. First antibacterial tests in animal models infected with *P. aeruginosa* have been successfully conducted. The complete characterization of carbohydrate specificity, affinity and atomic details of interaction between the two *P. aeruginosa* lectins and their ligands allowed for the design and synthesis of high affinity glycomimetics and glycodendrimers that can act as antiadhesive compounds [2].

References:

1. Imberty A, Wimmerova M, Sabin C and Mitchell EP. Structures and roles of *Pseudomonas aeruginosa* lectins. In *Protein-Carbohydrate Interactions in Infectious Disease* (Bewley, C. ed.), pp. 30–48, 2006; The Royal Society of Chemistry, Cambridge.
2. Imberty A, Chabre YM and Roy R. Glycomimetics and glycodendrimers as high affinity microbial antiadhesins. *Chemistry* 2008; **14**, 7490–7499.

IL 3.3–2
Glycation and post-translational processing of human interferon-gamma expressed in Escherichia coli

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Non-enzymatic glycosylation (glycation) is a multistep chemical reaction between free amino groups in biomolecules (mainly Lys ε-amino groups in proteins) and reducing sugars. It starts with formation of Schiff bases, which are further converted to Amadori products and advanced glycation end-products (AGEs). This process takes place in humans and causes severe complications in diabetic and uremic patients, whereas in normal subjects contributes to senescence and ageing. Because glycation is a slow process, it has always been regarded as typical for the long-living organisms affecting their long-living proteins (hemoglobin, crystalline, etc.) only. Surprisingly, we found that glycation takes place also in *E. coli* and affects both host and recombinant proteins (Mironova *et al*., *Mol. Microbiol.* 2001; **39**, 1061–1068). Employing fluorescent spectroscopy (to measure AGEs specific fluorescence), Western blotting (using AGEs specific monoclonal antibodies) and ESI-mass spectrometry, we proved that the recombinant human interferon-gamma (rhIFN-g) expressed in E. coli is glycated. The presence of reactive Amadori products and AGEs makes the protein chemically unstable. Due to this it is involved in spontaneous dimerisation (although devoid of Cys residues) and chemical proteolysis. Target sites of the latter were identified by ESI-MS and N-terminal sequencing. Our analysis revealed four major (Arg1402Arg141, Phe1372Arg138, Met1352Leu136 and Lys1312Arg132) and two minor (Lys1092Ala110 and Arg902Asp91) cleavage sites. Tryptic peptide mapping indicated that the covalent dimers originating during storage were formed mainly by lateral cross-linking of the monomer subunits. Bioassay showed that the proteolysis lowered rhIFN-g antiviral activity and the covalent dimerisation led to its complete abolishment. Our recent studies showed that other recombinant interferons (both alpha and beta) produced in E. coli are also glycated and that their immunogenicity and loss of activity is related with their glycation. Searching for inhibitors of glycation applicable during E. coli fermentation, we found that the most potent anti-glycating compounds are aminoguanidine, acetylsalicylic acid (ASA), vitamin B 1 (thiamine), vitamin B 6 (pyridoxamine), L-arginine, etc.

IL 3.3–3
Frontiers of human glycosylation disorders

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All cells are coated with a dense forest of sugar chains or glycans. About 1–2% of the human genome encodes proteins that synthesize or recognize a vast and complex array of glycans that are usually attached to proteins or lipids. In just over a decade, mutations in over 30 different genes were shown cause a spectrum of human genetic, mostly autosomal recessive, disorders. The clinical spectrum is nearly as diverse and heterogeneous as the glycans themselves. One group, called the Congenital Disorders of Glycosylation (CDG), is caused by mutations in genes that mostly affect the *N*-glycosylation pathway located in the endoplasmic reticulum and Golgi apparatus. Defects in monosaccharide precursor activation and interconversion, transport of activated nucleotide sugars into the Golgi, glycosyltransferases, glycosidases, chaperones, trafficking proteins, and pH regulators all contribute to the disease burden. Many of these disorders also affect multiple glycosylation pathways. As physician awareness of these rare disorders grew, more patients were identified, focusing interest in two areas: therapy and model systems to better understand the nature of the deficiencies. Nearly all patients have hypomorphic, rather than null alleles, making a traditional? gene knockout mouse? lethal *in utero*. It is challenging to find an appropriate, viable phenotypic model to study therapy. It requires the right combination of genetic deficiencies and environmental insults to tease out the subtle interactions and identify therapeutic opportunities. We will present a few examples of this and suggest where the diagnostic and therapeutic frontiers may lead us.

IL 3.3–4
When PNGase-F fails to deglycosylate native *N*-glycoproteins? NMR and modeling studies

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In glycoanalysis protocols, glycoprotein *N*-glycans are generally released with peptide-N4-(*N*-acetyl-β-glucosaminyl) asparagine amidase F (PNGase-F). As the enzyme is an amidase, it cleaves the NH-CO linkage between the Asn side chain and the Asn-bound GlcNAc residue. Usually, the enzyme has a low activity,

or is not active at all, on native glycoproteins. Stereochemical reasons for being inactive in case of native human chorionic gonadotropin α-subunit (αhCG) and native bovine pancreatic ribonuclease B (RNase-B) will be explained. For αhCG, bearing complex- and hybride-type *N*-glycans at its two *N*-glycosylation sites, holds that Asn52 is completely accessible to digestion by PNGase-F under native conditions, but Asn78 not. Using NMR spectroscopy and molecular modeling it could be demonstrated that the Asn78-linked GlcNAc residue is tightly packed against the protein, being an integral part of the structure of the α-subunit. The remainder of the *N*-glycan at Asn78 is largely extended in solution (as is the complete *N*-glycan at Asn52). RNase-B with oligomannose-type *N*-glycans at Asn34, can not be de-*N*-glycosylated under native conditions. However, native RNAse-BS, generated by subtilisin digestion of native RNase-B, which comprises amino acid residues 21–124 of RNase-B, is sensitive to PNGase-F digestion. NMR analyses indicated that the *N*-glycan at Asn34 is more mobile in RNase-BS than in RNase-B. MD simulations showed that the region around Asn34 in RNAse-B is not very flexible, whereby the α-helix of the amino acid residues 1–20 has a stabilizing effect. In RNase-BS, the α-helix formed by amino acid residues 23–32 is significantly more flexible.

IL 3.3–5
Enzymes in the synthesis of new unique carbohydrate structures and their mimetics

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We have previously described several interesting aspects of the use of beta-*N*-acetyl-hexosaminidases (glycosylhydrolase family 20) in oligosaccharide synthesis. These enzymes can catalyze 'reverse hydrolysis' and transglycosylation reactions yielding new oligosaccharide structures. Generally, beta-*N*-acetylhexosaminidases are able to hydrolyze both beta-GlcNAc and beta-GalNAc moieties (so called 'wobbling' specifity). This enzyme is able to 'wobble' also at other positions of glycon, namely at C-6 moiety. Modified carbohydrate donors and acceptors were prepared by standard chemical procedures or by a combination with another enzyme system, e.g., galactose oxidase, laccase or lipase/protease. Modification in this glycon part is tolerated usually not only for the hydrolytic mode of enzyme action, but also in the synthetic reactions. We have demonstrated that C-6 acylation or oxidation to aldehyde in the gluco- series creates substrates for beta-*N*-acetylhexosaminidase enabling the synthesis of non-natural glycosides (glycomimetics). Synthesis of some novel hexosamine derivatives prepared by enzymatic and/or chemical ways will be described. New modified substrates were tested for the cleavage by the beta-*N*-acetylhexosaminidases (panel of 20 fungal hexosaminidases) for inhibitory activity and also for the use in the synthetic reactions. Molecular modeling (docking of modified substrates) supported by the 'wet' experiments was used to probe active site of beta-*N*-acetylhexosaminidase from *Aspergillus oryzae*. Series of new glycosides and glycomimetics (non-natural carbohydrate structures) were tested for their immunomodulatory activity towards activating receptors of NK cells, such as NKR-P1 and CD69.

OP 3.3–1
The activity of glycosidases in tobacco leaves under stress conditions

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Generally, glycosidases degrade polysaccharides, oligosaccharides and saccharide chains of glycoproteins in plants. They are known to hydrolyse glycosides to change inactive storage form to active one. Some of them are considered as pathogenesis related protein expressed under stress conditions. In this work, we focused on a group of exoglycosidases in plants exposed to biotic stress. Tobacco plants (*Nicotiana tabacum* L.) infected by *Potato virus Y* and *ipt* transgenic plants with enhanced level of endogenous cytokinines were our experimental models. Transgenic plants exhibited lower photosynthesis, but were less sensitive to potyviral infection. We found that the activity of β-glucosidase (EC 3.2.1.21) was lower in both infected and transgenic plants, which was in agreement with an increase of inactive forms of cytokinines. The increase in activity of another glycosidase, β-hexosaminidase (EC 3.2.1.52), was caused particularly by potyviral infection. Both enzymes were purified and characterized considering substrate specificity, kinetic constants, pH and temperature stability.

Acknowledgements: This work was supported by the grants of Ministry of Education of the Czech Republic MSM0021620808 and 1M0505.

3.4. Cytochromes P450 and Xenobiochemistry

IL 3.4–1
Searches for cellular functions of new (and old) cytochrome P450 enzymes

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One of the central problems in biochemical research is elucidation of the functions of uncharacterized proteins. Reflecting the general case for all genes, fewer than one-half of the human cytochrome P450 (P450) proteins have established functions and the situation regarding these orphan P450s is even less clear with microbial P450s. We have a number of human and microbial P450s using heterologous expression, use of the tissue where the P450 is expressed as a source of substrates, and developed a battery of HPLC-mass spectrometry (MS) methods for interrogation of function, including substrate analysis of chromatographic data and 18O2-labeling/detectin. The latter approach (Anal. Chem. 79, 3355, 2207) was validated using P450 7A1 and human liver extracts. We used this approach to identify several fatty acids as endogenous hepatic substrates of P450s 1A2, 2C8, and 2C9. These approaches are now being applied to other human P450s, e.g. 2S1, 2S1, 20A1, and others. These approaches have been applied to the bacterial *Streptomyces coelicolor* P450 154A1 and a transgenic knockout. Using this analysis, we identified a substrate of possible terpenoid origin, characterized using UV, high resolution MS and UV and NMR spectroscopy. Other approaches include rapid screening methods to identify pro-carcinogens that might be activated by these orphan P450s.
Acknowledgement: Supported in part by USPHS grants R37 CA090426 and P40 ES000267.

IL 3.4–2
Structural genomics of human drug metabolizing P450 monooxygenases

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Crystal structures of the principal human drug metabolizing P450s reveal distinct active site architectures that underlie the individual contributions of each enzyme to xenobiotic oxidations. The more conserved and rigid structural features support heme binding and redox partner interactions, whereas flexibility of the substrate-binding site contributes to broad substrate recognition as well as substrate access to the active site cavity. P450/substrate interactions are largely hydrophobic leading to substrate selectivity based on predominately on size and fit. Chemical complementarities also contribute substrate positioning as well as enzyme selectivity. Although cavity size reflects generalized trends in substrate characteristics for each enzyme, it is also clear that relatively small substrates can be efficiently oxidized by enzymes with larger active site cavities. This can reflect specific interactions that position the molecule appropriately for catalysis as well as occupancy of the active site by more than one substrate molecule so that one molecule facilitates oxidation of the other. Further analysis of structures obtained for individual P450s complexed with structurally distinct substrates also demonstrates plasticity for some active sites, which can adapt in response to specific substrates. This promiscuous nature of xenobiotic metabolizing P450s complicates strategies for the prediction of substrate binding in silico. It is expected that additional structures together with advances in computational approaches will improve the potential for in silico applications. Acknowledgement: Supported by US NIH grant GM031001.

IL 3.4–3
Cytochrome P450s as biomarkers for individualised drug therapy: genetic and epigenetic aspects

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Human drug metabolising cytochrome P450 enzymes exhibit a pronounced interindividual variability in expression because of CNVs and activating or inactivating mutations. This affects drug therapy and influences response rates and occurrence of adverse drug reactions. Pharmacogenomic biomarkers can facilitate an individualized drug therapy and among them are mutations in the 450 genes. The gene expression is to a great extent also determined by gene methylation which regulates the expression of CYP1A1, CYP1A2, CYP1B1, CYP2E1 and CYP2W1. In addition in the CYP2 family the CYP2A6, CYP2C19, CYP2D6,, CYP2J2, CYP2R1, and CYP2S1 genes contain putative important CpG islands which suggest a potential role of DNA methylation in their regulation and micro RNA dependent control of translation. Recently CYP1B1 was shown to be controlled by miR 27b and putative micro RNA regulation motifs The miR-148a-dependent decrease in PXR protein attenuates the induction of CYP3A4 mRNA and protein levels indicating that this could contribute to the basis for the interindividual variation in CYP3A4 expression. Gene methylation determines the tumor specificity in CYP2W1 expression, is of importance for expression of CYP1B1 in prostate tumor tissue and is also of crucial importance for tissue specificity of P450 gene expression. Furthermore tumor associated gene methylation is of importance for control of CYP1A1 expression In the lecture an overview will be given about functionally important mechanisms for pharmacogenomics. epigenetic and post-transcriptional mechanisms controlling the expression of drug metabolising P450s will be given.

IL 3.4–4
Changing substrate specificity and activity of bacterial and mitochondrial P450 systems

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Besides playing an important role in drug metabolism, cytochromes P450 are pivotal for the biosynthesis of steroid hormones. Steroids play an important role as hormones in mammals. In addition to this, many steroids are interesting pharmaceutical target substances for the production of different types of drugs. Since selective transformations of steroids are a difficult task, the pharmaceutical industry has rationalized the importance of microbial steroid transformations. CYP106A2 from *Bacillus megaterium* ATCC 13368 is one of the few known bacterial steroid converting cytochromes P450 and hydroxylates many 3-oxo-delta-4-steroids mainly in 15 beta-position. Here we report on the creation of mutants of this enzyme with improved activity and changed selectivity of hydroxylation. To check mutants produced by directed evolution, a whole-cell screening system, where Adx and AdR (as redox partners transferring electrons from NADPH to the P450) were co-expressed in *Escherichia coli*, has been developed. We were able to select mutants with considerably improved activity and with changed selectivity of hydroxylation. This way, a system for creating libraries of steroid molecules with hydroxyl groups in different positions seems to be feasible. In addition, the regio-selectivity of steroid hydroxylation was changed in mitochondrial P450s. The results of these experi-

ments turned out to be helpful for the design of selective and specific inhibitors of the steroid hydroxylases studied. Overproduction of aldosterone leads to hypertension and congestive heart failure. Therefore, CYP11B2 became a new target for drug development. We established an effective screening system and were able to identify new specific and selective inhibitors of CYP11B2.

IL 3.4–5
Flexibility and plasticity of the structures of cytochromes P450 as a property determining their function

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Versatility of functions as well as of substrates of cytochromes P450 should be reflected in their ability to accommodate molecules of various size and shape. Active sites of cytochromes P450 hence differ in their flexibility and plasticity of the corresponding parts of the structure. Examples discussed here are cytochromes P450 known to metabolize various drugs and their analogs, namely, cytochromes P450 3A4, 2C9 and 2D6. Experimental data obtained by spectroscopic methods (absorption spectroscopy at high hydrostatic pressure and resonance Raman data) are correlated with results of molecular dynamic simulations carried out using the AMBER package with *parm99* force field. Starting structures were essentially based on these taken from the PDB database; simulations were made at normal (0.1 MPa) and high pressure (300 MPa). The results indicate a decrease in radius of gyration as well as the overall B-factor decrease under high pressure. A correlation has been found between the experimentally obtained coefficient describing a shift of the Soret band with pressure (related to compressibility of the active site) and coordination numbers of water molecules and backbone atoms in the active site around the axial ligand (carbon monoxide). The data show that the spectral changes under pressure reflect structural changes in the close proximity (< 0.5 nm) of the heme. The compressibility of the active site apparently increase with number of water molecules in the active site, and decrease with number of non-polar protein backbone atoms close to the heme. Raman spectral data reflect the situation in the active site by presence of heme vinyl vibrations assigned to two vinyl conformations, with two conformations present in the spectrum indicating a more flexible structure in the active site as e.g. in the case of CYP3A4. The data both experimental and obtained from the simulations hence document an essential necessity of plasticity/flexibility of enzyme active site for proper functioning of an enzyme, here cytochrome(s) P450.

Acknowledgements: Support from Czech Ministry of Education grants MSM6198959216 and LC512 is gratefully acknowledged.

SYMPOSIUM 4: CELLULAR AND SUBCELLULAR BIOCHEMISTRY

4.1. Cell Differentiation

IL 4.1–1
Epigenetic regulation of gene expression by the Myb Oncoprotein and the E2F-RB tumor suppressor complex

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The Drosophila Myb oncoprotein, the E2F2 transcriptional repressor, and the RBF and Mip130/LIN-9 tumor suppressor proteins reside in a conserved Myb-MuvB (MMB)/dREAM complex. We have found that Myb is required *in vivo* for the expression of Polo kinase and components of the spindle assembly checkpoint (SAC). Surprisingly, the highly conserved DNA-binding domain was not essential for assembly of Myb into MMB/dREAM, for transcriptional regulation *in vivo*, or for rescue of Myb-null mutants to adult viability. E2F2, RBF, and Mip130/LIN-9 acted in opposition to Myb by repressing the expression of Polo and SAC genes *in vivo*. Remarkably, the absence of both Myb and Mip130, or of both Myb and E2F2, caused variegated expression in which high or low levels of Polo were stably inherited through successive cell divisions in imaginal wing discs. Restoration of Myb resulted in a uniformly high level of Polo expression similar to that seen in wild-type tissue, whereas restoration of Mip130 or E2F2 extinguished Polo expression. Our results demonstrate epigenetic regulation of gene expression by Myb, Mip130/LIN-9, and E2F2-RBF *in vivo*, and also provide an explanation for the ability of Mip130-null mutants to rescue the lethality of Myb-null mutants *in vivo*.

IL 4.1–2
Understanding early haematopoietic development using human embryonic stem cell as a model

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Transplantation of haematopoietic stem cells (HSCs) in leukaemia treatment requires high quality bone marrow, mobilised peripheral blood or umbilical cord blood however these are in short supply. Directed differentiation of hESC offers an attractive alternative. The potential of hESC to differentiate towards haematopoietic lineages was investigated by co-culturing hESC with monolayers of cells derived from mouse aorta-gonad-mesonephros and fetal liver region or with stromal cell lines derived from these tissues (EL08.1D2, AM20.1B4 and UG26.1B6). The hESC derived differentiating cells were able to form early haematopoietic progenitors that peaked at day 18–21 of differentiation and corresponded to the highest CD34 expression. The hESC derived haematopoietic cells were capable of primary and secondary haematopoietic engraftment into immunocompromised NOD/LtSz-Scid IL2Rγnull recipients at levels higher (0.11–16.26%) than described previously. Large scale transcriptional analysis combined with blocking and stimulation experiments identified TGFβ1 and TGFβ3 as positive enhancers of human ESC haematopoietic differentiation. Addition of TGFβ1 or TGFβ3 to differentiating hESC in the absence or presence of stromal cells resulted in a significant increase in haematopoietic differentiation, thus suggesting a hitherto undiscovered role for TGFβ1 and TGFβ3 in human ESC differentiation to haematopoietic lineages. Overall, our data presents an important step towards deriving functional HSCs from hESC.

IL 4.1–3
Bone development and disease regulated by AP-1 (Fos/Jun)

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AP-1 proteins such as Fos and Jun are prototypic oncogenes regulating cell proliferation, differentiation and cell transformation in various organs. We have been defining the specific functions of various Fos proteins, e.g. Fos, Fra-1 and in particular Fra-2 in bone development and disease using gain-of-function and loss-of-function experiments in mice. The role of c-Fos as a differentiation-specific transcription factor for osteoclasts, the bone resorbing cells, is well established, whereas the functions of Fra-1 and Fra-2 are less well understood. It appears that Fra-1 mainly controls matrix gene expression (1), whereas Fra-2 is important for chondrocyte differentiation (2). We have recently shown that newborns lacking Fra-2 have increased size and numbers of osteoclasts *in vivo* likely caused by impaired LIF/LIFR signalling and hypoxia. Interestingly, newborns lacking LIF also display giant osteoclasts and LIF is a transcriptional target of Fra-2. Fra-2 and LIF-deficient bones are both hypoxic and express increased levels of HIF1α and Bcl-2. Furthermore, Fra-2 and LIF deficiency affects HIF1α through transcriptional modulation of the HIF-prolyl-hydroxylase PHD2 (3). Moreover, Fra-2 deficient osteoblasts display a differentiation defect *in vivo* and *in vitro* (unpublished), which appears to be cell autonomous and LIF-independent. Preliminary data demonstrating direct transcriptional regulation of osteoblast-specific genes such as collagen type 1 (col1α 2) and osteocalcin by Fra-2 will be discussed. These findings indicate that despite sequence homology, the Fos proteins c-Fos, Fra-1 and Fra-2 play specific roles in bone development and disease. The identification of the molecules/pathways operating downstream of Fos proteins as well as novel AP-1 target genes in osteoblast and osteoclast differentiation will certainly lead to new insights into the molecular mechanisms coordinating bone cell differentiation under normal and pathological conditions.

References:

1. Eferl R, Hoebertz A, Schilling AF, Rath M, Karreth F, Kenner L, Amling M & Wagner EF. *EMBO J.* 2004; **21**: 2789–2799.

2. Karreth F, Hoebertz A, Scheuch H, Eferl R & Wagner EF. *Development* 2004; **131**: 5717–5725.
3. Bozec A, Bakiri L, Hoebertz A, Eferl R, Schilling AF, Komnenovic V, Scheuch H, Priemel M, Stewart CL, Amling M & Wagner EF. *Nature* 2008; **10:** 454, 221–225.

IL 4.1–4
Origin and mode of function of bone resorbing osteoclasts

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As every tissue, bone is composed of cells and of an extracellular matrix and has to be renewed along life. Bone homeostasis is maintained through a finely tuned remodeling process involving degradation by osteoclasts and formation by osteoblasts. Osteoclasts derive from hematopoietic lineage. They are multinucleated cells, formed by fusion of monocyte/macrophage in the presence of M-CSF and RANKL. Throughout the differentiation process they express a series of markers, which characterize the osteoclast phenotype, but the hallmark of osteoclasts is their unique ability to resorb mineralized calcium apatite. We have shown *in vitro* that immature immune Dendritic Cells (DC), can transdifferentiate into resorbing osteoclasts bearing all characteristic and functional markers of osteoclasts We have postulated that transdifferentiation could specifically be induced in response to inflammatory conditions. Indeed, in our experiments, synovial fluid, from patients with rheumatoid arthritis, potentiate transdifferentiation whereas it has recently been shown that DC can transdifferentiate into osteoclasts *in vivo*. Results from our transcriptomic analysis comparing DC- versus monocyte-derived osteoclastogenesis has revealed that DC are more closely related to osteoclasts than are monocytes. From these data we have hypothesized that there must exist several osteoclast subpopulations and/or different osteoclatogenesis pathways from different progenitors, according to microenvironment conditions. Osteoclasts can adhere to several substrates on which they form specific distinct F-actin structures. When adherent on glass they exhibit podosomes formed by an F-actin core, surrounded by several focal adhesion proteins. Osteoclasts adhering to mineralized extracellular matrix become apico-basically polarized, and form a different actin-containing structure called sealing zone (SZ) that delineates the ruffled border, a site of active membrane traffic and transport, where protons and proteases are secreted in order to dissolve mineral. We have shown that podosome patterning dynamically evolves during osteoclastogenesis and in mature osteoclasts, they are arranged at the cell periphery as a characteristic belt reminiscent of the sealing. However, it remains to be understood how a bone adhering osteoclast activates its cellular machinery to become polarized, to condense its actin structure into a sealing zone and to resorb mineralized extracellular matrix.

OP 4.1–1
Role of reactive oxygen species as signal molecules in adult stem cells

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Consolidated evidence highlights the importance of redox signalling in the homeostasis of cell adaptative biology processes, like the balance between self-renewal and differentiation of the stem cells. In this study we investigated how changes in the oxidative metabolism may modify the fate of adult hematopoietic stem cells (HSC). In particular it is shown that circulating human HSC are endowed with a panel of constitutively active NADPH-oxidases (NOX) comprising the cell membrane-localized catalytic subunits of the NOX1, NOX2 and NOX4 isoforms together with the accessory subunits and a splicing variant of NOX2. It is suggested that NOX isoforms in HSCs act as environmental oxygen sensor, generating low level of reactive oxygen species (ROS) which likely serve as second messengers. In addition it is shown that NOX-produced ROS in HSCs stabilize under normoxic condition the hypoxia-inducible factor (HIF-1alpha). The control is exerted at post-translational level through the down-regulation of the von Hippel-Lindau protein which mediates proteasome-degradation of HIF. Although HIF is the master transcription factor mediating cell adaptation to hypoxia recent evidence supports its involvement in activation of signalling pathway as Notch and control of the expression of Oct4. Notch and Oct4 are transcription factors involved in regulating stem cell self-renewal and preservation of pluri/multipotency. Our findings are hence discussed in the context of the recruitment of progenitor/stem cells to injured ischemic tissues and in the control of the maintenance on the undifferentiated state in mobilized peripheral blood circulating HSC with the aim to provide applicative insights to cell-based therapy/regenerative medicine.

4.2. Biochemistry of Melanins and Melanosomes

IL 4.2–1

Chemical and biophysical properties of eumelanin and pheomelanin

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Melanin – one of the most common biological pigments – in human, is present in the skin, hair, eye, inner ear and some neurons of the midbrain. Although melanogenesis has not been completely determined, it is apparent that the process involves both enzyme-catalyzed and non-enzymatic reactions of key melanin precursors. The oligomeric/polymeric product is deposited on protein matrix of distinct pigment granules, the melanosomes. There are two main melanin pigments – eumelanin and pheomelanin, which are formed in the absence and presence of cysteine or glutathione, respectively. These melanins are built of different monomer units, they exhibit slightly different optical properties and distinct photochemical reactivity, and their paramagnetic properties are quite distinguishable. Both types of melanin are synthesized, to a different degree, in melanocytes of the human skin, choroid and iris of the eye, but only eumelanin is present in the retinal pigment epithelium. While eumelanin is commonly viewed as an efficient photoprotective agent, the biological role of pheomelanin remains ambiguous. Indeed, it was demonstrated that under some experimental conditions, pheomelanin could act as a photosensitizer. However, the exact molecular mechanisms by which eumelanin protects pigmented cells against damage induced by solar radiation and pheomelanin aggravates the photodamage, are not fully understood. This paper will critically review relevant data and discuss chemical and physical basis of the postulated biological functions of eumelanin and pheomelanin, particularly in the human eye.

Acknowledgement: Supported by the Ministry of Science and Higher Education (DS11).

IL 4.2–2

Signaling from human melanocortin 1 receptor variants associated with red hair and skin cancer

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The melanocortin 1 receptor (MC1R) is a G protein-coupled receptor (GPCR) expressed in melanocytes, where it stimulates melanogenesis and increases the ratio of black eumelanins to yellowish-reddish pheomelanins. MC1R belongs to the melanocortin receptor subgroup of GPCRs. These receptors signal primarily to the cAMP pathway, but they also mediate other signaling events such as activation of ERK1 and ERK2 MAP kinases. In the mouse, efficient MC1R signalling due to gain-of-function mutations is associated with eumelanogenesis and darker coats. Conversely, absent or poor signaling due to loss-of-function mutations or expression of endogenous peptide inhibitors results in the production of pheomelanins. Human *MC1R* is unusually polymorphic, and several variant alleles are associated with pheomelanin-rich red hair, abundant freckles, inability to tan efficiently upon sun exposure, high sensitivity to ultraviolet radiation-induced skin damage and increased skin cancer risk (the RHC phenotype). The RHC variants R151C, R160W and D294H are particularly penetrant and frequent. These variants signal efficiently to the ERK module, but poorly to cAMP. This disparate effect on signaling to ERK and cAMP challenges current concepts of functional coupling in the melanocortin receptor family. On the other hand, reduced signaling to cAMP from the R151C and R160W variants is due to aberrant intracellular trafficking with intracellular retention in different subcellular compartments. Analysis of the trafficking patterns of these alleles provides mechanistic insights on the functioning of arginine-based sorting motifs. These findings illustrate the potential of pigmentation phenotypes as indicators of general physiological processes used in other systems in mammals.

IL 4.2–3

Physiological and pathological functions of melanosomes and their exploitation

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In vertebrates melanin is synthesized in specialised membrane-bound intracellular organelles known as melanosomes. They contain the apparatus of melanogenesis and melanin is deposited on a protein matrix within these organelles. An important physiological role of melanosomes is the segregation of reactive intermediates of melanogenesis that are potentially hazardous to the cell. In mammals the biosynthesis of melanin occurs in two classes of cells: the retinal pigment epithelium and melanocytes. Melanocytes are specialized dendritic cells that either retain the melanosomes or distribute them to adjacent cells, as in the skin and hair. The physiological significance of fully-melanized melanosomes rests on the physical characteristics that endow melanin with (i) broad optical absorbance and photon/phonon conversion; (ii) facile redox exchange (iii) cation binding and (iv) affinity for polycyclic compounds. The visible absorbance of melanin is exploited in surface colouration for camouflage or display. The UV absorbance is involved in photoprotection. The redox properties of melanin permit the pigment to function as a scavenger or generator of free radicals. Cation binding by melanin may provide an excretory pathway for metals. Recent work has shown that catechols may play a role in intracellular calcium homeostasis. Exploitation of melanosomal properties has principally been in relation to the possibility of utilizing the melanogenic pathway as a targeting strategy for anti-melanoma chemotherapy where several approaches have been investigated. Melanosomal proteins are of diagnostic use in pathology and constitute potential targets for future immunotherapy.

IL 4.2–4

Mutator phenotypes in pigment cells and mechanism(s) of their DNA damage

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The existence of mutator phenotypes, the pre-cancer cells that may acquire large number of mutations, is becoming largely accepted. The mutator phenotypes can arise by simple (random) mutations that drive carcinogenesis by highlighting new emerging pathways that generate the hypermutability status. We propose that dysplastic naevus cells are the mutator type of cells. In these

cells, the increased rate of genetic changes may eventually lead to the transformation to melanoma. Many clinical observations agree that the presence of so called dysplastic naevi on the skin is an important risk factor for developing melanoma. Moreover, these pigmented naevi are also known as potential melanoma precursors. We provided some evidence that dysplastic naevus cells suffer from chronic oxidative stress. They produce in their melanosomes more phaeomelanin than do normal melanocytes. Melanosomes are specific sources of oxidative imbalance in pigment-forming cells. Normal melanocytes partly get rid of these oxidative 'packages' by transporting them to the keratinocytes. That is not possible in naevus cells. The accumulation of melanosomes in naevus cells can increase the risk of oxidative damage. Their preference for phaeomelanogenesis lowers the antioxidative capacity by increased consumption of thiols (cystein). Our experiments with comet assay show that nuclei of dysplastic naevus cells are more fragmented than nuclei of normal melanocytes. The use of formamido-pyrimidine-DNA nuclease revealed that the DNA of dysplastic naevus cells displayed also more oxidative DNA damage. We propose that the existence of chronic oxidative imbalance forms the basis for mutator phenotype of these pigment cells.

IL 4.2–5
Too much or too little BETA-catenin represses melanoblast proliferation

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During development, the canonical Wnt/b-catenin pathway governs cell fate, polarity, proliferation and death in various cell lineages. In the melanocyte lineage, Wnt/b-catenin signalling has been shown to induce melanoblast determination and differentiation. Little is known about the function of b-catenin and its association with other cellular mechanisms in the establishment of the melanocyte lineage. We have produced mice with various levels of b-catenin in melanoblasts, in a C57BL/6 background. Far fewer melanoblasts were generated in the absence of b-catenin production (Tyr::Cre/°;$bcat^{floxDex2\text{-}6/floxDex2\text{-}6}$ = $bcat^{null}$) than in wild-type conditions. bcat-null mice thus had a white trunk and a grey head. Surprisingly, the number of melanoblasts producing more b-catenin (Tyr::$bcat^{act}$/° = $bcat^{act}$) than normal was not higher, as expected, but lower. $bcat^{act}$ mice were hypopigmented. No apoptosis or transdifferentiation of melanoblasts was detected, suggesting an effect on proliferation. $bcat^{act}$ embryos had fewer melanoblasts than normal in both the dermis and the epidermis, whereas the number of $bcat^{null}$ melanoblasts was much lower than normal in epidermis but similar to that in the wild type in the dermis. These results account for the phenotypes of these two new mouse mutants. The full molecular picture remains unclear, but it is becoming evident that the four main targets of b-catenin (Mitf-M, Myc, cyclin D1 and Brn2) and their multiple interactions are involved in melanoblast proliferation.

OP 4.2–1
Suicide-inactivation of tyrosinase by catecholic substrates

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Tyrosinase (E.C. 1.14.18.1), a mono-oxygenase with a binuclear copper catalytic center, is the principal enzyme of melanogenesis and catalyses the oxidation to the corresponding *ortho*-quinones of both monohydric phenols (cresolase activity) and dihydric phenols (catecholase activity). The enzyme is characterised by suicide-inactivation in the presence of catecholic substrates. A mechanism to account for this has recently been proposed based on cresolase oxidation of catecholic substrates resulting in the generation of a zero-valent copper atom which is lost from the active site, a process known as reductive elimination. This unusual inactivation mechanism predicts certain features that are the topic of recent studies which have shown that cresolase activity is necessary for suicide-inactivation to be expressed. Marquardt optimisation of oximetric data obtained with the substrate, 4-methylcatechol, using a mathematical model of the oxidation kinetics, yields a ratio of comparative catechol oxidation and enzyme inactivation rates of 1:1530 and the model closely fits the inactivation data obtained in experiments in which successive tyrosinase additions are made. Structure-activity analysis permits a number of inferences to be made regarding the structural features of catechols affecting the inactivation, including substitutions that prevent cresolase activity and thus abolish the inactivation. Additional data are presented relating to other catecholic substrates that are consistent with the mechanistic model of suicide-inactivation advanced.

4.3. Extracellular Matrix and Cell Adhesion

IL 4.3–1
Deciphering the mechanisms of fibronectin fibrillogenesis and its role in angiogenic tumor progression

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The extracellular matrix (ECM) provides both an adhesive substrate and a platform for integrin-dependent signalling events that regulate a host of cellular functions. Altered matrix and cell-ECM interactions contribute widely to neoplastic, inflammatory and infectious diseases. Both genetic and pharmacological evidence points to fundamental role for fibronectin (FN) and its receptor alpha5beta1 integrin in vascular physio/pathology. Secreted FN is assembled into a 3D fibrillar network by an integrin-dependent process termed FN fibrillogenesis. Our recent findings in endothelial cells indicate that production and secretion of cellular FN isoforms is tightly coupled to fibrillar assembly and this process is determinant for integrin signalling, the establishment of endothelial cell polarity and plexus organization. Thus, elucidating the precise mechanisms that underlie FN fibrillogenesis should be important for determining rational strategies for more effective anti-antiangiogenic/anti-tumoral approaches. To this end, we have studied integrin-based signaling in angiogenic endothelial cells with a focus on Integrin-linked Kinase (ILK), a cytoplasmic partner of the beta integrin subunits. ILK and cytoplasmic adaptors of the PINCH and parvin families form a ternary complex termed IPP that localizes in integrin adhesions and drives focal to fibrillar adhesion maturation and FN fibrillogenesis. Molecular mechanisms underlying FN fibrillogenesis and functional dissection of the IPP complex will be discussed.

IL 4.3–2
Extracellular matrix and tumor stroma in cancer progression

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Epithelial tissues depend on normal stromal cells and the basement membrane for maintenance of tissue homeostasis. These interactions continue to be important in epithelial pathologies. During carcinogenesis tumor-associated cells of the stromal compartment express pro-proliferative paracrine signals for epithelial cells and stimulate angiogenesis. Therefore, understanding the mechanisms of the complex crosstalk between the cancerous epithelial cells and the tumor stroma might lead to novel approaches for cancer therapies which target the functions of the activated stromal cells. A prominent extracellular matrix protein specifically present in tumor stroma is tenascin-C (TNC). Interestingly, TNC was shown to be expressed around angiogenic vessels in many tumors and to promote angiogenesis in cell culture studies. Furthermore, TNC added to a fibronectin substratum stimulated cancer cell growth and migration *in vitro*. Therefore, TNC is a candidate molecule mediating the pro-tumorigenic effects of tumor stroma. Glioblastomas are highly invasive and aggressive brain tumors that show limited response to conventional therapies. We observed in human glioblastoma high Notch2 protein levels to coincide with expression of TNC. Expression of activated forms of Notch2 in glioma cells triggered RBPJk-dependent induction of TNC transcription. Furthermore, transfection of activated Notch2 increased endogenous TNC protein production. Since increasing amounts of TNC stimulated glioma cell migration, this may represent a mechanism for the invasive properties of glioma cells controlled by Notch and defines a novel oncogenic pathway in gliomagenesis to be targeted for therapeutic intervention in glioblastoma patients. Recently, we determined the presence of a novel tenascin family member, tenascin-W (TNW), in the stroma of breast and colon cancers and elevated levels of TNW were found in the sera of colon cancer patients. *In vitro*, TNW did not interfere with cancer cell adhesion to fibronectin, but promoted migration of breast cancer cells towards fibronectin. These data imply that TNW expression in the activated tumor stroma facilitates tumorigenesis by supporting the migratory behavior of cancer cells.

IL 4.3–3
Human Embryonic stem cells and bone regeneration

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The establishment of human embryonic stem cell (HESC) lines and recent progress in the area of medical research offer the possibility to understand early developmental processes, thus contributing to the improvement of strategies for regenerative medicine. Our work aims to grow HESCs in laboratory conditions and to investigate various mechanisms by which stem cells differentiate into bone producing cells, osteoblasts. We have earlier been able to show that differentiation of pluripotent HESC to an osteogenic lineage does not require initiation via embryoid bodies (EB) formation, and that HESCs produce a mineralized matrix possessing all the major bone markers in mono-layer cultures. Thereby, providing a suitable model for the development of bone regeneration strategies. Moreover, enhanced differentiation of HESCs, induced by genetic modification could potentially generate a vast number of diverse cell types. To date, attempts to promote osteogenesis by means of gene transfer into HESCs using the early bone 'master' transcription factor osterix (Osx) have not been reported. We attained HESC subpopulations expressing two significantly different levels of Osx, following lentiviral gene transfer. We show that the high Osx levels induced the commitment towards the hematopoietic-endothelial lineage-by up-regulating the expression of CD34 and Gata1. However, low levels of Osx up-regulated collagen I, bone sialoprotein and osteocalcin. Conversely, forced high level expression of the homeobox transcription factor HoxB4, a known regulator for early hematopoiesis, promoted osteogenesis in HESCs, while low levels of HoxB4 lead to hematopoietic gene expression.

IL 4.3–4
Extracellular matrix in bone and cartilage pathologies – Regulatory and structural roles of collagen XIII in bone and muscle

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The extracellular matrix plays a crucial role in controlling cell differentiation and function in multicellular animals. Collagens are known to be essential in maintaining the integrity of tissues. However, collagens can also serve as reservoirs for growth factors and can modulate cell signaling critical for tissue morphogenesis and homeostasis. Collagen XIII is a transmembrane protein, which is found in adhesive structures in tissues, and it is thought to serve structural roles. It can also be shed by proprotein convertases, and the shed ectodomain can affect the migration and proliferation of cultured cells. In human skeletal tissues collagen XIII is found in the periosteum, in the mesenchymal cells forming the reticulin fibres of the bone marrow and in the proliferative and hypertrophic chondrocytes of the growth plate. Findings with a novel gene-targeted reporter mouse line revealed periosteum and muscle fibres as the major sites of expression. The roles of collagen XIII in osteoblast differentiation, and in bone formation and remodeling will be discussed. In skeletal muscle, collagen XIII was identified as a previously unrecognized component of the neuromuscular junction. Results obtained with collagen XIII null mice indicate that collagen XIII affects both presynaptic and postsynaptic development in a manner that suggests specific roles at different developmental stages. Moreover, the results are suggestive of distinct roles for the transmembrane and shed forms of the collagen in the neuromuscular junction.

IL 4.3–5
Extracellular matrix in tooth enamel formation

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Dental enamel is the hardest mineralized tissue found in mammals. Covering the outer layer of teeth, this remarkable, natural bioceramic is designed to last a lifetime under extreme mechanical, chemical and physical stress in a microorganism-infested environment. Ultimately consisting of a complex hydroxyapatite (HAP) meshwork, enamel is formed by a controlled biomineralization process under the guidance of several structural proteins such as amelogenin, ameloblastin and enamelin, which are expressed and secreted by ameloblasts. The formation of enamel is a multi-step process: During the secretory stage, structural proteins accumulate and provide a three-dimensional scaffold for the initiation and elongation of HAP crystallites. During the transition stage, ameloblasts reduce their synthetic activity and HAP crystals grow primarily in width. The structural proteins, together with water and other organic components, are then removed by specific proteolytic enzymes such as matrix metalloproteinase-20 (MMP-20; enamelysin) and kallikrein-4 (KLK-4) during the final enamel maturation phase. We have recently discovered another enamel-specific protein, which we have named amelotin. Amelotin is predominantly expressed during the transition and maturation stages of amelogenesis. The secreted protein forms large aggregates that accumulate at the interface between the basal ameloblast cell surface and the mineralized enamel. We have conducted and present here several studies to characterize the amelotin protein biochemically and to elucidate its biological role in transgenic mouse models. Understanding the mechanism of enamel formation will not only help diagnose and treat related pathologies, but also assist in the development of novel biomaterials.

OP 4.3–1
The role of lumican in human colon cancer cells migration

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The interactions of cells with the extracellular matrix (ECM) proteins are essential for cells survival and motility. It has been reported that lumican, the member of small leucine-rich proteoglycans family of ECM, plays a regulatory role in cancer cells adhesion and migration. Remodeling of actin cytoskeleton, induced in response to the external stimuli, is crucial for cell motility and intracellular signal transduction. As we have previously shown, lumican decreases human melanoma A375 cells migration ability and induces actin cytoskeleton rearrangement (Radwanska *et al.*, 2008 *Life Sci.* 83). However, the level of lumican expression in colorectal cancer tissues was found to correlate with tumor metastatic capacity. We have focused on the role of lumican in colon cancer LS180 cells invasiveness and its effect on proliferation rate and actin cytoskeleton organization. Lumican expression level, as analyzed by real-time PCR and western blotting, was strongly elevated in selected LS180 cells sublines (EB3, 5W, 3LNLN) with higher metastatic potential. The transfection of LS180 cells with human lumican cDNA verified that the cells overexpressing lumican are characterized by increased ability to invade matrigel-coated Transwell filters. It was accompanied by these cells higher proliferation rate. The changes in actin cytoskeleton organization in lumican-transfected LS180 cells are followed by the changes of cell shape: the cells are spread and morphologically similar to the highly invasive sublines (5W and 3LNLN), presenting numerous cellular protrusions and actin stress fibers. These results confirm the correlation between lumican expression level and human colon adenocarcinoma cells invasive potential.

4.4. The Cell Nucleus: Structure, Function and Dynamics

IL 4.4–1
Using multi-dimensional proteomics to define the complete protein composition of mitotic chromosomes

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Mitotic chromosomes pose an intriguing problem for proteomic analysis, as they do not have a single defined protein composition. Upon nuclear envelope breakdown at prometaphase, numerous cytoplasmic and nuclear proteins associate with the chromosomes, some passively and some in order to perform a variety of essential functions. We have used a multidimensional SILAC approach to develop a strategy for identification of structural proteins of chromosomes. An initial mass-spectrometry based proteomic analysis of chicken DT40 cell mitotic chromosomes identified 2991 proteins in purified chromosomal fractions. Roughly 2000 of these are likely to be present in < 30 copies per chromatid. Of the identified proteins, 105 had previously been annotated as centromere proteins and 12 as telomere proteins. This large number of centromere proteins was due to the fact that our analysis identified multiple variant forms of a number of the proteins. Our subsequent analysis utilized three classifiers in a combination of novel SILAC-based approaches to sort proteins into groups. These included the comparison of the abundance of proteins found on chromosomes versus in post-chromosomal supernatants; the exchange of proteins between chromosomes and cytosol; and the dependence of protein association with mitotic chromosomes upon the presence of a functional condensin complex. Analysis of these three qualifiers in three dimensions identifies a space containing of true chromosomal proteins, within which lie 49 novel uncharacterized proteins. Our preliminary studies of this group to date have identified ten novel centromere proteins. This multi-dimensional profiling analysis can in principle be applied to any high-order structural complexes that can be enriched but for not purified to homogeneity.

IL 4.4-2
The mammalian genome: its duplication, organization and mobility

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We are interested on how genetic and epigenetic information is replicated every time a cell divides and how the epigenetic information is accessed and "translated" to define specific cell types with specific sets of active and inactive genes, collectively called the epigenome. We are using a combination of *in vivo* approaches including time lapse microscopy and photodynamic assays and, more recently, single molecule microscopy and particle tracking. Nuclear DNA is organized together with structural proteins into dynamic higher order chromatin structures. Chromatin can thus be broadly subdivided into eu- and heterochromatin, depending on its condensation state, transcriptional activity and the modification of associated chromatin organizing proteins. Whereas euchromatin is generally assumed to be actively transcribed and replicated early in S phase, heterochromatin is more condensed and replicates later during S phase. Using a combination of knock out cell lines and chemical inhibitors we are probing the epigenetic features regulating the timing of replication of facultative and constitutive heterochromatin in mammalian cells. It is unclear whether and how changes in the chromatin compaction state, in particular the heterochromatic state, affect the mobility of chromatin, its organizing proteins and the access of proteins to chromatin. To address these questions, we are using a combination of live-cell chromatin labels and high-speed single molecule tracking microscopy in living mammalian cells. Our results indicated that all nuclear subcompartments were easily and similarly accessible for an average-sized probe protein (streptavidin) and even condensed heterochromatin did neither exclude single molecules nor impede their passage. The only significant difference was a higher frequency of transient trappings in heterochromatin lasting though, only tens of milliseconds. The streptavidin molecules, however, did not accumulate in heterochromatin suggesting comparatively less free volume. These data suggest that genome metabolism may not be regulated at the level of physical accessibility but rather by local concentration of reactants and availability of binding sites. Finally, we labeled different chromatin subsets by fluorescent nucleotide incorporation during DNA replication and tracked their mobility throughout the cell cycle by particle tracking. We will discuss our chromatin mobility data and its interdependency of nuclear metabolism and topology.

IL 4.4–3
Nucleoplasmic lamin complexes regulate tissue progenitor cells

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Lamins are nuclear intermediate filament proteins in multicellular eukaryotes that form a scaffolding network at the nuclear envelope. While B-type lamins are ubiquitously expressed, A-type lamins are only expressed at later stages of development. Intriguingly, mutations in the LMNA gene were linked to human diseases, whose pathologies range from striated muscle defects to premature ageing syndromes. The molecular pathomechanisms, how mutations in lamins can give rise to the diverse pathologies are poorly understood. We show that in addition to the major lamin structures at the nuclear envelope a small pool of lamin A/C is located in the nuclear interior in proliferating cells in culture and in tissues. The specific lamin A-binding protein, lamina-associated polypeptide 2 alpha (LAP2α), is required for the localization of this lamin A/C pool in the nuclear interior. The intranuclear LAP2α-lamin A/C complex regulates retinoblastoma protein (Rb), a major cell cycle regulator protein involved in controlling proliferation and differentiation of cells. Knockdown of LAP2α in mice impaired the Rb pathway in postnatal regenerative tissues and caused accumulation of proliferating early progenitor cells in skin and intestine and of immature erythroblasts in the hematopoietic system due to an inefficient cell cycle arrest. Based on these findings, we propose that disease-causing mutations in lamins may disturb the balance between proliferation and differentiation of early progenitor cells, and impair tissue homeostasis in patients.

Acknowledgements: This study was supported by the Austrian Science Research Fund and the EURO-Laminopathies research project of the European Commission (Contract LSHM-CT-2005-018690).

IL 4.4–4
Protecting genome integrity by DNA damage signalling and repair

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The lecture will first provide some background information on DNA damage response (DDR) pathways and their biological significance, followed by examples from our recent work on the following issues: (i) evidence supporting the emerging role of the DNA damage signalling cascades in coordination of cell cycle checkpoints and DNA repair mechanisms, and including unpublished insights into the critical role of protein ubiquitination in key DDR processes, documented by identification of new DDR components through our pangenomic siRNA-based screens; (ii) novel insights into the spatio-temporal control and dynamics of responses to DNA double strand breaks – the most dangerous type of DNA lesion, emphasizing the distinct behavior of various classes of DDR components, as monitored by live-cell imaging approaches in human cell nuclei in real time; (iii) new data extending our concept of the DDR machinery as an inducible biological barrier against activated oncogenes and progression of human tumours, followed by a brief outline on emerging applications of these concepts for biomedicine, particularly personalized treatment of cancer.

OP 4.4–1
The dynamics of single mRNP nucleo–cytoplasmic transport and export in living cells

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Transcribed mRNAs are released from the transcription site and travel through the nucleoplasm en route to the nuclear pores. The *in vivo* kinetics of mRNP export on the single mRNA level are uncharacterized. We generated a human cell system that allowed us to follow *in vivo* as single mRNPs traveled through the nucleoplasm, and translocated through the nuclear pore to emerge in the cytoplasm. To detect real-time single mRNP export we generated several gene constructs that transcribed large mRNAs, based on different versions of the large human dystrophin mRNA. These genes were under the transcriptional control of the ecdysone-inducible system. In order to detect the mRNPs in single living cells, the 3′UTR of the mRNAs contained a series of 24 MS2 repeats. These were bound by YFP-MS2 proteins to yield tagged mRNP complexes, which were detected using live-cell fluorescence microscopy. Using rapid imaging, deconvolution and tracking techniques we could follow single mRNPs as they reached the nuclear envelope, unfolded, traveled through the pore, and restructured on the cytoplasmic side. We quantified the kinetics of single mRNP export, the diffusion properties of the traveling mRNPs before and after export, and the inter-chromatin regions in which they traveled. We examined these kinetic properties with respect to the presence or lack of an intron within the mRNA transcripts. Our data show that mRNA average transport time from the site of transcription to the nuclear envelope occured within ˜20 minutes, while a shorter time-scale (seconds) was required for mRNP restructuring and export.

IL 4.4–5
Keeping track of multiple forms of actin in- and outside the nucleus

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With the role of actin in chromatin remodeling, transcription and RNA processing now being widely accepted, the quest for its oligomeric/polymeric form(s) in the nucleus has become a major challenge (Jockusch *et al. Trends. Cell. Biol.* 2006; **22**: 391). Because of its high degree of structural plasticity, actin may assume forms in the nucleus that are distinct from those observed in the cytoplasm. To this end, we have shown with monoclonal antibodies (mAbs) that different forms of actin exist in different cellular locations (Schoenenberger *et al. J Struct Biol.* 2005; **152**: 157). Moreover, uncommon forms of actin like the lower dimer (LD), bipolar filaments, ribbons, tubes and sheets have been observed *in vitro* (Steinmetz *et al.*, *J Struct Biol.* 1997; **119**: 295). To identify and characterize the different oligomeric/polymeric forms of actin *in vivo*, in particular those in the nucleus, we chose to tailor form-specific anti-actin mAbs by repetitive antigen display on *de novo* designed virus-like peptide nanoparticles (Raman *et al.*, *Nanomed.* 2006; **2**: 95; Schroeder *et al. J Mol Biol*, in press). Immuno-fluorescence studies on cells revealed that several of these actin-form specific mAbs labeled actin-containing structures predominantly residing in either the cytoplasm or the nucleus. Also by repetitive antigen display, we obtained a polyclonal rabbit anti-actin serum that upon depletion with monomeric G-actin became exclusively LD-specific. Taken together, our results suggest that oligomeric/polymeric actin forms exist in the nucleus that clearly differ from their cytoplasmic counterparts and may require association with nucleus-specific actin binding proteins.

4.5. Function and Dysfunction of Mitochondria

IL 4.5–1
Human complex I: from gene cloning towards remedy

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Deficiencies of human complex I (NADH:ubiquinone oxidoreductase; E.C. 1.6.5.3), the first multi-protein enzyme complex of the mitochondrial oxidative phosphorylation system, are the most frequently encountered defects of mitochondrial energy metabolism. Affected patients often suffer from severe fatal multi-system disorder. Complex I consists of 45 subunits, 7 encoded by the mitochondrial genome, the remainder by the nuclear genome. In the past decade we have cloned most of the nuclear complex I structural genes, described the first nuclear mutations, proposed a model for complex I assembly and studied in detail the cell biological consequences of complex I disease. These advances have lead to greatly increased possibilities for definite diagnosis of complex I deficiency and have also made prenatal diagnosis possible for affected families. Despite this very significant progress there is still no cure for human complex I deficiency or other types of mitochondrial diseases. The pathophysiology linking the mutated complex I genes to the disease phenotype are poorly understood. In this lecture I will review the results of complex I genetic, biochemical and cell biological studies in man and mice and show how these are instrumental in the development of future therapies.

IL 4.5–2
Mitochondrial spare respiratory capacity and the life and death of neurons

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Mitochondrial dysfunction is implicated in many forms of cell death, particularly in the central nervous system. The mitochondria are required at the same time to generate ATP for the cell, sequester excess cytoplasmic Ca^{2+}, and both produce and detoxify superoxide free radicals. The electron transport chain and proton circuit are central in keeping these three balls in the air at the same time.We have investigated the bioenergetics of the *in situ* mitochondria in cultured neurons exposed to pathological glutamate concentrations to model glutamate excitotoxicity and have revised the conventional view that mitochondrial calcium loading results in increased oxidative stress that damages the mitochondrion and ultimately the cell. Instead, a central role is played under these conditions by limitations in mitochondrial and cellular ATP generating capacity. Sodium and calcium entering via the *N*-methyl-D-aspartate receptor impose a large energetic load on cells and can use the entire respiratory capacity of the *in situ* mitochondria. As a result, even modest restrictions in mitochondrial capacity – caused by low concentrations of electron transport chain inhibitors such as rotenone, as in models of Parkinson's disease; low concentrations of uncouplers, to test the so-called neuroprotective mild uncoupling hypothesis; or preexisting oxidative stress – greatly potentiate glutamate excitotoxicity. Our findings may lead to a reevaluation of the potential for mild uncoupling to provide a neuroprotective role in aging-related neurodegenerative disorders because the deleterious consequences of restricting ATP generating capacity greatly outweigh the negligible effect on oxidative stress.

IL 4.5–3
Sulfur toxicity in a human mitochondrial disorder

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Ethylmalonic encephalopathy (EE) is an autosomal recessive, invariably fatal disorder characterized by early-onset encephalopathy, microangiopathy, chronic diarrhoea, defective cytochrome oxidase (COX) in muscle and brain, high levels of C4 and C5 acylcarnitines in blood, and high excretion of ethylmalonic acid (EMA) in urine.ETHE1, a gene encoding a β-lactamase-like, iron-coordinating metalloprotein, is mutated in EE. Bacterial ETHE1-like sequences are in the same operon of, or fused with, RDS, the gene encoding rhodanese, a sulfurtransferase. Both ETHE1 and rhodanese are mitochondrial matrix proteins in eukaryotes.We created and characterized an Ethe1-less mouse that manifested the EE cardinal features. We found that thiosulfate was excreted in massive amount in urines of both $Ethe1^{-/-}$ mice and EE patients. High thiosulfate and sulfide levels were present in $Ethe1^{-/-}$ mouse tissues. Sulfide is a powerful inhibitor of COX and terminal β-oxidation, with vasoactive and vasotoxic effects that explain the microangiopathy in EE patients. Sulfide is detoxified by a mitochondrial pathway that includes a sulfur dioxygenase (SDO). SDO activity was absent in $Ethe1^{-/-}$ mice, whereas ETHE1 overexpression in HeLa cells and *E. coli* markedly increased it. Therefore, ETHE1 is a mitochondrial SDO involved in catabolism of sulfide, which accumulates to toxic levels in EE.The toxic mechanism underpinning EE makes effective therapy a realistic goal. Relevant to this issue is to understand the major source of H2S in ETHE1 mutant patients. Exogenous versus endogenous H2S production can orient rational therapeutic strategies, based for instance on drugs that reduce the H2S-producing bacterial contingent in the former case, or on H2S clearance by bone marrow transplantation in the latter.

IL 4.5–4
Mitochondria-targeted rechargeable antioxidant SkQ1 prevents senescence in mammals

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Cationic derivative of plastoquinone SkQ1 was synthesized and tested on BLM, mitochondria, cells and organisms. It is found that SkQ1 penetrates BLM and accumulates in mitochondria electrophoretically and due to high distribution coefficient (DC) in lipid/water systems. Assuming electric potential on plasma membrane and inner mitochondrial membrane as 60 and 180 mV respectively and DC = 13,000:1, one can predict that SkQ1 can accumulate in inner leaflet of inner mitochondrial membrane up to 130 min times. In mitochondria, 0.1 nM SkQ1 is shown to prevent lipid peroxidation accompanying aging of mitochondria *in vitro*. In cell cultures, SkQR1 specifically stains mitochondria and arrests ROS-induced apoptosis. SkQ1 increases the median lifespan of podospora, ceriodaphnia, drosophila and mouse

(in the latter case, by factor 2). In mammals, the effect of SkQ1 on aging is accompanied by inhibition of development of cataract, retinopathy, glaucoma, balding, canities, osteoporosis, involution of thymus, hypothermia, torpor, chromosome aberrations, peroxidation of lipids and proteins, etc. With drops containing 250 nM SkQ1, vision is restored to 67 of 89 animals (dogs, cats, and horses) that became blind because of retinopathies. Moreover, the SkQ1 pretreatment of rats decreases the ROS- or ischemia-induced heart arrhythmia. SkQs strongly reduce damaged area in myocardial infarction or stroke and prevents death of animals from kidney ischemia. In mice without p53, 5 nmol SkQ1/kg × day inhibit appearance of lymphomas to the same degree as million-fold higher dose of conventional antioxidant NAC. Thus, SkQ1 looks promising as tool to treat senescence and age-related diseases.

Reference:

1. Skulachev *et al. Biochim. Biophys. Acta.*, 2009, doi: 10.1016/jbbabio.2009.12.008

IL 4.5–5
Complex I of the respiratory chain, from physiological regulation to pathological hereditary dysfunctions

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The functional capacity of complex I is regulated by the cAMP cascade. Phosphorylation by the cAMP-dependent protein kinase (PKA) of the subunit of the complex encoded by the nuclear NDUFS4 gene promotes its mitochondrial import, enhances the NADH-ubiquinone oxidoreductase activity and prevents ROS production (1). The transcription factor CREB moves from the cytosol to mitochondria where, once phosphorylated by PKA, promotes the expression of the ND1, ND2 and ND6 mitochondrial genes of complex I (1). The NDUFS4 gene has been found to be a hotspot for mutations associated with mitochondrial encephalopathy. Mutations of this gene in neurological patients resulted in defective complex assembly, depression of the catalytic activity, suppression of cAMP-dependent regulation and accumulation of unproductive alternative transcripts (2). In a patient with PINK1 familial Parkinson, homoplasmic mutations in the ND5 and ND6 mitochondrial genes of the complex have been found, resulting in early onset of the disease (2).

References:

1. Papa S, De Rasmo D, Scacco S, Signorile A, Technikova-Dobrova Z, Palmisano G, Sardanelli AM, Papa F, Panelli D, Scaringi R & Santeramo A. Mammalian complex I: A regulable and vulnerable pacemaker in mitochondrial respiratory function. *Biochim. Biophys. Acta.* 2008; **1777**: 719–728.
2. Papa S, Petruzzella V, Scacco S, Sardanelli AM, Iuso A, Panelli D, Vitale R, Trentadue R, De Rasmo D, Capitanio N, Piccoli C, Papa F, Scivetti M, Bertini E, Rizza T & De Michele G. Pathogenetic mechanisms in hereditary dysfunctions of complex I of the respiratory chain in neurological diseases. *Biochim. Biophys. Acta.* 2009; Jan 10; [Epub ahead of print]

IL 4.5–6
Respiratory active mitochondrial supercomplexes

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The structural organization of the mitochondrial respiratory complexes as four big independently moving entities connected by the mobile carriers CoQ and cytochrome c has been challenged recently. Blue-Native gel electrophoresis reveals the presence of high molecular weight bands containing several respiratory complexes and suggesting an *in vivo* assembly status of these structures (respirasomes). However, no functional evidence of the activity of supercomplexes as true respirasomes has been provided yet. We have observed that: (1) supercomplexes are not formed when one of their component complexes is absent; (2) there is a temporal gap between the formation of the individual complexes and that of the supercomplexes; (3) some putative respirasomes contain CoQ and cytochrome c; (4) isolated respirasomes can transfer electrons from NADH to O2, that is, they respire. Therefore, we have demonstrated the existence of a functional respirasome and propose a new structural organization model that accommodates these findings.

4.6. RNA Turnover and Quality Control

IL 4.6–1
SMG6 is the catalytic endonuclease that cleaves mRNAs containing nonsense codons in metazoa

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Messenger RNAs harbouring nonsense codons (or PTCs) are degraded by a conserved quality-control mechanism known as nonsense-mediated mRNA decay (NMD), which prevents the accumulation of truncated and potentially harmful proteins. In *D. melanogaster*, degradation of PTC-containing messages is initiated by endonucleolytic cleavage in the vicinity of the nonsense codon. The endonuclease responsible for this cleavage has not been identified. Here, we show that SMG6 is the long sought NMD endonuclease. First, cells expressing an SMG6 protein mutated at catalytic residues fail to degrade PTC-containing messages. Moreover, the SMG6-PIN domain can be replaced with the active PIN domain of an unrelated protein, indicating that its sole function is to provide endonuclease activity for NMD. Unexpectedly, we found that the catalytic activity of SMG6 contributes to the degradation of PTC-containing mRNAs in human cells. Thus, SMG6 is a conserved endonuclease that degrades mRNAs terminating translation prematurely in metazoa.

IL 4.6–2
Running rings around RNA: a bacterial Ro and a Y RNA function in noncoding RNA maturation and degradation

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The Ro autoantigen is a ring-shaped protein that is present in many animal cells and also some prokaryotes. In animal cell nuclei, Ro binds misfolded noncoding RNAs and likely functions in RNA quality control. Structural studies have revealed that the single-stranded 3′ ends of misfolded RNAs insert into the Ro central cavity, while associated helices contact a basic platform on Ro's outer surface. Because the binding of Ro to misfolded RNA is largely sequence–nonspecific, Ro may scavenge RNAs that fail to bind their specific RNA-binding proteins. Since Ro can associate with a variety of RNAs that contain both a single-stranded 3′ end and helices, it is likely to have additional roles in cells. Consistent with this idea, Ro contributes to survival after ultraviolet irradiation in both mammalian cells and the stress-resistant eubacterium D. radiodurans. All species that contain Ro also contain one or more ˜100 nt RNAs called Y RNAs that bind to the outer surface of the ring. Studies in bacteria have revealed that one role of Y RNAs is to regulate access of other RNAs to Ro. To learn more about Ro and Y RNAs, we have been examining their roles in *D. radiodurans*. Previously, we found that Ro and two exonucleases are required for efficient maturation of 23S rRNA in this bacterium. We have now obtained evidence for an additional role for Ro. Evidence will be presented that Ro and a Y RNA form a complex with an exonuclease that functions in stress-induced RNA degradation. Taken together, our data suggest that binding of Ro to its various RNA targets may enhance RNA decay by exonucleases.

IL 4.6–3
RNA-protein cross-linking and deep sequencing reveal novel ncRNAs and Pol III substrates for the nuclear RNA surveillance machinery

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The RNA binding proteins Nrd1 and Nab3 function in transcription termination by RNA Pol II, particularly on snRNA and snoRNA genes, mediated via interactions with the CTD. They also participate in nuclear RNA surveillance and ncRNA degradation, acting together with the exosome and the Trf-Air-Mtr4 polyadenylation (TRAMP) complexes. To better understand the signals for surveillance and ncRNA degradation, we identified *in vivo* binding sites for Nrd1, Nab3 and Trf4 using cross-linking and cDNA sequencing (CRACS) techniques in combination with Solexa sequencing. Analysis of several million target sequences over the entire yeast genome identified (1) expected substrates; (2) unknown but anticipated substrates; (3) unexpected targets. Expected targets included snRNAs, snoRNAs, characterized ncRNAs and the *NRD1* and *NAB3* mRNAs that are known to be autoregulated. Unknown but anticipated targets were novel ncRNAs, and we identified several hundred potential ncRNAs that lie antisense to protein coding genes. Numerous mRNAs were also apparently targeted the nuclear RNA surveillance system, but there was little overlap between these and the putative antisense transcripts, suggesting that mutually exclusive expression may be common. The most notable unexpected targets were many transcripts generated by RNA Pol III, which were surprising because all previous data had linked Nrd1 and Nab3 to only to surveillance of RNA Pol II transcripts. Identified targets included truncated, polyadenylated 5S rRNA and many different tRNA precursors. These were generally unspliced and/or 3′ unprocessed, often with non-templated poly(A) tails characteristic of TRAMP activity and in some cases lacking RNA editing events, demonstrating that defective pre-tRNAs are targeted by the nuclear surveillance machinery. Northern analyses demonstrated that unspliced pre-tRNA accumulated in strains depleted of Nrd1 or Nab3, confirming their functional roles in pre-tRNA surveillance. These observations reveal an unexpected degree of overlap between nuclear surveillance of Pol II and Pol III transcripts.

IL 4.6–4
Molecular mechanisms of RNA degradation by the exosome

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The eukaryotic RNA exosome is an essential exonuclease responsible for the processing and degradation of a wide range of transcripts. It is a multi-protein complex whose components bear similarities with those of nucleases found in archaea and eubacteria. Structural studies of bacterial polynucleotide phosphorylase (PNPase), archaeal and human exosomes exosomes have revealed a common ring-shaped architecture, indicative of a shared

ancestry. However, the catalytic mechanisms have diverged. The archeal exosome degrades RNA via a phosphorolytic activity residing in one of its subunits. We have previously shown that the RNA substrate is threaded through the central channel of the *S. solfataricus* exosome, towards the cavity where the active site is located. The equivalent eukaryotic core is inactive and relies on the binding of additional subunits, the hydrolytic nucleases Rrp44 and Rrp6, to catalyze RNA degradation. In this work, we have addressed the question of whether the S. cerevisiae exosome core, lacking the phosphorolytic active site, has evolved into a simple docking platform for recruiting nucleases, or whether it also plays an active role in substrate binding.

IL 4.6–5
Structural rearrangements in transfer-messenger RNA in the process of trans-translation – protein synthesis quality control system in bacteria

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Trans-translation is a process which switches the synthesis of a polypeptide chain encoded by a nonstop messenger RNA to the mRNA-like domain of tmRNA. Initially trans-translation was described as a mechanism for the removing from the cell potentially hazardous incomplete proteins by addition to them encoded by tmRNA signal peptide that recognized by proteases. Now trans-translation seems to be involved in translational control. It has been shown that mRNAs that are known to promote ribosome stalling such as transcripts containing cluster of rare codons or poor termination signal also cause trans-translation. Thus tmRNA performs general translation quality control: any translation arrest with empty ribosomal A-site is treated as a potentially hazardous event, and the probability of triggering trans-translation apparently depends on the duration of arrest. The mRNA whose translation has caused the arrest and the protein synthesized from this mRNA are degraded. Although significant progress has been made in studying of trans-translation the molecular basis of this process is not well understood. We have developed an approach which allows isolation of the tmRNA-ribosomal complexes arrested at a desired step of tmRNA passage through the ribosome, obtained tmRNA-ribosomal complexes with an equimolar ratio of tmRNA to the ribosome and blocked at the position of the second, forth, fifth and eleventh codon in mRNA-like domain and examined tmRNA structure using biochemical and structural approaches. Computer modeling has been used to develop a model for spatial organization of the tmRNA inside the ribosome at different stages of trans-translation and to propose the mechanism of the template switching process.

OP 4.6–1
The importance of RNase II in RNA recognition, RNA binding and RNA degradation

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RNA degradation plays a fundamental role in all biological processes, since the fast turnover of mRNA permits rapid changes in the pattern of gene expression. Ribonucleases (RNases) are essential enzymes that process and degrade RNA, being one of the main factors responsible for the determination of the levels of functional mRNA in the cells. *Escherichia coli* RNase II is the prototype of a widespread family of exoribonucleases and RNase II homologues can be found in all kingdoms of life. Our structural and biochemical data together allowed explaining the dynamic RNA degradation by RNase II, and elucidated RNA translocation and enzyme processivity. We have performed a functional characterization of several highly conserved residues located in the RNB catalytic domain in order to address their precise role in the RNase II activity. Our results showed that although not directly involved in the RNA binding, highly conserved aspartates 201, 207 and 210 are contributing to the stabilization of the RNA-protein complexes. Moreover, we showed thatthese residues are not equivalent in their role in catalysis, confirming that Asp209 is in fact the only critical residue for RNase II activity. We have also identified Tyr253 as the residue that is responsible for setting the end-product of RNase II, accentuating the importance of this residue in the stabilization of the 3′-end of the RNA molecule. We also analyzed which residues were responsible for substrate specificity, and verified that both Glu390 and Tyr313 play an important role in the discrimination of RNA *versus* DNA. Our results also show that Arg500 is crucial for RNase II activity but not for RNA binding. Interestingly, the substitution of Glu542 by Ala lead to a 120 fold increase of the exoribonucleolytic activity, and improved significantly substrate binding, turning RNase II into a super-enzyme. RNase II is still an enzyme full of surprises. Rrp44/Dis 3 is RNase II-family enzyme that is the catalytic subunit of the exosome, a multiprotein complex involved in RNA processing degradation and surveillance. Recently we have actively participated in the discovery that Rrp44 includes a PIN domain that acts as an endonuclease. All the results here stated have improved substantially the model for the mechanism of RNA degradation, and will be crucial for the fully understanding of the processes of RNA maturation, quality control and RNA decay mediated by RNase II.

4.7. Nervous System Stem Cells

IL 4.7–1
Human difference starts with neural stem cells

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Although the basic principles of brain development may be similar in all vertebrates, the modifications of developmental events during millennia of mammalian evolution produce not only quantitative but also qualitative changes to its cellular structure and synaptic circuitry. The origin of these species-specific distinctions can be traced to either the new or phylogenetically conserved genes that act at the time of the neural stem cell's exit from the mitotic cycle and generate a different outcome, depending on the evolutionary context. Thus, our strategy has been to explore and compare early embryonic development of the crowning achievement of evolution, the cerebral cortex, in rodents, non-human primates and human. We have found that paradoxically, the duration of the neuronal stem cell cycle is about four times longer in human and macaque monkeys than in rodents, posing logistical problems in constructing a larger neocortex. There are also marked differences in the onset of the divergence of the neural stem and the radial glial cells that in primates express GAFP at the start of corticogenesis and some of which stop dividing while serving as guides for migrating neurons in the large and convoluted cerebrum. We have also identified in human a class of early generated predecessor cortical cells, as well as a population of thalamic neurons that originate from the ganglionic eminence that have not been observed in rodents. In addition, subclasses of cortical interneurons that in primates originate in the subventricular zone of the dorsal telencephalon and the subpial granular layer in the neocortical marginal zone do not exist in rodents. Furthermore, corticoneurogenesis in primates is completed before birth, but it is followed by overproduction and elimination of synapses during prolonged childhood, puberty and adolescence. Finally, lack of neuronal turnover and neurogenesis in the adult primate cortex occurs concomitantly with their diminished capacity for regeneration. Thus, the search for causes and designs of therapies for human neuropsychiatric disorders needs to take into consideration these species-specific distinctions.*Supported by NINDS, NIMH, NIDA and the Kavli Institute for Neuroscience at Yale.*

IL 4.7–2
Adult neurogenesis, neurodegeneration, and aging

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Adult hippocampal neurogenesis contributes to hippocampal function by allowing a particular type of structural plasticity, presumably necessary to allow life-long adaptation of the mossy fiber connection to the functional demands encountered by the individual. As far as we know today, the new neurons add flexibility to the learning response and are particularly relevant in re-learning situations. At the same time, activity regulates adult neurogenesis. With age, adult neurogenesis decreases but remains present at very low levels throughout life. Activity counteracts this decrease and maintains an increased potential for adult neurogenesis, which can be used, if appropriate stimuli are present. From this it appears that adult neurogenesis might contribute to a structural reserve, a "neurogenic reserve" in the adult hippocampus. Neurodegenerative disorders appear to interfere with this process but on the other hand the potential for plasticity contributed by adult neurogenesis allows compensation in cases of degeneration.

IL 4.7–3
Specification and integration of cerebellar GABAergic neurons

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The local GABAergic network in the cerebellum is composed by different types of cortical and deep nuclear inhibitory interneurons, generated from late embryonic to early postnatal life, according to an inside–out sequence. Unlike other cerebellar cell types, which derive from fate-restricted progenitors, precursors of inhibitory interneurons share a common lineage and maintain their multipotency up to late phases of postnatal development. However, the mechanisms giving rise to the different interneuron classes remain unknown. Namely, it is not clear whether mature interneuron phenotypes are specified in germinal zones or at their final destination sites in the cortex or nuclei. In order to clarify this point, we analysed the proliferation and the migratory pathways of interneuron precursors in wild type and transgenic Pax2-GFP and GAD67-GFP mice, that finely characterize this cell population during earlier and later stages of development, respectively. We found that inhibitory interneuron precursors exclusively proliferate in the periventricular zone and along the folial white matter tracts of postnatal cerebellum, migrating to the cortex as post-mitotic cells. To ask whether transplanted precursors entrain in the same developmental mechanisms we considered their behaviour at short time-intervals after graft in hosts of different ages. Donor cells integrate in the host proliferative sites and follow the usual migratory routes of the local inhibitory interneurons, joining a final layer position which is strictly dependent on the host age. In particular, grafted interneurons consistently acquire positions and phenotypes that match those adopted by host interneurons born on the day of transplantation. Interestingly, donor cells acquire host-specific identities even when grafted as postmitotic neurons, indicating that fate specification of cerebellar interneurons occurs after the progenitors have left the cell cycle. On the whole, our results indicate that grafted interneuron precursors fully integrate into the host neurogenic mechanisms in which different phenotypes are generated by temporally patterned signals acting on cells that remain multipotent even after leaving the cell cycle.

IL 4.7–4
Lack of adult brain neurogenesis and its effects (or lack of thereof) on mouse learning and memory

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Adult brain neurogenesis is phenomenon by which new neurons are being added to the adult brain in a process that has been suggested to affect brain plasticity, i.e. an ability to respond differentially to changes in an external environment. We have reported on knockout mice that due to the lack of cell cycle regulatory protein, cyclin D2 miss the adult brain neurogenesis almost completely (Kowalczyk *et al.*, *J Cell Biol* 2004). We have extended

the analysis of the newly born neurons in the adult brains of those animals and observed in cyclin D2 KO hippocampi < 10% of BrdU-positive cells and even less of doublecortin-positive early neurons as compared to their wild-type siblings. To investigate the effect of this most severe impairment of adult brain neurogenesis on learning and memory, cyclin D2 KO mice and their wild type siblings were tested in several behavioral paradigms including those in which the role of adult neurogenesis has been postulated. The knockout mice showed no impairment in sensorimotor tests with only sensory impairment in an olfaction-dependent task. However, KO mice showed proper procedural learning as well as learning in a context (including remote memory), cue and trace fear-conditioning, Morris water maze, novel object recognition test, and in a multifunctional behavioral system - Intellicages. Cyclin D2 KO mice also demonstrated correct reversal learning. Our results suggest that adult brain neurogenesis is not obligatory for learning, including the kinds of learning where the role of adult neurogenesis has previously been strongly suggested.

IL 4.7–5
Stem cells and treatment of brain and spinal cord injury

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Mesenchymal stem cells (MSCs), olfactory ensheathing glia (OEGs) and neural progenitor cells (PNCs) have the capacity to migrate towards lesions and induce better regeneration. MSCs, OEGs or PNCs labeled in culture with iron-oxide nanoparticles were transplanted into rats in a stroke model or with a spinal cord compression lesion (SCI). *In vivo* MRI, used to track their migration and fate, proved that MSCs, OEGs as well as PNCs migrated into the lesion. We compared the effects of implanted PNCs, OEGs or MSCs with the implantation of a mononuclear fraction of bone marrow cells (BMCs) or the injection of granulocyte colony-stimulating factor (G-CSF). Seven days after SCI, the rats received either PNCs, MSCs, OEGs, BMCs or G-CSF intravenously or intraspinally. All implanted animals had significantly smaller lesions and higher BBB scores. In other studies, hydrogels or nanofiber scaffolds seeded with stem cells were implanted into rats with SCI. In both acute and chronic SCI, implants reduced scar formation, bridged the lesion and increased functional recovery. Autologous BMC implantation was also used in a Phase I/II clinical trial in patients with acute and chronic SCI (n = 34). The results show that implantation is safe and has a beneficial effect if administered in the first 4 weeks after injury, close to the injury site. We conclude that stem cells, with or without scaffolds, improve regeneration by the rescue and replacement of local neural cells, by bridging gaps after SCI and by mechanically supporting ingrowing cells and axons.

OP 4.7–1
Drosophila cells with neurotrophic factor genes influences a development of mammalian neural tissue

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A possibility to direct behaviour of mammalian neural stem/progenitor cells by co-culturing with embryonic tissue bearing human neurotrophic factor genes (NGF,BDNF or GDNF) has been studied. *In vitro* co-culture system contains dorsal root ganglion (DRG) of newborn rat and embryonic nerve cells bearing humanneurotrophic genes, that were located 0.2–0.5 mm apart of DRG. Locally implantedcells could direct a growth of rat DRG axons. The outgrowth (directed toward transfected cells) initiates after 1–2 hours, while in control it was started on the third/forth day. These findings suggest that co-cultures of rat DRG and genetically modified cells provides a powerful tool to study survival and integration of neuronal population. Other experiments analyzed effects of expression of neurotrophic factors on preventing glial scar formation after xenotransplantation. HEK293 were transfected with GDNF expressing vectors, and the cells were transplanted into caudatum–putamen of CBA mice. Transplanted cells survived in recipient brain for 18 days. Immunohistochemical analysis of brain sections stained for the scar (actin, fibronectin, and collagen type IV) showed that GDNF decrease glial post-traumatic reaction. Other genetic construct has temperature-regulated *Drosophila* HSP70 promoter with BFP reporter gene in mouse ESC; fluorescence appeared only at 39°C. Both strategies of gene activation could be used in gene therapy experiments.

Acknowledgement: The work has been supported by Rosnauka 02.512.11.2224, RFBR 07-04-12120, 07-04-00835.

SYMPOSIUM 5: SIGNAL TRANSDUCTION

5.1. Cytoskeleton Dynamics

IL 5.1–1

Mechanism of actin-dependent nuclear positioning in cell polarization and migration

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In many directionally migrating cell types, the centrosome is positioned between the nucleus and the leading edge and this is thought to direct membrane traffic toward the leading edge. Surprisingly, in fibroblasts, centrosome reorientation results from active movement of the nucleus away from the leading edge, while the centrosome remains stationary in the cell center (Gomes *et al*., *Cell* 2005). Cdc42 regulates myosin activation and actin retrograde flow that are necessary for nuclear movement, but how actin might engage the nucleus was unclear. Using siRNA and dominant negative approaches, we found that the outer and inner nuclear membrane proteins, nesprin2G and SUN2, respectively, were necessary for nuclear movement. Rescue experiments showed that the actin-binding CH domains of nesprin2G were essential for nuclear movement. Imaging studies revealed that nesprin2G and SUN2 accumulated along moving actin cables that contacted the nucleus. FRAP experiments showed that nesprin2G within these arrays was immobilized relative to bulk nesprin2G. We refer to these novel membrane assemblies as TAN lines for transmembrane actin-associated nuclear lines. TAN line movement was coincident with nuclear movement and treatments that disrupted TAN lines inhibited nuclear movement. Nesprin2G TAN lines still formed in SUN2 depleted cells, but in these cells, TAN lines moved over an immobile nucleus. Interfering with either nesprin2G or SUN2 also inhibited directional migration into wounds. Our results establish a novel molecular mechanism for coupling actin filaments to move nuclei and suggest that migrating cells may actively determine the position of their nucleus for efficient cell migration.

IL 5.1–2

Centrosome dynamics in *C. elegans* embryos

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Centrosomes duplicate once per cell cycle by duplication of their centrioles. Centrioles then assemble different components, in the form of pericentriolar material, which act as down stream effectors of centrosome function. In *C. elegans* embryos, centrosomes are required to build a mitotic spindle. Our work shows that the size of the centrosome sets the length of the mitotic spindle. During development, centrosome size varies, and this is apparently partially responsible for setting spindle length during early development. I will discuss ways in which embryos set the size of the centrosomes.

IL 5.1–3

Cell-matrix adhesions as mechano-chemical environmental sensors

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Cell adhesion to the extracellular matrix is a complex process, regulated at multiple levels, and at a wide range of spatial and temporal scales. Cells can differentially sense and respond to different chemical signals induced by specific components of the extracellular matrix, as well as to a variety of physical cues, including external or internal forces, surface rigidity, topography and fine texture. These structural and signaling activities are executed by multi-molecular protein complexes known, collectively, as the 'integrin adhesome network'. In this lecture, I will describe our attempts to unravel the molecular structure and signaling activities of different types of integrin adhesions, in order to define their roles, and the possible mechanisms underlying the effects of micro- and nano-scale perturbations on the adhesion process. In my talk, I will particularly focus on siRNA-based screens for molecules that participate in the organization and signaling activity of these adhesions, as well as on correlated microscopy, in which the same specimens are examined by fluorescence microscopy and cryo-transmission electron microscopy. Applying novel experimental approaches to studies of focal adhesions may provide new insights into the molecular organization and nano-scale structure of these cellular adhesion sites, and their sensory and signaling activities.

IL 5.1–4

Pushing with actin in cell migration

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Eukaryotic cells initiate movement by extending cytoplasmic processes as sheets (lamellipodia), rods (filopodia) or blebs, singly, alternately or in combination. Of these, lamellipodia appear essential for the migration process. The extension of lamellipodia and filopodia is based on the polymerisation of actin, with the insertion of actin monomers at the interface between filament plus ends and the leading membrane front. The currently propagated model of lamellipodia protrusion is one in which actin filaments form branched, dendritic arrays, whereby the Ap2/3 complex initiates and stablises the branch sites. Primary evidence for this model comes from electron microscopy of platinum replicas of cytoskeletons that were dehydrated and critical point dried. We have applied electron tomography to cytoskeletons dried in deep negative stain, or vitreously frozen, to reinvestigate lamellipodia architecture. Our findings show: (i) that a high degree of three dimensionality is preserved in the negatively stained preparations; (ii) that the double helical structure of actin filaments can be resolved in negative stain tomograms of the

lamellipodium cytoskeleton, indicative of a high degree of structural preservation; (iii) actin filaments are not branched in lamellipodia. The features of actin filament arrays in lamellipodia will be presented and the implications discussed.

OP 5.1–1
Characterization of MUF1, a binding partner of atypical Rho GTPases of the RhoBTB family

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Rho proteins have been implicated mainly in the regulation of the cytoskeleton, but also in vesicle trafficking, phagocytosis and transcriptional activation. RhoBTB proteins constitute a subfamily of atypical Rho GTPases represented by three isoforms in vertebrates. They consist of a GTPase domain followed by a proline-rich region and two BTB domains. BTB domains, including those of RhoBTB, are involved in the formation of cullin 3-dependent ubiquitin ligase complexes therefore the function of RhoBTB proteins seems to be distinct from that of the classical Rho GTPases. In our working model RhoBTB proteins play a role in targeting of substrates for ubiquitinylation and degradation via cullin 3-dependent ubiquitin ligase complexes, but the substrates remain unknown. As a first step towards understanding the function of RhoBTB we performed a two hybrid screening on a mouse brain cDNA library and identified MUF1 (LLR41) as a potential interaction partner. We confirmed the interaction using immunoprecipitation. MUF-1 is ubiquitously expressed, with the highest expression in testis. MUF1 is a leucine rich repeat containing protein that, interestingly, also contains a BC-box that serves as a linker in multicomponent, cullin 5-based ubiquitin ligases. This raises the question whether MUF1 and RhoBTB3 are together involved in multiprotein complexes containing cullin 3 and cullin 5 simultaneously. We envision two possible models: (i) MUF1 is ubiquitylated by a Cul3 complex dependent on RhoBTB, which acts as the substrate specific adaptor. (ii) RhoBTB is ubiquitylated by a Cul5 complex dependent on MUF1, which acts as the substrate specific adaptor.

5.2. Reactive Oxygen Species in Cell Signalling

IL 5.2–1

Gas sensing by potassium channels: oxygen, carbon monoxide and redox

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The enzymatic breakdown of cellular heme by the activity of hemeoxygenases leads to the endogenous production of carbon monoxide. This gas is a potent second messenger with a steadily growing number of identified molecular targets, including ion channels such as the large-conductance, calcium- and voltage-activated potassium (BK_{Ca}) channel (Williams *et al., Science* 2004; **306**: 2093–2097) and the amiloride-sensitive epithelial sodium channel (Wang *et al., Proc Natl Acad Sci U S A* 2009; **106**: 2957–2962). Hemeoxygenases are oxygen-dependent enzymes, and their production of carbon monoxide is limited by oxygen availability, suggesting that they can act as ion channel oxygen sensors. Carbon monoxide activates the human BK_{Ca} channel alpha-subunit (KCNMA1) in a calcium-dependent manner, and this activation is completely absent when the C-terminal tail of BK_{Ca} is substituted by an homologous region of mSlo3 (Williams *et al., Pflugers Arch* 2008; **456**: 561–572). Furthermore, a chimera in which the so-called 'calcium bowl' region of BK_{Ca} is swapped with the homologous region of mSlo3 is also completely insensitive to carbon monoxide. Within that region, sequence alignment of the BK_{Ca} channel alpha-subunit with mSlo3 shows that there is only one cysteine which is unique to the BK_{Ca} alpha-subunit. Mutagenesis of that single cysteine residue (to glycine) greatly attenuates both the oxygen- and carbon monoxide-sensitivity of BK_{Ca} channels. This cysteine is also redox-sensitive, suggesting that redox and gas signalling converge at a single residue within the C-terminal tail of the BK_{Ca} channel alpha-subunit. Furthermore, using low concentrations of cyanide, as a substituent for metal ions in a potential transition metal cluster, we observed that carbon monoxide regulation of BK_{Ca} channels in inside-out membrane patches was ablated. Taken together, these observations lend support to the idea that BK_{Ca} channels might bind carbon monoxide in a manner analogous to the transition metal-dependent co-ordination of this gas by several well characterised enzymes, including carbon monoxide dehydrogenase (Jeoung & Dobbek, *Science* 2007; **318:** 1461–1464). Such a mechanism may go some way to explaining the molecular basis of how this particular ion channel demonstrates enzyme-linked oxygen and carbon monoxide sensitivity.

IL 5.2–2

Oxygen and reactive oxygen species as signals for wound repair

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In the United States, chronic wounds affect 6.5 million patients. It is claimed that an excess of US$25 billion is spent annually on treatment of chronic wounds and the burden is rapidly growing due to increasing health care costs, an aging population and a sharp rise in the incidence of diabetes and obesity worldwide. One factor that is centrally important in making the wound environment receptive to therapies is correction of wound hypoxia. Wound tissue oxygen not only provides energy for tissue repair but it also supports reactive oxygen species (ROS) production required for wound healing. The wound fluid is the most oxidant-rich fluid in the body. At the wound site, phagocytic cells generate oxidants to fight infection and cleanse the wound tissue. Lower level oxidants generated by non-phagocytic cells, on the other hand, support numerous processes including cell proliferation and migration necessary for angiogenesis as well as re-epithelialization. Recent observations in our laboratory resulted in the first publication demonstrating that cellular redox signaling is regulated by microRNAs (miRs). miRs are small noncoding RNAs that regulate gene expression at the post-transcriptional level by either degradation or translational repression of a target mRNA. Encoded in the genome of most eukaryotes, miRs have been proposed to regulate specifically up to 90% of human genes through a process known as miR-guided RNA silencing. The significance of miR in regulating cutaneous wound healing will be presented.

IL 5.2–3

The cellular functions of eukaryotic GSH and thioredoxin pathways revisited

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The tripeptide GSH is considered as having a unique cellular redox buffering function, capable of maintaining reduced the thiol-redox cellular balance, and also to eliminate electrophilic compounds including ROS and RNS species. This function is in keeping with the characteristics of GSH of being the most abundant low molecular thiol of eukaryotic cells and having a relatively low redox potential. Nevertheless, redox buffering might not explain all GSH cellular functions, and in particular its essential nature in both yeast and mammals. We have used *S. cerevisiae* as a model eukaryote to explore the essential function(s) of GSH, exploring the cellular defects ensuing (i) the deletion of the GSH biosynthetic gene γ-glutamyl cysteine synthase (*GSH1*) that leads to GSH depletion and cell death, and (ii) an artificial increase in its concentration up to 80 mM, which also eventually leads to cell death. Data gathered using a combination of global and cell biological approaches lead to the conclusion that the essential requirement of GSH is due to its role in the maturation of cytoplasmic Fe-S clusters proteins. Toxicity of GSH at elevated concentrations also associates with a major alteration of iron metabolism, which also relate to defective assembly of Fe-S at monothiol Grxs, and to a major block of secretion due to ER reductive stress. Surprisingly, both GSH cellular depletion and its presence at toxic concentrations do not appear to alter thiol-redox control in the cytoplasm. We will discuss the respective cellular functions of the GSH and thioredoxin pathways in eukaryotes.

IL 5.2–4

Hypercholesterolemia-induced oxidative stress related chances in the brain of rabbits

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Hypercholesterolemia, a major risk factor for age related diseases such as atherosclerosis and Alzheimer's disease (AD).

Changes in human plasma cholesterol levels results from the interaction between multiple genetic and environmental factors. The accumulation of excess cholesterol in blood vessels leads to atherosclerosis. Studies show that differential expression of oxidative stress proteins, lipid metabolism related enzymes and receptors response to atherogenic diet. Excess brain cholesterol has been associated with increased formation and deposition of amyloid-β peptide from amyloid precursor protein which may contribute to the risk and pathogenesis of AD. More than 50 genes have been reported to influence the risk of late-onset AD. Several of these genes might be important in cholesterol metabolism and transport. On the basis of these results, an *in vivo* study has been carried out. Rabbits were fed with cholesterol supplemented diet or cholesterol supplemented diet plus alpha tocopherol, after 4 weeks brains were removed. Formation of malondialdehyde in serum, a marker of lipid peroxidation, was tested. Protein carbonylation, HNE-proteins and 3-nitrotyrosinated proteins and proteosome function evaluated in the brain of hypercholestrolemic rabbits. The results indicate a cellular mechanism for hypercholesterolemia induced AD similar changes will be discussed.

Acknowledgements: This work is supported by COSTB35 Action, TUBITAK SBAG-COSTB35-5(106S121), MU BABKO-SAG-DKR-290506-0091 and the Braun Stiftung.

IL 5.2–5
Nox family of NADPH oxidases: sources of reactive oxygen

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Reactive oxygen species (ROS) play a central role in many physiological processes including host defense, hormone biosynthesis, fertilization and cellular signalling. When exposed to foreign pathogens, phagocytic cells – such as neutrophil granulocytes – produce large amounts of superoxide, the precursor of ROS. ROS have a pivotal role in the killing of the invading microorganisms. ROS operate like a 'double edged sword', meaning that they are essential for killing the foreign pathogens, however they are also harmful to the host tissues, and their overproduction has been implicated in the pathogenesis of several inflammatory diseases, including inflammatory bowel disease, rheumatoid arthritis and kidney diseases. In phagocytic cells, the precursor of ROS is superoxide (O_2^-), produced by the NADPH oxidase enzyme, which transfers one electron from NADPH to molecular oxygen. The 'heart' of this enzyme is a heme-binding, flavoprotein Nox2 (formerly known a $gp91^{phox}$), which catalyzes the final steps of electron transfer. It was known for decades that besides phagocytes, several other types of mammalian cells synthesize and release ROS, which can serve various biological functions, including oxygen sensing, vascular regulation, hormone synthesis and fertilization. The enzymatic basis of non-phagocytic ROS-production was unknown. In the last few years, several enzymes were identified at the molecular level, which are now thought to be responsible for ROS production observed in diverse tissues. These enzymes show a high degree of homology to the phagocytic NADPH oxidase and are now designated the Nox family of NADPH oxidases. The Nox family of NADPH oxidases consists of the following members: Nox1, Nox2, Nox3, Nox4, Nox5, Duox1 and Duox2. The discovery of this enzyme family has revolutionized ROS-research since we can now assign a specific source of ROS in several tissues, including colon, kidney, heart, lung, thyroid and immune organs. In my lecture I will discuss our current knowledge on six new members of the Nox family, with a special emphasis on Duox enzymes.

OP 5.2–1
Role of Noxes and ROS in Resveratrol-mediated inhibition of Akt phosphorylation in Ang II or EGF-activated vascular smooth muscle cells

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Resveratrol (RV) inhibits the angiotensin (Ang) II- and epidermal growth factor (EGF)-induced phosphorylation of Akt in vascular smooth muscle cells (VSMC). For the Ang II-induced transactivation of the EGF-receptor NADPH oxidase (Nox)-derived reactive oxygen species (ROS) are important and also the EGF-receptor is reported to use ROS to transduce growth signals. We therefore examined whether RV inhibits Akt phosphorylation in Ang II or EGF-activated VSMC via an antioxidant mechanism including Nox proteins. We found increased intracellular ROS level in Ang II- but also in EGF-activated VSMC. The same concentration of RV (50 μM) that inhibits Akt kept ROS even below control levels in Ang II-and EGF-activated VSMC. To investigate, whether Nox1 or Nox4 is pivotal for Ang II or EGF signaling we inhibited Nox1 by a specific blocking peptide leading to inhibited Akt phosphorylation after Ang II but not after EGF stimulation. A Nox4 knock-down by siRNA did not block either signal. Moreover, pre-treatment with the antioxidant N-acetyl cystein (NAC) and the flavoprotein inhibitor DPI inhibited Ang II-but not EGF-mediated Akt phosphorylation. This confirms that Ang II signaling requires ROS to transactivate the EGF receptor. The signaling downstream of the EGF receptor towards Akt, however, seems to occur largely independent from ROS. These data suggest that RV inhibits EGF signaling to Akt in a redox-independent manner whereas Ang II-mediated Akt phosphorylation could be affected by inhibition of ROS originating from Nox1. However, there must be an additional activity of RV since the Nox1 knock-down cannot mimic the high specificity of RV towards Akt.

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5.3 Gaseous Signaling

IL 5.3–1
Mitochondria, nitric oxide and cell bioenergetics

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At physiological concentrations nitric oxide (NO) inhibits mitochondrial cytochrome c oxidase in competition with oxygen. We have developed a technique based on visible light spectroscopy and used it to demonstrate that endogenous NO enhances reduction of the electron transport chain, thus enabling cells to maintain their VO_2 at low oxygen concentrations. This favours the release of superoxide anion, which initiates the transcriptional activation of NF-kB as an early stress signalling response. We have also used this technique to demonstrate that NO is inactivated by cytochrome c oxidase in its oxidised state and have proposed that cessation of such inactivation at low oxygen concentrations may account for hypoxic vasodilatation. Many cells respond to a decrease in oxygen availability via stabilisation of hypoxia-inducible factor-1a (HIF-1a), whose accumulation is normally prevented by the action of prolyl hydroxylases. We have found that inhibition of mitochondrial respiration by low concentrations of NO leads to inhibition of HIF-1a stabilisation. This prevents the cell from registering hypoxia at low oxygen concentrations, which would otherwise result in upregulation of defensive genes, including those for glycolysis and angiogenesis. Furthermore, inhibition of mitochondrial respiration in hypoxia leads to redistribution of available oxygen toward non-respiratory oxygen-dependent targets. In addition to its interaction with cytochrome c oxidase, NO can signal for mitochondrial biogenesis via a cyclic GMP-dependent mechanism. Furthermore, increases in NO beyond physiological levels lead to persistent inhibition of other key enzymes in the mitochondria and this may account for NO-dependent initiation of cell pathology.

IL 5.3–2
Designer nitric oxide synthases for understanding the production of gaseous signals

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Nitric oxide synthases (coded by three NOS genes) are dimeric enzymes that contain heme, flavins (FAD and FMN), tetrahydrobiopterin, and a single tetrahedrally coordinated Zn atom, all of which are required for the production of nitric oxide (NO). The heme-binding (oxygenase) domain and the flavin-binding (reductase) domains are joined by a calmodulin-binding sequence necessary for the catalytic function of NOS. The molecular conformations of these dimeric structures are unknown due to the lack of holoenzyme structures. The quest for the intrinsic and extrinsic factors that control NOS function has been facilitated by molecular dissection and genetic engineering of the structures, permitting production of the separate domains of the enzymes, which have provided insights into their regulation. The interactions initiated by calmodulin binding in the case of the constitutive NOS isoforms, neuronal (NOSI) and endothelial (NOSIII), will be discussed with particular attention to the intrinsic molecular inserts, the autoregulatory insert in the FMN-binding domain and the C-terminal extensions. The production of NO and uncoupled reduced oxygen species under various conditions will be addressed. A model of these interactions will be presented as a plausible explanation of the mechanism of time- and structure-dependent production of the gaseous signaling molecule, NO.

Acknowledgements: Supported by US National Institute of General Medical Sciences GM081568 and GM052419 to BSSM (The Robert A. Welch Distinguished Professor in Chemistry, AQ-0012).

IL 5.3–3
Nitric oxide–carbon monoxide interaction and oxidative stress in obesity and diabetes

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We examined the effect of interaction of HO-1 gene expression and NOS protein during an increase in oxidative stress in obese/diabetic models. We hypothesized that upregulation of HO-1-derived CO reduces oxidative stress and increases eNOS, peNOS via an increases in pAMPK-pAKT cross talk in obese diabetic mice. Obese, ob, lean mice were divided into groups compromising lean, lean-inducers of HO-1, L-4F-treated, ob, ob-L-4F-treated. Food intake, blood glucose levels, CO, eNOS, pAMPK-pAKT and insulin receptor phosphorylation. Subcutaneous fat tissue (SAT) and visceral adipose tissue (VAT) were determined by MRI. The effect of HO-1 expression-derived CO and bilirubin on adiponectin levels and adipogenesis was also examined in human adipocytes stem cells. Both SAT and VAT global volumes decreased in ob-L-4F treated animals. Decreased levels of eNOS, peNOS, pAKT and pAMPK in vascular endothelium in ob mice were reversed by HO-1 expression. Upregulation of HO-1 produced adipose remodeling and increases peNOS levels in adipocytes. The anti-obesity effects of HO-1-derived CO, bilirubin are manifest by a decrease in visceral fat content with reciprocal increases in EC-SOD, eNOS, peNOS, adiponectin, pAMPK and pAKT. The increase in peNOS resulted in restoration of vascular function and increased phosphorylation of insulin receptors with improved insulin sensitivity.

IL 5.3–4
Carbon monoxide-releasing molecules: anti-ischemic and anti-inflammatory properties

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Heme oxygenase-derived carbon monoxide (CO) serves as signaling mediator in a wide array of physiological functions to the extent that the beneficial effects observed when small amounts of CO gas are administered to mammalian organisms may be exploited for therapeutic purposes. In this context, the development of carbon monoxide-releasing molecules (CO-RMs) represents a pharmaceutical stratagem for the safe delivery of CO in the treatment of various pathological disorders. Transition metal carbonyls and boranocarbonates have been identified as ideal scaffolds for the synthesis of water-soluble compounds that release controlled amounts of CO within biological systems. Specifically, CORM-3 (ruthenium tricarbonyldichloro glycinate) and CORM-A1 (sodium boranocarbonate), which possess different chemical reactivities and kinetics of CO release, have been tested

in various models of disease. The results collected to date indicate that CO-RMs are pharmacologically active as they exert vasodilatory, anti-ischemic and anti-inflammatory effects and can protect tissues against oxidative stress. Although the mechanism of action of CO-RMs remains to be fully elucidated, we have proposed that a dynamic interaction of CO with specific intracellular metal centers may be the common denominator for the diversified beneficial effects mediated by this gaseous molecule. Circumstantial evidence points to mitochondria as plausible, and perhaps, preferential targets of the signals transduced by CO. Thus, CO-RMs may help to identify cellular components that are responsive to CO and facilitate the therapeutic delivery of this gas in a safe, measurable and controllable fashion.

IL 5.3–5
Endothelial cells - new territory for heme oxygenase-1 biochemistry

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Heme oxygenase-1 (HO-1) possesses potent pro-angiogenic properties in addition to well-recognized anti-inflammatory, antioxidant and anti-apoptotic effects. Angiogenic factors, such as VEGF and SDF-1, mediate their activities through induction of HO-1, making it an attractive target for therapeutic intervention. However, the real significance of HO-1 in the vessels remains to be assessed in human subjects. To better understand whether HO-1 is merely a marker of oxidative injury or a prerequisite mediator of endogenous protection against more severe insults, we compared primary human endothelial cells of different HO-1 promoter variants, checking their angiogenic potential, inflammatory response, and sensitivity to oxidative stress. A (GT)n repeat in the human HO-1 promoter shows length polymorphism and can modulate the level of gene transcription. We propose classification of the alleles into three groups: S (most active, $GT \leq 23$), M (moderately active, GT = 24–28), and L (least active, $GT \geq 29$). Presence of S allele was associated with higher basal HO-1 expression and stronger induction in response to oxidants or inflammatory mediators. Cells carrying S allele displayed highest concentration of total glutathione and more favorable oxidative status determined by GSH: GSSG ratio. Accordingly, they survived better when exposed to oxidative stress. Moreover, they proliferated more efficiently in response to VEGF, although the VEGF-induced migration and sprouting of capillaries were not influenced. Presence of S allele was also associated with higher basal or LPS-stimulated production of TNF, IL-1β, IL-6, ICAM-1 and E-selectin. Thus, the lesson from promoter polymorphism supports a cytoprotective, proangiogenic and anti-inflammatory role of HO-1 in the vessel wall in humans.

5.4 Signalling in Plants

IL 5.4–1
Cytokinin signal perception and transduction

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Cytokinins are a class of plant hormones that regulate numerous developmental and physiological processes. The cytokinin signal is perceived by membrane-located sensor histidine kinases which transfer the signal through a His-Asp-phosphorelay into the cell nucleus. There response regulator proteins are activated and function as transcription factors. Among higher eukaryotes this signaling system is unique to plants. It was shown that the three cytokinin receptors of Arabidopsis differ in their ligand binding specificities. Steps have been made towards structural characterization of the ligand-binding CHASE domain. Domain swap and promoter swap experiments revealed that signal recognition and regulation of receptor expression contribute to signal specification. Protein interaction maps have been established for many of the ca. 30 proteins of the signaling system. Immediate early target genes of the signal pathway were identified and progress towards identification of cis regulatory elements will be reported. The functions of the pathway in plant development and the perspective of its modulation for biotechnological purposes will be discussed and exemplified.

IL 5.4–2
Plant phospholipases and phosphatidic acid-mediated signaling in stress responses

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Phospholipases produce various lipid messengers, such as phosphatidic acid (PA), lysophospholipids (lysoPLs), free fatty acids, and diacylglycerol (DAG). The activation of phospholipases often occupies a critical and early step in a specific signaling cascade. Phospholipases are grouped into four major classes, phospholipase D (PLD), phospholipase C (PLC), phospholipase A_2 (PLA_2), and phospholipase A_1 (PLA_1), according to the site of hydrolysis of glycerophospholipids. Within each class, the enzymes can be further divided into families or subfamilies accordingly to their sequence similarities, biochemical properties, and biological functions. Distinct differences exist in the occurrence and functions of various phospholipases between plants and animals. PLD constitutes a major phospholipase family in plants. Plants contain more genes and types of PLDs than animals do, and different PLDs and their derived PA are involved in a wide range of signaling processes in plants. Each of the dozen Arabidopsis PLDs characterized displays distinguishable properties in activity regulation and/or lipid preferences. The molecular and biochemical heterogeneities of the plant PLDs play important roles in the timing, location, and amount of PA produced. PLD-catalyzed production of PA has been shown to play important roles in plant growth, development, and response to various stresses, including drought, salinity, freezing, and nutrient deficiency. PLD and PA affect cellular processes through different modes of action, including direct target protein binding and biophysical effects on cell membranes. A better understanding of the mechanism by which specific phospholipases and lipid messengers mediate given plant responses will provide insights into the molecular processes that connect the stimulus perception on membranes to intracellular actions and physiological responses.

IL 5.4–3
Regulation of epidermal cell-pattern by secretory peptides

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Regulation of the number and placement of cells is critical for proper development of multicellular organisms. During development of plant leaf epidermis, a protodermal cell first makes a fate decision of whether or not to be the meristemoid mother cell (MMC), which undergo asymmetric cell division forming a meristemoid and its sister cell. A meristemoid eventually differentiates into guard cells of a stoma, and the sister cell either undergoes additional asymmetric cell division or differentiate into a pavement cell. We found two closely related secretory peptides that regulate epidermal cell placement or number. EPF1 is expressed in the meristemoids, guard mother cells and guard cells. It regulates the plane of asymmetric cell division in a cell neighboring to a cell that expresses EPF1. This in turn results in proper placement of epidermal cells. EPF2 is expressed in MMCs and meristemoids, and negatively regulates successive formation of MMC. This feedback-regulation limits epidermal cell-density. The coding sequences are responsible for the difference in the functions of EPF1 and EPF2.

IL 5.4–4
Regulation of cellular polarity

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Development in multicellular organisms depends on the ability of individual cells to transport small molecules in a polar fashion. In plants, polar auxin transport and distribution are required for almost every aspect of plant development. The Rho associated small GTPases ROPs are master regulators of cell polarity. In my talk I will discuss some the mechanisms of ROP membrane targeting and partitioning and the involvement of a ROP associated protein in the regulation of cell polarity and polar auxin transport.

IL 5.4–5
Cellular mechanism of polar auxin transport

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Cell-to-cell polar auxin transport plays a key role in control of spatial and temporal aspects of plant growth and development. Auxin polar transport machinery consists of a balanced system of passive diffusion into cells of non-dissociated auxin molecules, as well as activities of auxin importers and exporters. For physical–chemical reasons, it is the activity and subcellular localization of auxin exporters (efflux carriers) which is crucial for both quantity and direction of auxin flow, respectively. At present, in Arabidopsis there are two main candidates for auxin exporters – plant-specific PIN-FORMED proteins (PINs) and multidrug-resistance-like P-glycoprotein transporters (MDR/PGPs/ABCB), and one main candidate family for auxin importers (influx carriers) – permease-like AUX1/LAX proteins. We have transformed tobacco cells of BY-2 line (*Nicotiana tabacum* L., cv. Bright-Yellow 2) with various genes of *PIN*-, *MDR/PGP/ABCB*- and *AUX1*-type. After their overexpression, we have characterized

concomitant changes in cell morphology and development together with biochemical characteristics of intracellular auxin accumulation. Detailed kinetic characterization of auxin transport using various auxins and their structurally-related analogues, selected on the basis of their ability to induce the auxin-responsive reporter DR5rev::GFP in the roots of Arabidopsis seedlings, revealed that the auxin transport machinery is specific to those compounds which were able to induce DR5rev::GFP reporter and showed other auxin-like properties (as e.g. growth-regulatory activity in suspension-cultured tobacco cells, inhibition of endocytosis, etc.). On the basis of quantitative data measured by the auxin-accumulation assay, we have constructed a mathematical model of auxin flow through a single cell; this model has a potential to predict auxin concentrations in cell compartments and it can complement the already existing models of auxin flow on the tissue and organ levels.

Acknowledgement: This work was supported by the Ministry of Education, Youth and Sports of the Czech Republic, project LC06034.

OP 5.4–1
Exo84 subunit of exocyst tethering complex in *Arabidopsis*

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The exocyst is an octameric protein complex crucial for polarized secretion in yeast and animal cells. It is an effector of Rho and Rab GTPases, tethers vesicles to their target membranes and precedes the SNARE module assembly. Recently it wash shown that the exocyst functions as a complex in plant cells, however, the question whether the Exo84 subunit (critical for yeast and *Drosophila* exocyst) is part of the complex was not completely answered in this study. The Exo84 family is present in Arabidopsis; we revealed structural similarity of the Exo84 proteins between plant and Opisthokonta using bioinformatics approach. We localised subunits of the exocyst complex using GFP fusions; the Exo84 localises to membrane associated spots which partially co-localise with microtubules and Golgi apparatus. We present a phylogenetic and genetic analysis of the Arabidopsis Exo84 family. *Arabidopsis* encodes three Exo84 homologues. Public microarray data implicate distinct functions of these homologues in plant development. Using T-DNA insertion mutants we show that all members of the Exo84 family are fundamental for Arabidopsis development. Moreover, concluding from the severity of the insertion phenotypes, we propose that some of the T-DNA mutant alleles of the Exo84b subunit might interfere with proper exocyst assembly/action. These might prove useful in future work as the precise mode of action of the exocyst complex and the inter-exocyst subunit relations are not completely understood.

Acknowledgement: Grant support – V.Z. MSMT Kontakt ME841, MSM LC06034 "EMOROST", MSM LC06004 and MSM0021620858. J. F. was supported by the US National Science Foundation (#IBN-0420226).

5.5. Small GTPases in Cell Function and Disease

IL 5.5–1
Spatio-temporal control of small G protein function during phagocytosis

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Phagocytic uptake is a highly ordered and stringently controlled process that allows the internalization of particles over 300 nm in diameter (e.g. cell debris, microorganisms and apoptotic cells). Driven by the local three-dimensional re-organisation of the actin cytoskeleton and plasma membrane, it is orchestrated by small GTP-binding proteins. The best-studied phagocytic receptors are the Fcγ receptor and Complement receptor 3 (aka CR3, Mac-1, αMβ2 integrin). Phagocytosis through these two types of receptors differ in terms of specific subsets of G proteins involved, as shown in the past using dominant negative and/or bacterial toxins, and ultrastructures formed. We have recently used RNA interference and cells derived from knock-out animals to obtain a better understanding of the specific Rho proteins involved during phagocytosis and clarify their role in local actin polymerization. How the function of Rho proteins at the membrane of phagocytic cups is regulated in space and time, and how this relates to other actin-dependent cellular processes will be the focus of the presentation.

IL 5.5–2
Effects of Rho-deamidating and Rho-gluosylating bacterial protein toxins on G2-M transition and cytokinesis

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The permanent renewal of epithelial surfaces is one of the host defence mechanisms to limit bacterial colonization within the mammalian host organism. Two microbes that colonize epithelial surfaces are *Clostridium difficile* (colon) and uropathogenic strain of *E. coli* (UPEC, urothelium). Both microbes produce protein toxins that target low molecular weight GTP-binding protein of the Rho subfamily. The *C. difficile* toxins Toxin A (TcdA) and Toxin B (TcdB) mono-glucosylate and thereby inactivate RhoA, Rac1, and Cdc42. Cytotoxic necrotizing factors (CNF) deamidate and thereby constitutively activate Rho proteins. Recent reports by several labs provide evidence on the role of Rho proteins in G2M transition and cytokinesis. We here analyze the toxins' effects on G2-M transition and cytokinesis in Hela cells synchronized using the thymidine double block technique. We show that either activation of Rho proteins by *E. coli* CNF1 or inactivation of Rho proteins by TcdB results in a comparable outcome i.e. delayed G2-M transition and inhibited cytokinesis. Modulation of Rho activity by the toxins thus limits proliferation of epithelial cells. This leads to the model that toxins inhibit the renewal of epithelial surfaces, in order to favor the colonization of the producing microbe.

IL 5.5–3
Roles of Rho GTPases in cell adhesion and migration

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Rho GTPases are found in all eukaryotes and regulate actin and microtubule dynamics, thereby contributing to multiple cellular functions, including cell migration, vesicle trafficking, and transcriptional regulation. We are investigating the roles of the 20 different mammalian Rho family members and their interacting partners in leukocyte adhesion to and migration across endothelial cells, and in cancer cell migration and cell–cell adhesion. For example, we have shown that Rac1 and Rac2 each play distinct roles in regulating macrophage shape, cytoskeletal organisation and invasion, but that Rac proteins are not essential for macrophage migration. In addition, we are studying the regulation and function of Rnd proteins, which are less well-characterised members of the Rho family and are unusual in that they do not hydrolyse GTP. Instead, we have found that Rnd3/RhoE is regulted by expression levels, phosphorylation and localisation. The mechanisms underlying the roles of different Rho proteins in mammalian cell migration and adhesion will be discussed.

IL 5.5–4
Rho GTPases and cell adhesion: upstream and downstream signaling

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Rho GTPases regulate many cell activities including cell adhesion, cell migration and the organization of the cytoskeleton. With respect to adhesion, a reciprocal relationship exists in which Rho proteins regulate the assembly and disassembly of cell-matrix and cell–cell adhesions, but in which engagement of many cell adhesion molecules also regulates the activities of Rho proteins. We have studied how integrin-mediated adhesion regulates RhoA activity. Initially, integrin engagement activates Src leading to the phosphorylation and activation of p190RhoGAP. This depresses RhoA activity, allowing cells to spread. Over time, integrin engagement stimulates RhoA activity, which promotes the generation of traction forces on the underlying matrix. Pursuing how integrin engagement stimulates RhoA activity, we have found that integrin engagement activates the guanine nucleotide exchange factors Lsc and LARG. With cell–cell adhesion we are studying how Rac1 regulates the integrity and permeability of endothelial junctions. Surprisingly, Rac1 has been found to both increase and decrease endothelial permeability depending on the source of stimulation. With sphingosine-1-phosphate, many groups have shown that activation of Rac1 leads to decreased endothelial permeability and enhanced barrier function, whereas with VEGF the activation of Rac1 increases permeability and decreases barrier function. Examining the basis for VEGF-induced permeability, we have found that, downstream from VEGF, Rac1 stimulates NADPH oxidase to generate reactive oxygen species (ROS). In turn, ROS elevate the tyrosine phosphorylation of junctional proteins, decreasing junctional stability and increasing permeability.

OP 5.5–1
Activation of endogenous RhoG by epidermal growth factor (EGF)

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RhoG belongs to the Rac-like subgroup of Rho GTPases and is involved in diverse cellular functions, like macropinocytosis, caveolar endocytosis, neurite outgrowth and cell survival. The current understanding of the upstream regulation of RhoG is limited, as only a few guanine-nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs) for RhoG have yet been described. Furthermore, stimuli like serum treatment or integrin engagement which are known to activate other Rho GTPases were described not to influence RhoG activity. Here we show that stimulation of epithelial cells with epidermal growth factor (EGF) induces a strong and rapid increase of the endogenous level of active RhoG (RhoG.GTP). The kinetics of RhoG activation after EGF treatment parallel the previously described activation of Rac1 upon EGF treatment. Interestingly, activation of RhoG by EGF appears to be specific to this growth factor, as we did not observe RhoG activation after VEGF-treatment of endothelial cells or PDGF-treatment of fibroblasts, even though Rac1 was activated by these growth factors. Further, we found that the activation of RhoG and Rac1 do not depend on each other, as each GTPase is still activated by EGF when the other one is knocked-down by RNA interference. Using a set of siRNAs which target GEFs known to activate RhoG or appear likely to have exchange specificity for RhoG, we found that the activation of RhoG by EGF is complex and regulated by multiple GEFs. Our current research focuses on understanding the underlying signaling pathways and on characterizing biological functions regulated by EGF induced RhoG activity.

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5.6. Signaling in Host-Pathogen Interactions

IL 5.6–1
Calcium signaling and Shigella invasion of epithelial cells

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Shigella, the causative agent of bacillar dysentery, induces its internalization into epithelial cells by locally reorganizing the actin cytoskeleton. Bacterial invasion is determined by a T3SS. Upon cell contact, two T3S substrates the IpaB and IpaC proteins assemble into a pore-forming translocator in host cell membranes that allows the injection of bacterial effectors inside host cells. Initial bacterial signaling has been linked to the carboxyterminal domain of IpaC and activation of Src family kinases (Mounier *et al*., 2009), but the precise sequence of signaling events leading to actin polymerization is still unclear. During invasion, *Shigella* induces transient increases in global intracellular Ca^{2+} concentration in a T3SS-dependent manner, that are not required for bacterial invasion (Tran Van Nhieu *et al*., 2003). This has been recently confirmed in unpublished collaborative work from the proponents of this proposal, showing that cell treatment with thapsgargin, an inhibitor of the ATPase involved in the replenishing of intracellular Ca^{2+} pools, in the absence of extracellular Ca^{2+} abrogate bacterial-induced variations in global intracellular concentration, but does not inhibit *Shigella* invasion. Interestingly, however, preliminary results indicate that *Shigella* invasion induces a redistribution of the inositol 1,4,5-trisphosphate (IP3) receptor (IP3R). Furthermore, expression of an IP3-5-phosphatase that prevents variations in IP3 levels or siRNA knocking out of IP3Rs resulted in inhibition of *Shigella*-induced actin reorganization during invasion. Because IP3-signaling is required for local Ca^{2+} increase, these results suggest that local but not global Ca^{2+} increases regulate cytoskeletal reorganization during bacterial invasion.

IL 5.6–2
Role of α-hemolysin induced Ca^{2+} signaling in the progression of uropathogenic *E. coli* infection within the urinary tract

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The study of bacteria-host interaction is the key to understanding the complexity of microbial pathogenesis. By combining the power of multiphoton-based live animal imaging with the precision of micro-puncture, we have developed a system that allows us to visually track a progressing infection from the first interactions between host and pathogen. The live model allows for study in the most clinically relevant setting with the full complement of integrating factors accounted for. Using GFP^{+} expressing clinical uropathogenic *E. coli* (UPEC) we have followed the progressing pathophysiology of pyelonephritis, in high resolution, within a single live animal. A combination of the intravital imaging with complementary physiological and molecular techniques has revealed a previously undescribed innate vascular response to mucosal infection. The extremely rapid host response to mucosal infection was highlighted by the triggering of a cascade of events within 3–4 hours of infection. Epithelial signalling produced an increase in cellular O_2 consumption and affected microvascular flow via the induction of clotting, causing localized ischemia. Subsequent ischemic damage dramatically affected pathophysiology. Infection with an isogenic mutant strain lacking expression of the exo-toxin α-hemolysin (Hly) displayed a dramatic delay in the onset of vascular dysfunction. Hly is known to induce Ca^{2+} oscillations in primary renal proximal tubule cells, the same cells they come into contact with in our *in vivo* model. These oscillations were shown to induce inflammatory signalling, a known factor in the initiation of the clotting cascade. These results give an *in vivo* role to *in vitro* findings, demonstrating down-stream effects of this signalling which are only observable in this living model. Suppression of this clotting response by heparin treatment resulted in fatal urosepsis. This work describes a rapid vascular response as a new factor in the early innate response to bacteria–host interaction in the kidney. Bacterial virulence factors, such as Hly, are shown to influence the kinetics of this response *in vivo*.

IL 5.6–3
Cellular regulation of calcium; a life and death messenger

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Calcium is a prominent cellular messenger that has roles in processes as diverse as fertilisation, gene transcription, metabolism and cell death. Cellular calcium signals vary in their spatial properties, kinetics, amplitude and frequency. No two cell types display exactly similar calcium signals. Rather, cells employ components from a substantial tool kit of possible calcium signalling elements. By expressing various components from the tool kit, cells can generate calcium signals with characteristics that fit the cells' physiological functions. Although specific, the calcium signalling proteome employed by a single cell type is not generally invariant. Depending on prevailing conditions, forms of stimulation or stress, the calcium signalling proteome within cells can be remodelled, leading to calcium signals with distinctive properties. Furthermore, although calcium signalling proteomes are designed to fit the physiological functions of a particular cell type, they can be hijacked by pathological processes and thereby promote deleterious outcomes. A well-known example of remodelling of a calcium signalling proteome occurs during cardiac hypertrophy and heart failure. Cardiac myocytes are post-mitotic, so the contractile portion of the heart cannot grow through cell division. Rather, when the body is subject to increased haemodynamic demand, the cardiac myocytes enlarge (hypertrophy) and express proteins not found in control conditions. Depending on the stimulus causing hypertrophy, this change in myocyte function can be beneficial (more blood pumping) and reversible. However, with prolonged haemodynamic demand, or with other triggers, hypertrophy can take adopt an irreversible pathological form that culminates in heart failure.

IL 5.6–4
Calcium signalling and viral infections

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Viruses need cell machinery for their multiplication and "survival". We first investigated the role of calcium signalling in the biological effects of two viral proteins involved in liver carcinogenesis. HBV X expression was found to be associated with elevation of cytosolic calcium, and decreased calcium in mitochondria, while ER calcium remains unchanged. HBV X-induced elevation of cytosolic calcium follows HBV X-induced caspase 3 mediated cleavage of PMCA (Plasma Membrane Calcium ATPase), decreasing its calcium pumping activity. This mechanism modulates the level of cytosolic calcium potentially leading to apoptotic cell death. We then showed that HCV core expression determines ER stress which, in turn, induces ER calcium depletion, caspases and Bax activation, and apoptotic cell death. Thus, two viral proteins involved in liver carcinogenesis were shown to deregulate the control of cell viability by modifying calcium signalling. We have then tested the hypothesis that modulation of subcellular calcium concentrations by specific drugs can have an impact on HCV protein expression and HCV replication. We studied the Huh7 cell line stably expressing the full length HCV genome and a more recent system to generate infectious virus particles in cell culture by modulating intracellular calcium concentrations with CsA (3 μM during 4 h) and DEBIO-025 (0.3 μM). The amount of viral RNA was evaluated by real-time quantitative RT-PCR (GAPDH normalization). Aequorin measurements confirmed that the virus, independently from all treatment, disturbs intracellular calcium concentrations. Pharmacological modulation of calcium homeostasis was shown to affect HCV replication showing an impact of intracellular calcium modulation on viral multiplication.

OP 5.6–1
Dissecting signal transduction pathways on host–*Plasmodium* interactions

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During evolution, parasites have adapted to subvert the host endocrine regulation to coordinate its cell cycle and proliferation. We want to understand how *Plasmodium* signalling pathways interact with one another to put in action a response during its complex life cycle. We have proposed a novel mechanism that allows the intracellular protozoan *Plasmodium* to use host melatonin and its derivatives to synchronize intraerythrocytic malaria parasites in a Ca^{2+}-dependent manner (Hotta *et al*, 2000; Beraldo and Garcia 2005). We have investigated the function of a RACK orthologue in *Plasmodium*. In mammalian cells RACK is responsible for stabilizing PKC in its active form as well as to bind to IP3 receptors and modulate calcium release. We reported previously that *Plasmodium falciparum* expresses an ortholog of RACK1, named PfRACK (Madeira *et al*, 2003). This protein is constitutively expressed along the intraerythrocytic cyle, and can be considered a target for antimalarial drug development. We have used codon-optimized form of the PfRACK gene to express this protein in the HEK 293 and PC12 mammalian cell lines and primary hepatocytes. Endogenous RACK1 expression was also knocked down. By time-lapse confocal microscopy, we observed that ATP (100 μM)-induced Ca^{2+} signals were reduced by 83.3 + 3.8% in HEK 293 cells expressing PfRACK and by 89.6 + 7.8% in cells expressing PfRACK and treated with siRNA for mammalian RACK1. We also performed experiments using a permeable caged InsP3. Upon 2-photon encaging, the InsP3 failed to increase Calcium in cells expressing PfRACK. Confocal immunolocalization showed partial co-localization of PfRACK with its mammalian ortholog.

5.7. Plasma Membrane and Cell Signaling

IL 5.7–1
Lipid rafts and membrane organization

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The lipid raft concept introduces into membrane organization the capability of dynamic sub-compartmentalization. Our present concept of lipid rafts is that they are dynamic nano assemblies of sphingolipids, cholesterol and proteins that dissociate and associate on a rapid timescale. These assemblies can be induced to coalesce to form raft clusters and these are the platforms that function in membrane trafficking, cell polarization and signaling. Amazingly, the plasma membrane can also be induced to separate into two phases of micrometer size in which the raft phase includes transmembrane proteins and excludes the transferrin receptor. A difficult issue in understanding membrane structure and function is how membrane proteins interact with lipids. With respect to understanding how proteins associate with rafts it is fairly straight-forward to understand that proteins with attached saturated acyl chains such as GPI-anchored proteins can be associated with liquid-ordered raft assemblies. Transmembrane proteins, however, do pose a problem. When they cross the bilayer the transmembrane domains should disrupt the packing of the liquid-ordered domain. The EGF receptor is one of the best studied receptors and is involved in membrane signaling and cancer. The EGF receptor has been shown to associate with rafts and this association modulates its activity. Moreover, gangliosides seem to modulate the receptor activity. We have now directly shown that the purified EGF receptor reconstituted into proteoliposomes is inhibited by the ganglioside GM3. This ganglioside abolishes the autophosphorylation activity of the purified EGF receptor in proteoliposomes with a liquid-ordered lipid compostion. Our working model is that binding of raft lipids make transmembrane proteins raftophilic.

IL 5.7–2
Plasma membrane signalosomes – structure-function relationships

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The earliest known biochemical event that occurs after ligand binding to the multichain immune recognition receptors (MIRRs) is tyrosine phosphorylation of the receptors and formation of signaling units – signalosomes. In mast cells and basophils activated via the high-affinity IgE receptor (FcεRI), this step is mediated by Src family protein tyrosine kinase (PTK) Lyn. The mechanism of this step has been extensively studied; consequently, three major models are considered. (i) The transphosphorylation model is based on observations that small fraction of Lyn kinase is weakly, albeit constitutively bound to FcεRI. When FcεRI is aggregated, Lyn kinase bound to one receptor can phosphorylate the immunoreceptor tyrosine-based activation motifs (ITAMs) on an adjacent receptor and initiate thus the signaling pathway. (ii) An alternative model suggests that Lyn is not pre-associated with the FcεRI but instead is separated from it into membrane microdomains called membrane rafts; this prevents FcεRI phosphorylation. After activation, the aggregated FcεRI associates with membrane rafts, and only this fraction of the FcεRI is tyrosine phosphorylated. This model is supported by experiments in which FcεRI in activated cells exhibited enhanced association with detergent-resistant membranes (DRMs). (iii) The third model postulates that FcεRI triggering is regulated by an interplay between PTKs and protein tyrosine phosphatases (PTPs). Cell activation initiates a local increase in reactive oxygen species (ROS) which react with cysteine in the active site of PTPs; in this way the enzymatic activity of PTPs is inhibited. This leads to a local change in the equilibrium between PTKs and PTPs, and enhances phosphorylation of the receptor. This model is supported by experiments indicating that ROS induces rapid phosphorylation of the receptor in the absence of its aggregation, as determined by electron microscopy on isolated membrane sheets. Importantly, ROS-induced phosphorylation of the receptor is observed even in the absence of its association with DRMs, suggesting the presence of preformed receptor/PTK/PTP complexes. A variation of this model presumes decreased access of PTPs, but not PTKs, to the ITAMs as a result of activation-induced conformational changes of the receptor. Our results based on analysis of tyrosine phosphorylation of the FcεRI in antigen- or pervanadate-activated cells support the third model.

Acknowledgement: Supported by project 301/09/1826.

IL 5.7–3
Lateral compartments in the yeast plasma membrane: how are they formed and what are they good for?

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The plasma membrane of *S. cerevisiae* is subdivided into at least two stable lateral compartments. One consists of 50–80 patches of about 300 nm in diameter and is called MCC (Membrane Compartment of Can1, the arginine permease). The other one, MCP (Membrane Compartment of Pma1, the H+-ATPase) fills the space in between the MCC patches (1,2). Nine membrane proteins and 12 cytosolic ones are localized within or in close vicinity to the patches; the latter holds also for the so-called 'eisosome' (3,4). MCC is enriched in ergosterol; the localization of several MCC proteins is affected by the membrane potential (5). Besides lipid biosynthetic gene products, we identified two proteins (Nce102 and Pil1) in a genome-wide screen (3), which are essential for MCC pattern formation. Punctuate dynamic components of the endocytotic machinery, never coincide with the MCC pattern. We show that MCC protects its residents from turnover/degradation (3). Before internalization, the MCC proteins have to leave the area of the patch. Investigations of the plasma membrane by various electron microscopic approaches showed that the MCC patches coincide with short rod-shaped invaginations, originally described by Moor and Mühlethaler in 1963 (6), when freeze fracturing was invented. These invaginations had remained mysterious membrane differentiations until today.

References:

1. Malinska *et al. Mol Biol Cell* 2003; **14:** 4427.
2. Malinska *et al. J Cell Sci* 2004; **117:** 6031.
3. Grossmann *et al. J Cell Biol* 2008; **183:** 1075.
4. Walther *et al. Nature* 2006; **439:** 998.
5. Grossmann *et al. EMBO J* 2007; **26**: 1.
6. Moor and Mühlethaler. *J Cell Biol* 1963; **17:** 609.

IL 5.7–5
Dynamics of Ras and Src membrane interactions in live cells and their role in signaling

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We studied the role of the dynamic interactions with the plasma membrane in signaling by two oncogenic proteins, Ras and Src. To investigate the role of the polybasic cluster in K-Ras4B (K-Ras), we combined live cell microscopy and FRAP beam-size analysis. We show that the cationic amphiphilic drug chlorpromazine (CPZ) modulates the membrane association of oncogenic GFP-K-Ras(G12V) (but not H-Ras) by enhancing its exchange with the cytoplasm. This increases K-Ras(G12V) in the cytoplasm and internal membranes. The cellular response to this dislodgement is cell-type specific and depends on the internal membranes to which K-Ras(G12V) is targeted; targeting to endosomes arrests the cell cycle, while targeting to mitochondria induces apoptosis. Thus, by disruption of charge-based interactions, CPZ alters K-Ras localization, leading to cell-specific effects on its signaling outcome. To investigate the roles of different elements (kinase, SH2, SH3 domains) in Src membrane interactions, we employed FRAP beam-size analysis on a series of c-Src-GFP proteins with mutations in specific domains, together with assays that determine the ability of the mutants to generate and bind to phosphotyrosyl-proteins. We show that wild-type Src displays lipid-like membrane association, while constitutively active Src-Y527F interacts transiently with membrane-associated proteins. These interactions require Src kinase activity and SH2 binding, but not SH3 binding. Overexpression of paxillin, a largely cytoplasmic Src substrate, competes with membrane phosphotyrosyl-protein targets for binding activated Src. We show that the interactions of Src with lipid and protein targets are dynamic, and that Src kinase and SH2 domain cooperate in the membrane targeting of Src.

Symposium 6: Organism – The Network of Interactions

6.1. Mitochondrial Membranes, Apoptosis and Cancer

IL 6.1–1
Model membranes as valuable tools for mechanistic studies of BCL-2 protein function

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BCL-2 family proteins are the main regulators of the intrinsic pathway of apoptosis, which primarily function by modulating the mitochondrial outer membrane permeabilization (MOMP) process triggering release into the cytoplasm of apoptogenic factors such as cytochrome c. Core components of the BCL-2 family are proapoptotic BAX-type proteins which directly cause MOMP, and antiapoptotic BCL-2-type proteins which inhibit MOMP. Numerous factors that interact with and regulate core BCL-2 family proteins have been identified, among which members of a third BCL-2 subgroup (BH3-only proteins), components of the mitochondrial morphogenesis machinery, and mitochondrial lipids stand out. However, the inherent complexity of the BCL-2-interacting network constitutes a major hurdle to elucidate the exact mode of action of individual BCL-2 regulatory factors in studies with intact cells. Fortunately, several groups, including ours, have shown that basic aspects of the BCL-2-regulated MOMP process can be reconstituted *in vitro*, using purified recombinant proteins and compositionally-defined model membranes. Here, we will summarize the information gathered in our laboratory from studies using these simplified systems, concerning how the function of core BCL-2 family proteins is modulated by specific regulatory factors (proteins and lipids). Additionally, insights on the mechanism of action of promising anti-cancer agents targeting the BCL-2 system will be discussed.

IL 6.1–2
Various modes of cell death induced by DNA damage

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In dividing cells DNA damage caused by genotoxic insults results in the activation of cell cycle checkpoints followed by DNA repair. p53, being a 'guardian of genome' is activated by stress and, depending on the severity of damage, it triggers either G1 or G2 arrest or cell death. In addition to several known p53 targets, such as Bax and PUMA, PIDD appears to be a crucial target gene that leads to either activation of NFκB-dependent cell survival, or apoptosis. The apoptotic pathway includes caspase-2, which is activated within the PIDDosome complex, and causes cytochrome c release and caspase activation. Hence, PIDDosome-mediated caspase-2 activation might be an important link between DNA damage and the engagement of the mitochondria-mediated apoptotic pathway. In addition to PIDDosome, caspase-2 is able to use the CD95 DISC as an activation platform and the recruitment of caspase-8 to this complex is required for efficient activation of both enzymes. We prove that caspase-2 and caspase-8 act upstream of cytochrome c release and that they contribute to cleavage of proapoptotic BH3-only Bcl-2 family member Bid. In the experimental system used, the DISC is aggregated through a distinct and p53-dependent upregulation of CD95. Finally, we provide evidence for a direct interaction between these two enzymes and show that caspase-8 is able to mediate proteolytical cleavage of caspase-2. The observed functional link between caspase-8 and -2 within DISC complex might be a second mechanism for caspase-2 activation in response to DNA damage. Investigation of the contribution of p53 and caspase-2 to apoptotic cell death and mitotic catastrophe (MC) induced by DNA damage in carcinoma cells revealed that both functional p53 and caspase-2 are required for the apoptotic response, which was preceded by translocation of caspase-2 to the cytoplasm. In the absence of functional p53, DNA damage resulted in caspase-2-independent MC followed by necrosis. In these cells apoptotic functions could be restored by transient expression of wt-p53. Hence, in this experimental model p53 appeared to act as a switch between apoptosis and MC followed by necrosis-like lysis.

IL 6.1–3
Mitochondrial fission-fusion and apoptosis

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Bax and Bak promote apoptosis by perturbing the permeability of the mitochondrial outer membrane and facilitating the release of cytochrome c by a mechanism that is still poorly defined. During apoptosis, Bax and Bak also promote fragmentation of the mitochondrial network, possibly by interfering with the mitochondrial fusion machinery. It has been proposed that Bax/Bak-induced mitochondrial fission may be required for release of cytochrome c from the mitochondrial intermembrane space. However, we have found that Bcl-xL, as well as other members of the apoptosis-inhibitory subset of the Bcl-2 family, can antagonize Bax and/or Bak-induced cytochrome c release but fail to block mitochondrial fragmentation associated with activation of these proteins. These data argue that Bax/Bak-induced fragmentation of mitochondrial networks and cytochrome c release are distinct and separable events. Our observations suggest that the Bcl-2 family perform an ancillary function as regulators of mitochondrial fission and fusion events, independent of their role in apoptosis.

IL 6.1–4
Beyond fusion: multiple roles for mitochondria-shaping proteins

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Mitochondria are complex organelles whose internal structure and cytosolic organization is controlled by a growing number of 'mitochondria-shaping' proteins. These include mitochondrial proteins like Mitofusin (Mfn) 1 and 2, Optic Atrophy 1 (Opa1); as well as cytosolic ones like the dynamin-related protein 1 (Drp1) and its receptor on the outer mitochondrial membrane Fis1. These proteins influence not only the shape of mitochondria, but also the function of the organelle and integrated cellular signalling cascades, including apoptosis. We undertook a genetic approach to elucidate function and regulation of these proteins.

Our data indicate that Opa1 and Parl, an inner membrane rhomboid protease, regulate the cristae remodelling pathway of apoptosis. Similarly, excess fission promoted by Fis1 results in alteration in the shape of the cristae, mitochondrial dysfunction and ultimately death. Surprisingly, apoptosis depends on adequate levels of Ca^{2+} in the endoplasmic reticulum (ER), strengthening the interaction between these two organelles in controlling signalling cascades like apoptosis. The role of mitochondria-shaping proteins in the cross-talk between mitochondria and ER is further substantiated by the finding that Mitofusin 2 regulates the shape of the ER and more importantly, the physical and functional connection between ER and mitochondria.

OP 6.1–1
Signaling pathways activated by photodynamic inducers of apoptosis in cancer cells

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Oxidative stress, such as photodynamic therapy (PDT), can act as an apoptosis inducer. In PDT, light-absorbing molecules (photosensitizers), which are relatively innocuous in the absence of light, upon illumination become activated and pass on their energy to molecular oxygen, causing localized oxidative damage leading to cell death. In the present study we investigated apoptotic pathways activated by various ethylene glycol porphyrin derivatives depending on their intracellular localization. Some derivatives, although primarily localized in lysosomes, induced mitochondria-driven apoptosis. The induction of apoptosis was accompanied by immediate and sustained activation of p38 mitogen-activated protein kinase (MAPK), connected with formation of reactive oxygen species. Moreover, inhibition of p38 MAPK by specific inhibitors and by the p38a dominant-negative mutant as well as deletion of the p38a gene (MEFs-KO) protected cells from apoptosis, thus suggesting direct involvement of the p38 kinase pathway in the initiation of this process. The other porphyrin derivatives were found to be localized in endoplasmic reticulum (ER) and the impact of ER stress induced by photodynamic treatment of cancer cells was investigated. *In vitro* study has been supplemented by *in vivo* results demonstrating high potential of these compounds in anticancer treatment.

Acknowledgements: This work was supported by grants of the Ministry of Education of the Czech Republic (LC06077), Grant Agency of the Czech Republic (203/09/1311) and Grant Agency of the Academy of Sciences of the Czech Republic (KAN200200651).

6.2. Virus–Cell Interactions

IL 6.2–1

Intracellular trafficking of retroviral proteins

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The mechanism by which retroviral Gag molecules are transported through the cytoplasm to the site of budding remains a poorly understood process in retrovirus assembly. Previous studies have shown that for Mason–Pfizer Monkey Virus (M-PMV), a *betaretrovirus*, immature capsids are assembled at the pericentriolar region of the cell and that trafficking to the budding site at the plasma membrane is dependent on an active vesicular transport system. Targeting of assembly to the pericentriolar region is dependent on a short N-terminal 18 amino acid sequence, the Cytoplasmic Targeting and Retention Signal, in Gag that binds to the TcTex-1 light chain of the microtubule dynein motor. In this way translating polysomes are concentrated in the centriolar region of the cell. A single amino acid substitution, R55Y, in Gag induces a major conformational change in the protein so that the CTRS is sequestered and TcTex-1 binding does not occur. This mutant now assembles immature capsids at the plasma membrane (PM). In order to further identify the role of cellular components in the transport of capsids from the cytoplasmic site of assembly to the plasma membrane, we have constructed a Gag–GFP fusion and have utilized live-cell imaging to further investigate mutations within *gag* known to alter intracellular transport of the precursor or immature capsids. The results of these experiments show capsids being formed at the pericentriolar site, as well as egress of these capsids from this site toward the plasma membrane. Preliminary analyses of particle movement are consistent with a role for microtubules in this process, although a switch to actin filaments appears to be necessary to reach the PM. Budding of M-PMV from the cell requires wrapping of the immature capsid with the PM. This process involves the host ESCRT machinery and phosphoinositol-triggered exposure of an N-terminal myristate from each Gag molecule followed its insertion into the lipid bilayer. Mutations that inhibit myristate exposure block budding. Thus, while retroviruses usurp key trafficking pathways within their host to efficiently target and assemble viral components, their study has uncovered novel processes that previously were unrecognized.

IL 6.2–2

TRIM5 and innate immunity to HIV-1

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TRIM5, a member of the tripartite motif protein family, is a host restriction factor that mediates a capsid-specific block to retroviral infection. Given that TRIM5 is located within a cluster of interferon-stimulated genes, we sought evidence for functional links between TRIM5-mediated restriction and innate immunity to HIV-1. We found that TRIM5 is induced by Type I interferons and PRR agonists in THP-1 cells and monocyte-derived dendritic cells and macrophages. The induction kinetics indicated that TRIM5 induction by PRR agonists is dependent on type I interferon signaling. Indeed, disruption of the interferon alpha receptor-ISGF3 axis ablated TRIM5 induction by PRR agonists. Furthermore, knock-down of IRF3 abolished the induction of TRIM5 by PRR agonists but not by Type 1 interferons. As TRIM5 mRNA was induced by type I interferons and PRR agonists, we reasoned that reducing the level of TRIM5 expression would rescue HIV-1 from the inhibitory effect of the Type I interferon response. Indeed, silencing of TRIM5 attenuated the inhibitory effect of Type I interferons and PRR agonists on HIV-1 transduction, independent of capsid-specificity. These experiments have been extended further to show that TRIM5 has capsid-independent effects on signaling pathways that lead to the antiviral state.

IL 6.2–3

HIV-1 assembly and its inhibition

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HIV-1 assembly is directed by the viral Gag polyprotein which assembles into an immature shell at the plasma membrane concomitant with initiation of the budding process. Completion of budding and virus release also involves the cellular ESCRT machinery. During or after budding, proteolytic cleavage of Gag polyproteins by the viral protease leads to separation of individual subunits MA, CA, NC and p6 and two short spacer peptides. This triggers disassembly of the immature Gag shell and assembly of the mature cone-shaped capsid required for viral infectivity. We have analyzed the structure and mechanism of HIV-1 budding as well as the structures of immature, mature and partly matured particles providing a revised model for HIV-1 assembly and maturation. We have also developed *in vitro* assembly systems faithfully mimicking the structure of the immature and mature shell and leading to higher resolution structural models. Using purified CA protein, we have selected peptides that block assembly *in vitro* and, if modified, in tissue culture, and have analysed their mode of binding and mechanism of action. Based on these results, inhibition of immature and mature assembly may be considered a promising target for antiviral therapy against HIV-1.

IL 6.2–4

ERVWE1/Syncytin-1 – an extreme example of interaction between retroviruses and host cells

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Sequences of retroviral origin represent about 8% of the human genome. There are at least 31 families of human endogenous retroviruses (HERVs) which derived from independent infections of the germ line by exogenous retroviruses during the evolution of the human lineage. The infectious retrovirus founding the contemporary HERV-W family entered the genome of a Catarrhine ancestor 25–40 million years ago. The spread of the HERV-W

family into the genome essentially results from autonomous and non-autonomous events of intracellular retrotransposition of transcriptionally active copies. The HERV-W family contains a unique locus, termed ERVWE1, which encodes an envelope glycoprotein expressed in the placenta. This envelope, also dubbed Syncytin, exhibits fusogenic properties *in vitro* and is directly involved in primary trophoblast differentiation. In addition, the functional conservation of the ERVWE1 locus among Hominoids strongly suggest that this retroviral locus has been recruited to play a role in placental physiology. The identification of env ERVWE1-specific traits suggests mechanisms underlying the process of evolution from a retroviral genome toward a bona fide cellular gene.

IL 6.2–5
The polyoma virus middle T-antigen acts as an active growth factor receptor analogue

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Viruses frequently encode mitogenic proteins whose role is to provide suitable conditions for replication of the viral genome. One of these, the middle T-antigen (MT) of polyoma virus, is a potent oncogene that can transform established cell lines to an oncogenic phenotype. It achieves this by mimicking a growth factor receptor by binding signaling proteins, including PP2A, the src-family, ShcA, PI3K and PLCγ1. This activates the MAP kinase and Akt pathways resulting in uncontrolled proliferation. MT assembles this multi-protein complex on the inner face of cell membranes. Reaching this location is mediated by two elements in MT, the PP2A binding site and a 22 hydrophobic residue region near the C-terminus. We show that in the absence of PP2A binding, MT no longer reaches the plasma membrane, but is instead concentrated on cytoplasmic membranes near to the nucleus. Deletion of the hydrophobic region generates a MT species that does not associate with membranes, binds only PP2A, and fails to transform. Although specific sequence motifs in the hydrophobic region do not seem to be required, some mutants associate with membranes and all the binding proteins but fail to transform. Epitope tags demonstrate that the C-terminus of MT is exposed on the outside of the cell in transforming mutants, but not in defective ones. This suggests that MT must reach the external membrane of the cell to transform, and that receptor mitogenic signalling might have similar restrictions. The requirements for oncogenic signalling will be discussed, together with the other constituents of MT complexes.

6.3. Molecular Immunology

IL 6.3–1
Signaling amplification at the immunological synapse

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The immunological synapse (IS) is a dynamic structure, formed between a T cell and one or more antigen-presenting cells (APCs), characterized by lipid and protein segregation, signaling compartmentalization, and bidirectional information exchange through soluble and membrane-bound transmitters. In addition, the IS is the site where signals delivered by the T cell receptors, adhesion molecules, co-stimulatory receptors are decoded and integrated. This complex process is required to tune TCR signaling and T cell activation thresholds, allowing T cells to interpret the context of antigen presentation, recognize infectious stimuli and finally decide between activation and tolerance. We study the molecular mechanisms underlying the costimulatory strategies at the IS. Our group has been proposed that CD28, the main costimulatory molecule for naïve T lymphocytes, amplifies TCR signal by inducing membrane rafts rearrangement and recruitment into the IS. We have shown that after physiological stimulation CD28 interacts with and recruits into the IS filamin-A, that organizes TCR and CD28 signaling by controlling cytoskeletal remodelling. We have described the T cell costimulatory properties of chemokines released as soluble immuno-transmitters by APCs at the IS. The T cell chemokine receptors CCR5 and CXCR4 are recruited into the IS and enhance T cell proliferation and cytokine production. Interestingly, whereas CXCR4 is constitutively expressed in T cells, CCR5 expression is induced by antigenic or inflammatory stimuli. We have demonstrated that CXCR4-CCR5 costimulation depends on receptor heterodimers. We are analizing how LFA-1, besides its classical role in promoting adhesion, prepares and facilitates TCR signaling by controlling mitochondrial localization. It has been reported that mitochondria accumulate under the IS in response to TCR stimulation, to allow proper TCR-induced calcium influx. We found that LFA-1 induces localization of mitochondria at the IS. This process is antigen-independent and is enhanced by the presence of chemokines in the T cell environment. However, TCR triggering stabilizes mitochondria at the IS and it is important to sustain their recruitment in time.

IL 6.3–2
Activation signals for naive and class-switched memory B cells

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Distinct immunoglobulin (Ig) isotypes can be expressed as integral part of the B cell antigen receptor (BCR) or become secreted as soluble immune effectors upon terminal differentiation of activated B cells into plasma cells. Newly generated B cells harbor an IgM-BCR. Following class switch recombination antigen-experienced B cells express a BCR of the IgG, IgE or IgA class. These B cells constitute the main reservoir from which memory B cells become established. A hallmark of class-switched memory B cells is their rapid activation and differentiation into antibody-secreting plasma cells upon antigen recall even in the absence of costimulation by T helper cells. In marked contrast, naïve B cells require BCR signals plus BCR-extrinsic costimulation. However, all BCR classes utilize the canonical Ig-alpha/Ig-beta subunit for signaling via the immunoreceptor tyrosine-based activation motif (ITAM) in their cytoplasmic domains. The lecture will focus on the molecular mechanisms of BCR signal initiation and downstream signal processing in a BCR class-specific manner. I will describe the role of ITAM and non-ITAM phosphorylation events and how the resulting phosphotyrosine residues in different BCR subunits couple to the generation of the intracellular second messengers as well as nuclear responses. Here I will highlight the critical importance of enzymatically inert adaptor proteins such as SLP-65, Grb2 or Dok-3. These scaffolding proteins can exert positive and/or negative regulatory signaling functions by controlling the multi-molecular assembly of BCR 'signalosomes' and their precise subcellular localization in the absence or presence of antigen stimulation. This provides the necessary signal plasticity that allows the B cell to properly respond to antigen encounter in a developmental stage-specific manner.

IL 6.3–3
Fooling the antigen-presenting phagocyte: molecular tricks and signaling of *Bordetella* adenylate cyclase toxin

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Bordetella pertussis adenylate cyclase toxin (CyaA) binds the alphaMbeta2 integrin receptor CD11b/CD18 (Mac-1 or CR3) of myeloid phagocytic cells and delivers into the cytosol of host phagocytes its catalytic adenylate cyclase (AC) enzyme domain. This binds intracellular calmodulin and subverts cellular signaling by unregulated conversion of ATP to cAMP. Independently from translocation of the AC domain into cells, the CyaA can also permeabilize cell membranes by forming small cation-selective pores. We have recently characterized the third activity of CyaA, its capacity to promote influx of calcium ions into cells concomitantly with translocation of the AC domain across cell membrane, forming a new type of calcium entry path that is not regulated by cAMP and is insensitive to specific inhibitors of known calcium channels. An intriguing manifestation of CyaA receptor binding and AC translocation-mediated Ca^{2+} entry into cells consists in relocalization of the CD11b/CD18 receptor from the bulk of the membrane into cholesterol and sphingolipid-rich membrane regions, known as lipid rafts or lipid microdomains. From microdomains the CyaA translocates the AC into cell cytosol and produces cAMP that causes transient inactivation of the small GTPase RhoA, thus provoking massive actin cytoskeleton rearrangements and ruffling of the phagocyte membrane. Loss of oxidative burst and block of macropinocytosis and complement mediated phagocytosis follow, disabling bactericidal capacities of phagocytes. I will discuss in the lecture our current understanding of structure–function relationships and molecular mechanisms underlying these subversive activities of CyaA.

IL 6.3–4
Role of TC21 in homeostatic antigen receptor signaling

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Peripheral T and B cell pools remain remarkably constant in size throughout life thanks to strict homeostatic mechanisms to prevent antigen-independent growth, which could provoke the development of lymphomas. The T and B cell antigen receptors (TCR and BCR) transmit low-grade signals necessary for the survival and maintenance of the mature cell populations. Another important mediator of antigen receptor survival and homeostatic signals is the phosphoinositide 3-kinase (PI3K). Genetic studies in mice show that PI3Ks, especially class IA PI3Ks, participate in lymphocyte homeostasis. We show here that TC21, a small GTPase, interacts constitutively with the TCR and BCR through their immunoreceptor tyrosine-based activation motifs (ITAMs). Expression of a dominant negative TC21 mutant in T cells provokes a rapid decline in cell viability. More importantly, $TC21^{-/-}$ mice have fewer mature T and B cells, possibly as a result of deficient homeostatic proliferation and impaired survival. B cell defect in $TC21^{-/-}$ mice specially affects the marginal zone B cell population and the germinal center response to antigen. We show that TC21 is overexpressed in several lymphoid malignancies, suggesting that deregulation of TC21 activity participates in lymphomagenesis. Finally, we demonstrate that TC21 is necessary to recruit the p110delta catalytic subunit of PI3K to the TCR and BCR. Consequently, we propose TC21 directly links antigen receptors to PI3K-mediated survival pathways.

6.4. P53 New Functions, New Targets, New Controls

IL 6.4–1
Single nucleotide polymorphisms in the p53 pathway

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The p53 protein and the signal transduction pathway controlled by that protein responds to a wide variety of stress signals which can disrupt the fidelity of DNA replication and cell division. To prevent these errors or mutations the p53 pathway responds by initiating a process of cell cycle arrest, senescence or apoptosis which either permits repair of these errors or kills the clone of cells that contain these mutations. Central to this pathway are a series of proteins that respond to the stress signals and regulate the levels and activity of the p53 pathway. We have identified a number of single nucleotide polymorphisms (SNP) in those genes that regulate p53 activity and functions and these SNPs can play a role in the incidence of cancers in a population, the age of onset of cancers and the response to therapy. Some of the haplotypes containing these SNPs appear to be under positive evolutionary selection pressures in some human populations. The reason for this appears to be the role of p53 in the implantation of embryos into the uterus and the impact of some of these SNPs upon the fecundity of mice and humans. This process is mediated by the p53 regulated gene, Leukemia Inhibitory Factor or LIF, a cytokine that is essential for the implantation of embryos. SNPs in the p53 gene, the MDM-4 gene and the LIF gene regulate the efficiency of implantation of embryos in humans. It could well be that the p53 protein is also involved in the surveillance of developmental abnormalities.

IL 6.4–2
Functions of mutant p53 in promoting invasion

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In the majority of tumours normal function of p53 is abrogated either by loss of the pathways that allow activation of wild type p53 or by the expression of mutant forms of p53. Although these cancer associated mutations in p53 result in loss of wild type transcriptional activity, studies in mice and cells have shown convincingly that expression of mutant p53 is not equivalent to loss of p53, and strongly support a gain-of-function for mutant p53 in promoting metastasis and proliferation. As half of all human cancers express a mutant form of p53, we have sought to elucidate the mechanisms by which mutant p53 enhances the metastasis observed *in vivo*. We have found that expression of mutant p53 in human cell lines lacking wild type p53 can promote their ability to invade in an *in vitro* matrigel assay. In wound healing assays, these mutant p53 cells show a pronounced loss of directionality in comparison to vector control cells that move persistent into the wounded area. Loss of directionality and increased invasion has previously been associated with increased integrin-dependent signalling through the RHO-ROCK pathway. Interestingly, mutant p53 expressing cells show a marked activation of this pathway. Inhibition of alpha5/beta1 integrin-dependent signalling or ROCK activity can clearly prevent the loss of directionality and promote invasion. This signalling is also dependent on the EGF receptor, which can interact with alpha5/beta1 integrin via RCP (rab coupling protein) and facilitate invasion. Studies designed to examine whether this effect of mutant p53 results from the acquisition of novel transcriptional activities, or modulation of the transcriptional functions of the p53-relatives p63 or p73, are in progress. In summary, our results demonstrate that mutations in p53 result in increased invasiveness of cells by enhancing integrin/EGF receptor-dependent signalling towards cofillin. These findings open the possibility that blocking alpha5/beta1 integrin and/or the EGF receptor will have therapeutic benefit in mutant p53 expressing cancers.

IL 6.4–3
Identification and characterization of p53-associated 'gene signatures' involved in cellular transformation

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As it is well accepted that malignant transformation is a stepwise process, it is challenging to discover which of these steps involve the p53 protein. To that end we have established several *in-vitro* transformation models in which normal cells were transformed into cancer cells by well-controlled genetic alterations. In our experiments, we have immortalized various human primary cells of lung and prostate origin and engineered into them several defined cancer associated genetic alterations. These included inactivation of the p53 tumor suppressors by several methods, over-expression of mutant p53, over-expression of the Ras oncogene and various combinations of these modifications. As a result we have obtained transformed cells that are capable of developing into tumors in mice, suggesting that the *in-vitro* developed system represents authentic model of cancer development. To characterize the gene networks that are associated with the defined malignant steps, we have used a genome-wide approach, which permits the identification of gene signatures that are associated with the individual steps of malignant transformation. In general it seems that the clusters that we have identified and analyzed using this *in-vitro* model agree with specific steps of transformation and thus may serve as specific hallmark signatures of tumorigenesis.

IL 6.4–4
Regulation of p53 tumor suppression

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Mdm2 and Mdm4 are critical inhibitors of the p53 tumor suppressor. Loss of *Mdm2* or *Mdm4* in mice leads to embryo lethal phenotypes that are p53-dependent. We have tested the hypothesis that *Mdm2* and *Mdm4* haploinsufficiency also contributes to differences in p53 activity and in tumor outcome. While *Mdm2* and *Mdm4* heterozygous mice are normal, they show an increased p53 response with regards to ionizing radiation and a delay in tumor onset of B-cell lymphomas. Moreover, *Mdm2 Mdm4* double heterozygous mice die shortly after birth due to lack of hematopoiesis, and this phenotype is rescued by deletion of a single *p53* allele. Lastly, we have generated mice with a single nucleotide polymorphism (SNP) in *Mdm2* that mimics the human *MDM2* SNP that was recently identified. This SNP alters Mdm2 levels, p53 activity, and a tumor phenotype. These data emphasize the exquisite sensitivity of the p53 pathway to changes in *Mdm2* and *Mdm4* gene dosage.

IL 6.4–5
The p53 mRNA

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Control of p53 expression takes place on a post transcriptional level. Mdm2 plays a key role by controlling p53 rate of synthesis and degradation. The capacity of Mdm2 to carry out its dual function requires interactions between the N-termini of Mdm2 and p53 and between the p53 mRNA and the C-terminal RING domain of Mdm2. The same RNA sequence that binds Mdm2 encodes the amino acids that interacts with Mdm2, suggesting that the control of p53 synthesis and degradation has evolved in parallel from the same genomic sequence. Single silent point mutations identified in human cancers reduce the affinity for the p53 mRNA to Mdm2 and suppresses p53 activity. This illustrates how single silent nucleotide polymorphism can affect the encoded protein. The p53 mRNA is translated from two sites of initiation giving rise to full length p53 (p53FL) and p53/47 lacking the first 40 amino acids that includes the Mdm2 binding site and transactivation domain (TA I). The physiological role of this isoform, or any p53 isoform, has been obscure. Induction of stress to the endoplasmic reticulum (ER) and triggering of the unfolded protein response activates cap-independent expression of p53/47 and the induction of a specific set of gene products that give rise to a defined cell biological outcome. The ratio of p53FL:p53/47 remains high even after ER stress and the interesting question of how relatively small amounts of p53/47 can induce a dominant phenotype in the presence of much larger amounts of the full length protein will be discussed.

OP 6.4–1
A study of the stability of wild type and mutant p53 complexes with different DNA substrates

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The tumor suppressor protein p53 is a transcription factor that plays a critical role in cell cycle regulation and differentiation and its function is closely related to maintaining genetic integrity of the cells and to defense against malignant transformation (1). Biological functions of p53 are closely connected with its ability to interact with DNA. The importance of DNA binding for tumor suppressor function of p53 is emphasized by the fact that most of the mutations in the p53 gene are located in the protein core DNA binding domain, which mediates sequence-specific DNA binding. We studied interactions of wild type (wtp53) and mutant p53 (mut p53) with different DNA substrates (supercoiled, sc; or linear, lin DNA containing or lacking specific p53 target sequence – p53CON) upon exposure to increased salt concentrations using an immunoprecipitation assay on magnetic beads. Complexes of wild type or mutant p53 with scDNA, captured at the beads via DO-1 antibody mapping to its N-terminus (amino acid aa 21–25), were remarkably stable in increasing ionic strenght (at least up to 400 mM KCl). On the contrary, complexes of wtp53 with linDNA lacking p53CON were unstable at ≥150 mM KCl and those of linDNA containing p53CON at ≥200 mM KCl. Immunoprecipitation with Bp53-10.1 antibody, binding to the p53 C-terminal DNA binding site (aa 375–379) (2), resulted in a formation of salt-unstable complexes of mut p53 with sc and linear DNA containing or lacking p53CON at ≥100 mM KCl. Using the same antibody, wtp53 complexes with lin DNA lacking p53CON were destabilized at ≥150 mM KCl while those with lin DNA containing p53CON at ≥190 mM KCl.

Acknowlegement: This work was supported by GA ASCR (IAA500040701), GACR (204/07/P476) and MEYS CR (LC06035).

References:

1. Flores ER and Jacks T. in GP Zambetti (ed), Springer Science + Business Media, Inc., New York, 2005; 187–198.
2. Fojta M, Pivonkova H, Brazdova M, Nemcova K, Palecek J and Vojtěšek B. *Eur J Biochem* 2004; **271**: 3865–3876.

SYMPOSIUM 7: TRENDS IN BIOTECHNOLOGY

7.1. Nanosensors and Nanomachines

IL 7.1–1
Physiologically active nanofibrin

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Fibrinogen, a soluble plasma protein, after conversion into fibrin forms an insoluble fibrin polymer (clot), which prevents the loss of blood upon vascular injury and contributes to subsequent fibrinolysis and wound healing through the interaction with various proteins and cell types. Fibrinogen is inert in the circulation while fibrin is highly reactive due to exposure of its numerous protein- and receptor-binding sites; among them are several tPA- and plasminogen-binding sites located in fibrin(ogen) alphaC-, P-, and coiled-coil-D domains. Binding of tPA and plasminogen to these sites results in conversion of the latter into active fibrinolytic enzyme plasmin and degradation of fibrin clot (fibrinolysis). To study the role of the alphaC-domain in initiation of fibrinolysis, we expressed alphaC-fragment corresponding to this domain in bacterial system and refolded it into a compact structure by standard procedures. However, because this fragment mimics the inactive conformation of two alphaC-domains in fibrinogen, it failed to bind tPA and plasminogen and stimulate activation of the latter in solution. To mimic the active conformation of the alphaC-domains in fibrin, where they form alphaC polymers, we identified conditions in which the alphaC-fragment formed short oligomers (8–12 molecules); however, the oligomers were unstable. To increase their stability, we expressed and refolded an extended version of the alphaC-fragment that formed similar oligomers, and covalently cross-linked such oligomers with factor XIIIa. The resulting product, alphaC-nanofibrin, which was highly soluble and stable in solution, exhibited functional properties of polymeric fibrin. Namely, in ELISA and SPR experiments, alphaC-nanofibrin, in contrast to the monomeric alphaC-fragment, bound both tPA and plasminogen and in the chromogenic substrate assay drastically increased the rate of activation of the latter into plasmin. Further, it significantly facilitated integrin-dependent endothelial cell binding, spreading, and signaling. Thus, alphaC-nanofibrin represents a nanoparticle that preserves some structural and physiological properties of polymeric fibrin. It can be utilized for studying the adhesive properties of a fibrin clot using single molecule manipulation techniques. It may also be used as a component for a nanosensor measuring plasmin(ogen) activity.

IL 7.1–2
Nanotechnology and protein arrays: recent developments

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IL 7.1–3
Machines made from DNA

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DNA is a wonderful material for nanoscale construction: it is a structural material whose self-assembly can be programmed by making use of its information-carrying capability, and its hybridization or hydrolysis can be used as to provide energy for molecular devices. I shall describe our work on self-assembled molecular machinery made from DNA, including active cages and the construction of a free-running bipedal molecular motor.

References:

1. Yurke B, Turberfield AJ, Mills AP, Simmel FC and Neumann JL A DNA-fuelled molecular machine made of DNA. *Nature* 2000; **406**: 605–608.
2. Turberfield AJ *et al.* DNA fuel for free-running nanomachines. *Physical Review Letters* 2003; **90**: 4.
3. Goodman RP *et al.* Rapid chiral assembly of rigid DNA building blocks for molecular nanofabrication. *Science* 2005; **310**: 1661–1665.
4. Erben CM, Goodman RP and Turberfield AJ. Single-molecule protein encapsulation in a rigid dna cage. *Angew. Chem. Int. Ed.* 2006; **45**: 7414–7417.
5. Bath J and Turberfield AJ. DNA nanomachines. *Nature Nanotech* 2007; **2**: 275–284.
6. Green SJ, Bath J and Turberfield AJ. Coordinated chemomechanical cycles: a mechanism for autonomous molecular motion. *Phys Rev Lett* 2008; **101**: 238101.

IL 7.1–4
Surface plasmon resonance biosensors

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Optical affinity biosensors are devices that incorporate a biological recognition element which specifically recognizes a particular analyte and an optical transduction system which allows observation and quantification of the interaction between the analyte and the biomolecular recognition element. In the last decade we have witnessed development of numerous optical transduction methods, including both label-based methods such as fluorescence spectroscopy and label-free methods such as optical interferometry, spectroscopy of guided modes of optical waveguides, and surface plasmon resonance. Label-free optical biosensors represent a unique technology that enables the direct observation of molecular interaction in real-time and the rapid and sensitive detection of a wide variety of chemical and biological species. Optical biosensors based on surface plasmon resonance (SPR) represent the most advanced and mature optical label-free biosensor technology. This paper reviews the present state of the art and recent advances in the development of SPR sensors (1–2) and presents selected results of SPR sensor research at the Institute of Photonics and Electronics, Prague. The developments discussed in detail cover advances in SPR sensor instrumentation (mobile SPR sensors for field use, SPR sensor platforms for parallelized observation of biomolecular interactions) and applications for study of molecular interactions and detection of chemical and biological analytes related to medical diagnostics (hormones, antibodies), environmental monitoring (endocrine disrupting compounds) and food safety (pathogens and toxins).

References:

1. Homola J. Surface Plasmon Resonance Based Sensors, Springer, 2006.
2. Homola J. *Chemical Reviews* 2008; **108**: 462–493.

IL 7.1–5
Nano devices for next-generation DNA sequencing techniques

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The availability of ultra-deep sequencing of the genomic DNA will in the near future transform the biological and medical fields, in the analysis of the causes of a disease, in the development of new drugs, as well as in diagnostics sequencing. It may become a promising tool in analysis of mental and developmental disorders like schizophrenia and autism. It is anticipated that DNA sequencing of whole genomes for clinical purposes using these new technologies will likely occur in the next couple of decades. At present it seems more acceptable to sequence and analyse genomes of seniors who reached high age in a very good health. The proposed Human Microbiome Project (also called Second Human Genome Project), analyzing the collection of microbes in and on the human body, will contribute to understanding human health and disease. Genomics, Proteomics and Medical research benefit from recent advances and novel techniques for high-throughput analysis (e.g. DNA and Protein microarrays, quantitative PCR, mass spectrometry, novel DNA sequencing techniques, others). Devices with short DNA sequence readings (25–50 bases) have already found many applications, as described above. But for genomic sequencing, and for analysis of the ever more important structural genetic variations in genomes (e.g. copy number variations CNVs selected by the journal Science as the most important scientific discovery of the year 2007, also chromosomal translocations, inversions, large deletions, insertions and duplications), it would be a great advantage if the sequence reading length on the original single DNA molecule could be increased, to several thousands bases and more, per second. Ideally, the goal would be the sequence determination of a whole chromosome from one single original DNA molecule. Hopes for future in this direction may give novel developments in several physics techniques (e.g. AFM, electron microscopy, soft X-rays, various spectroscopic techniques, nano-pores and nano-edges, to name some of them), with many improvements needed and under intense development.

Reference:

Ansorge WJ. Next-generation DNA sequencing techniques. New Biotechnol 2009; doi:10.1016/j.nbt.2008.12.009.

OP 7.1-1
Quick screening methods for quantification of oligonuclotides and antibodies bound to gold nanoparticles

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Colloidal gold nanoparticles (GNPs) armed with oligonucleotides, either alone or together with antibodies, are increasingly used for sensitive detection of DNA or proteins. An important step in optimizing the sensitivity and/or reproducibility of GNP-based assays is the quantification of oligonucleotides bound to the particles. To this end, several techniques, including real-time PCR with fluorescent beacon probes, have been described. However, these techniques are either insensitive or require expensive probes. Here we report on a SYTO9-based real-time PCR method for quantification of oligonucleotides bound to GNPs. In some experiments, rabbit polyclonal antibody specific for selected antigen was bound to 30 nm GNPs (30GNPs), followed by binding of thiol-capped oligonucleotides. To detect gold-bound oligonucleotides, the particles were pelleted and resuspended directly in 1x PCR master mix containing SYTO9 fluorescent dye. Routinely, 40 cycles of PCR were monitored using real-time Mastercycler Realplex. No inhibitory effect of 30GNPs on SYTO9-based real-time PCR was detected. An inverse relationship was found between the amount of oligonucleotides bound to 30GNPs and the Ct values obtained. Calibration curves were constructed using free thiol-capped oligonucleotides. Membrane dot-blot procedure was used for selection of appropriate antibodies. Briefly, antigen was diluted in borate buffer and 10 μl drops were spotted on a nitrocelulose membrane, followed by blocking in 1% bovine serum albumin. 30GNPs covered by oligonucleotides and antibodies were spotted on the membrane and allowed to absorbe for 5 min. After washing, the membrane was stained by a silver enhancer kit. Gray spots refer to antigen-antibody interactions. Rabbit antibodies bound to 30GNPs were detected with commercial 5 nm GNPs armed with goat-anti rabbit IgG (GaR_5GNPs) by electron microscopy. When properly prepared, 30GNPs with bound rabbit antibodies were surrounded by GaR_5GNPs forming rosette-like structures. The combined data indicate that SYTO9-based real-time PCR offers a rapid, simple and inexpensive method for mapping the surface coverage of oligonucleotides bound to GNPs. In combination with detection of antibodies bound to the same particles it makes it possible to monitor the properties of nanogold conjugates. Such data are important in standardization of bio-barcode amplifications and other assays used for detection of proteins. Supported by KAN200520701.

7.2 Photosynthesis and Solar Energy Conversion

IL 7.2–1
How nature solved its energy problem

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An enzyme emerged about 2.5 billion years ago which would dramatically change the chemical composition of our planet and set in motion an unprecedented explosion in biological activity. This enzyme used solar energy to power the thermodynamically and chemically demanding reaction of water splitting. In so doing it provided biology with an unlimited supply of 'hydrogen' needed to convert carbon dioxide into the organic molecules of life. In a nutshell, when biology learnt to split water using sunlight it had solved its energy problem allowing life to prosper and diversify on an enormous scale as witnessed by the extent of the fossil fuel reserves and the variety of living organisms which constitute present day biomass. Therefore the energy we derive from the burning of fossil fuels, biomass and other organic substances (food) originates from solar energy. The questions are now, can we continue to exploit the photosynthetic process through increased use of biomass as an energy source and, more importantly, can we address the energy/CO_2 problem by developing new photochemical technologies which mimic the natural system? After all there is no shortage of water for this reaction and the energy content of sunlight falling on our planet well exceeds our needs.

IL 7.2–2
How conformational switching in plant light-harvesting complexes regulates photosynthesis

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Regulation of photosynthetic light harvesting, which protects plants from the damaging effects of excess light, occurs by the rapid and reversible transformation of the photosystem II light-harvesting complexes (LHCII) from a state of efficient light utilisation to one in which absorbed excitation energy is dissipated as heat. This transformation is known as non-photochemical quenching (NPQ). Similar to many biological regulatory mechanisms, NPQ arises by a change in protein conformation, which turns LHCII into a quencher of chlorophyll excited states. Using femtosecond spectroscopy on strongly quenched aggregates of LHCII we were recently able to identify the quencher as lutein L1 in the structure of LHCII. The speed of NPQ formation *in vivo* suggests the involvement of only a subtle rearrangement of the pigments of LHCII. However, whilst the spectroscopic differences between the unquenched and quenched states of LHCII have been analysed in detail, little is known about the dynamics of the conformational change. We have analysed fluorescence emission of single, purified LHCII complexes, using single-molecule spectroscopy, a powerful experimental technique for studying molecular properties that are otherwise obscured by ensemble averaging. I will show that LHCII trimers have the inbuilt capacity to spontaneously, rapidly and reversibly switch into two different states: the first is a quenched state and the second state is characterised by a red-shifted emission peak (see figure below). Both states reflect properties of what is observed both in LHCII aggregates and in some states of NPQ *in vivo*. The relative rate of forward and backward switching therefore establishes whether the population of complexes is, on average, unquenched or quenched, red-shifted or not. Moreover, subtle manipulation of the switching rates is sufficient to reproduce the various extents of NPQ observed in plants under different physiological conditions.

IL 7.2–3
Biological and biomimetic water photolysis

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The water oxidising enzyme of photosynthesis is in large part responsible for converting solar energy into the high energy chemicals that sustain life on the planet. The excess energy stored has accumulated as the fossil fuels. This solar-driven enzyme also put the oxygen into the atmosphere and thus made the biosphere aerobic with all the associated repercussions for the planet (O_2-based respiration, multicelluar life, UV screening by ozone etc). It is also one of very few catalysts known that is able to oxidise water at close to its thermodynamic optimum. As such it is the focus of much attention with a view to producing bio-inspired catalysts that have potential uses for more efficient (and sustainable) energy conversion processes: such as (i) H_2 production by electrolysis or by photolysis and (ii) in electricity production in fuel cells. We have applied biophysical, biochemical and molecular biological methods to investigate the photochemistry and molecular enzymology of this remarkable enzyme. The active site is made up of four high valence Mn ions and a calcium ion. In parallel to the biochemical and biophysical studies, we (in association with the University of Paris at Orsay) also design, synthesize and study chemical models that are aimed at mimicking certain features of the water oxidising enzyme. Mn-based catalysts linked to photochemical charge separating systems have been constructed and characterised. Some of the properties of these photo-catalysts will be reported.

Acknowlegement: Supported by EU network Solar H2.

IL 7.2–4
Combining biology with technology for the sustainable production and use of fuels

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Energy transduction is fundamental to biology; biological organisms have evolved elegant machinery for energy capture and utilization. Parallels between technological and biological energy transduction suggest potentially fruitful research into biological, hybrid and artificial systems for efficient energy conversion. These parallels also set the stage for a major challenge facing hybrid constructs: coupling electromotive force (emf) provided by human technology to the phenomenal catalysts for the synthesis of energy-rich fuels found in natural systems (1). Addressing this challenge, several bio-inspired constructs for solar energy conversion have been assembled. In one, a photobiofuel cell uses artificial reaction centers, visible light and two enzymes to reform biological substrates such as ethanol into hydrogen (2). This system interfaces redox chemistry with emf at two junctions, and demonstrates two methods for coupling biological redox catalysts with emf. In a second system, solar-driven water oxidation using an IrO_2 catalyst replaces the anodic organic substrates, and an external boost of about 0.3 V allows the electrons to combine

with protons, producing hydrogen at the cathode (3). Another construct uses a bio-inspired molecular heterojunction-based polymer material to assemble a photovoltaic device (4). The control of energy flow in biological systems is crucial to their stability. We have developed energy and electron transfer-based models for incorporation of these features into artificial systems (5,6). Conducting the high oxidation potential necessary for water oxidation from sensitizer to catalyst presents challenges in electronic coupling and thermodynamics to control the flow of electrons. We have recently reported a bio-inspired system which accomplishes this by mimicking the elegant redox relay found in water-oxidizing photosynthesis (7).

References:

1. Chem Soc Rev 2009; **38**: 25.
2. JACS 2008; **130**: 2015.
3. JACS 2009; **131:** 926.
4. Chem Mat 2008; **20**: 135.
5. Nature Nano 2008; **3**: 280.
6. PNAS 2006; **103** 5343.
7. JACS 2008; **130**: 10466.

Acknowledgments: US DOE, NSF.

OP 7.2–1
Electrokinetic properties of pea thylakoid membranes

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The crucial role of the membrane structure in clarifying the mechanism of static magnetic field (SMF) action on biological structures was studied. The electrophoretic mobility of the thylakoid membranes as a model system was determined after exposition with SMF (2T) in dependence of iron content in culture media as follows: an iron sufficient medium ('Fe-sufficient' variant), an iron-deficient medium ('Fe-starved' variant) and an excess of iron supply medium ('2 × Fe' variant). We report the generation of light-induced increase in surface electrical charge of 'Fe-sufficient' thylakoids due to a strong aggregation and increase in lipid peroxidation of pea thylakoids after SMF pre-treatment. SMF altered the membrane potential (a significant increase in fast phase of LS) in 'Fe-starved' thylakoids upon exposure at near saturated light intensity. This light treatment induced a decrease in the production of thiobarbituric acid – reactive substances, marker for lipid peroxidation in 'Fe-starved' and '2 × Fe' SMF pre-exposed thylakoids. These results suggest the decay in charge recombination during electro-transport reactions. We have used atomic force microscopy (AFM) to scan the surface topography of the thylakoid membrane under physiological conditions as well as after lectin treatment. The AFM images were acquired using a Multimode, Nanoscope V (Veeco Instruments, Santa Barbara, CA, USA), employing an E scanner. The probe was DNP-S (Veeco Probes) and was mounted on a fluid cell for imaging in physiological conditions. The imaging was performed in tapping mode at 0.5 Hz scanning rate and resolution of 131 072 pixels. Imaging three-dimensional thylakoids without and in the presence of phytohemagglutinin (PHA-M) molecules revealed a height reduction of the thylakoid volume. There was a change in the topography of the scanned surface. The thylakoid height changed from 246.626 nm in isotonic buffer solution before lectin exposure to 202.449 nm after 0.1 ng/ml PHA-M treatment. Consequently, a change in the curvature of the membrane could be expected due to cross-link molecular reorganizations in the plane of the membrane.

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7.3. Plants for the Future

IL 7.3–1
Improving plant cell walls for bio-energy

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Plant biomass is an interesting alternative source for energy. Plant cell walls are composed of polysaccharides that can be converted to glucose and further fermented to bio-ethanol. However, the aromatic cell wall polymer lignin, which makes up about 20% of the cell wall, limits the access of cellulases to the cellulose microfibrils. Therefore, lignin needs to be degraded prior to saccharification, a process which consumes large amounts of energy and chemicals. The importance of lignin in biomass quality has been the main driver for research in this field. By genetic engineering, plants can be tailored that have an altered cell wall structure enabling a more efficient conversion to bio-ethanol. Research over the last decade has mainly focused on understanding the lignin biosynthesis pathway. These studies have shown that the lignin polymer, which is composed of various phenylpropanoids, is plastic in its composition and structure, and that it readily incorporates products from incomplete monolignol biosynthesis. This allows engineering lignin structures that are more easily degraded prior to saccharification. It now also becomes clear that genetic modification of monolignol biosynthesis in the cell wall has wide-ranging consequences on a number of metabolic processes, such as starch metabolism and photosynthesis, and that it affects the biosynthesis of the other cell wall macromolecules, such as hemicellulose. Molecular insight into these pleiotropic effects is essential if we want to design cell walls for end use applications.

IL 7.3–2
Functional analysis of genes related to secondary metabolism in plants

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Plant-derived secondary metabolites constitute an extremely diverse resource for drugs currently available on the market and for the discovery of active principles which are effective in new indication areas. The classical plant-based pharmaceutical production has been notoriously hampered by low yields, lack of reproducibility and the high complexity of plant extracts. Utilization of cell culture systems for biotechnological production offers many benefits but requires optimization through genetic engineering to reach economic feasibility. We have applied a comprehensive profiling approach based on functional genomics, which is independent of prior sequence knowledge i.e. applicable to medicinal plants, to monitor elicitor-induced changes in the transcript and metabolite profiles of cell and organ cultures. In a single experiment the behaviour of hundreds of known and unknown differentially expressed genes and metabolites can be followed simultaneously. The integration of gene expression profiles and the metabolite accumulation profiles through correlation network analysis allows the visualization of gene-to-metabolite networks and the prioritization of candidate genes for further characterization. Functional analysis is mainly achieved by homologous or heterologous overexpression in *Agrobacterium* induced hairy root systems and utilizing again metabolic profiling tools such as LC-MS and NMR for monitoring the effects. Integration with metabolic profiling provides direct insight on which genes are involved in biosynthetic pathways of relevant secondary metabolites at the structural or regulatory level while at the same time novel compounds through combinatorial biosynthesis may be discovered. This strategy has so far been employed to several medicinal plant species including *Catharanthus* and *Hyoscyamus* and the tobacco model.

IL 7.3–3
The potential of genetically modified plants for phytoremediation

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Phytoremediation, nowadays called phytotechnology, is a green and promising solution to problems of environmental contamination, since it entails the use of higher plants for uptake, sequestration, detoxification or volatilisation of inorganic and organic pollutants from soils, water, sediments and possibly air. Immediately after discovering that plant physiological properties can be exploited for remediation, scientists tried to improve these characteristics: they started by exploring species and ecotypes found in the natural environment, and then considered application of breeding techniques. Development of breeding approaches led to the search for markers and genes to be used in selection of useful properties, and this has produced hundreds of papers and book chapters on identification, cloning, characterisation, mapping of genes which can be involved at some level in specifying the plant properties useful in phytoremediation. Based on this knowledge, the possibility of creating transgenic plants with increased capabilities was taken into consideration. Even within the never ending debate surrounding environmental release of GM plants, the scientific community thinks in general that GM plants beneficial for the environment will be considered more favourably by the public opinion. After nearly 20 years of research, several examples of transgenic plants for application to phytoremediation have been described, but none have reached commercial existence. Three main approaches have been developed: (i) transformation with genes from other organisms (mammals, bacteria, etc.); (ii) transformation with genes from other plant species; (iii) overexpression of genes from the same plant species. Results have rarely been encouraging, and sometimes even contrary to expectations. This review will illustrate the main examples with a critical discussion of what we have learnt from them.

IL 7.3–4
New tools for the phytoremediation of xenobiotics

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Plants have a surprising capacity to metabolize and detoxify man-made chemicals entering the environment as pollutants and crop protection agents. We have termed the biosystem which recognizes, transports and biotransforms such xenobiotics the 'xenome' and using a combination of chemical biology and molecular genetic approaches we are functionally characterizing and engineering key xenome components for applications in crop protection, phytoremediation and metabolic/industrial chemical

biotransformations. In this presentation, the engineering of xenome enzymes to metabolise (i) herbicides and (ii) pollutants to improve plant performance in crop protection and remediation will be presented. In the first example, we will demonstrate how forced evolution approaches can be used to enhance the detoxification of diphenyl ether herbicides by glutathione transferases. In the second example, the molecular basis for the activity of a type 1 glucosyltransferase *N*-conjugating aniline pollutants has been determined and a surprising observation made concerning the role of this enzyme in controlling the metabolism and toxicity of pollutants. Finally, the potential to modify the expression of remediating enzymes and associated pathways using herbicide safeners will be presented. This will include new insights into how safeners function to accelerate the detoxification of xenobiotics and the potential for using this ability to remediate contaminants in the environment.

IL 7.3–5
Plant biochemistry as a source of new anticancer drugs

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In malignant cells, altered expression of cyclin-dependent kinases (CDKs) and their modulators, including overexpression of cyclins and loss of expression of CDK inhibitors, results in deregulated CDK activity, providing a selective growth advantage. In contrast to CDKs governing the transitions between cell cycle phases, transcriptional CDKs, including cyclin H-CDK7, and cyclin T-CDK9 (pTEFb), promote initiation and elongation of nascent RNA transcripts by phosphorylating the carboxy-terminal domain (CTD) of RNA polymerase II. Because of their critical role in cell cycle progression and cellular transcription, as well as the association of their activities with apoptotic pathways, the CDKs comprise an attractive set of targets for novel anti-cancer drug development. Our research focused on the primary mechanism of action of plant hormones cytokinins (N^6-substituted adenine derivatives) in cell division cycle has showed that natural plant cytokinins are also rather non-specific inhibitors of various protein kinases. Surprisingly, among aromatic cytokinin derivatives, we have discovered a compound, 2-(2-hydroxyethylamino)-6-benzylamino-9-methylpurine, named 'olomoucine' (OC), which specifically inhibits some CDKs at micromolar concentration. One of the inhibited kinases, the $p34^{cdc2}$/cyclin B kinase, assumed to be a key mitotic factor, which is highly conserved and strongly implicated in cell cycle transitions in all eukaryotic cells. The total lack of the inhibitory effect of olomoucine on major kinases, such as cAMP- and cGMP-dependent kinases, protein kinase C, and others, suggests that OC might be a useful tool for cell cycle regulation studies. The design and inhibitory activity of OC was further improved by modifications at positions 2, 6, and 9, i.e., the positions that control binding to CDK1. This led to discovery of novel specific CDK inhibitors named roscovitine, olomoucine II and purvalanol A (Fig. 1), which display an enhanced inhibitory activity toward CDK1, a higher selectivity toward some CDKs, an increased antimitotic activity at the G1/S and G2/M points of the cell cycle, and stronger and more selective antitumour effects. The compounds are also effective *in vivo* and one of them is already in clinical trials in USA and Europe (roscovitine Seliciclib®; Cyclacel Pharmaceuticals Ltd, UK). Seliciclib® is currently in Phase IIb clinical trials as a single therapy in multiple myeloma as well as two other B-cell hematological malignancies: B-cell chronic lymphocytic leukemia and mantle cell lymphoma. An additional Phase IIb clinical trial is in progress investigating the effects of Seliciclib® in patients with non-small cell lung cancer in combination with gemcitabine and cisplatin. Recently, we have developed also first CDK9 inhibitors and discovered whole range of new structural motifs for development of CDK inhibitors which are derived from anticytokinin structures.

OP 7.3–1
Preparation of geneticaly modified plants with enhanced metal tolerance and accumulation

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A large number of sites worldwide are contaminated by heavy metals as a result of human activities, leading to elevated risk for health and the environment. Although phytoremediation may offer a viable solution to this problem, the presence of heavy metals may inhibit plant growth and the concentration of metals could be limiting for the application of phytoremediation. Therefore, one option is to genetically engineer fast-growing species to improve their metal tolerance and metal-accumulating capacity. The γ-glutamylcysteine synthetase (γ-ECS) and the glutathione synthetase (GS) are key enzymes in glutathione (GSH) biosynthesis. GSH is not only the direct precursor of phytochelatins (PCs), but glutathion itself is also believed to play important roles in the detoxification of many heavy metals. The aim of the present study is to overexpress *Saccharomyces cerevisiae gsh1* gene for γ-ECS and *gsh2* gene for GS in *Nicotiana tabacum*, as a model plant, and a species of technological use, *Linum usitatissimum*, which is an annual plant species widely cultivated in temperate climates, to obtain plants with enhanced Cd accumulation and tolerance. Several vectors were designed for *Agrobacterium* mediated transformation, pNOV1 and pNOV2 contain *gsh1* gene and *gsh2* gene, pNOV12 contains both *gsh1* and *gsh2* genes. Each gene was flanked by Rubisco small subunit light-inducible promoter RbcS from *Chrysanthemum sp.* and at 3′ end by RbcS transcriptional terminator. Special vector was constructed for promoter expression study harboring *gus* gene for β-glucuronidase. Transient expression with tobacco leaves was carried out applying this vector and proving promoter activity. A method was designed for determination of glutathion and phytochelatins using RP-HPLC. For this purpose aseptically grown flax was stressed in 14 days' period by different concentrations of $CdCl_2$. Analysis of leaves extracts indicated the presence of GSH, PC2, 3, 4 and 5.

Acknowledgement: This work is funded by grants MSMT 1M06030, MSM 6046137305 and Z 40550506.

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7.4. Biochemistry of Phytoceuticals in Plant and Mammalia

IL 7.4–1
How plant cells survive their own toxins-controlled gene expression and dynamic compartmentation in secondary metabolism

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The secondary metabolism of plants provides an unlimited diversity of bioactive molecules, including established and prospective drugs. Secondary biosynthesis poses a burden to the producing cell as it competes for energy sources and building blocks and may generate cytotocic products. Thus, evolution of secondary metabolites is not only driven by their advantagous functions (pathogen defense, signaling etc.) but also selects for mechanisms to minimize the risk of self-intoxication. As a result, biosynthesis is embedded in programs of cell specialization and cellular ultrastructure: (i) genes encoding biosynthetic enzymes are often silent until activated by specific stressors, (ii) intermediates and products are compartmented via metabolons and membrane flow, (iii) cytotoxic products remain under the control of detoxifying enzymes. These principles are exemplified with the benzophenanthridine alkaloids, cytotoxic phytoalexins of the Papaveraceae. In the model plant *Eschscholzia californica*, biosynthetic genes are selectively activated via a novel signal path that operates beyond the ubiquitous, jasmonate-dependent control of defense genes and thus avoids hypersensitive cell death. Pathogen contact activates a G-protein controlled phospholipase A2 thus giving rise to the signal intermediate lysophosphatidylcholine. This compound modulates vacuolar H^{+}/Na^{+} antiporters, and the resulting pH shift in the cytoplasm activates gene transcription. The signal path is blocked by accumulated alkaloids. The overproduced benzophenanthridines are excreted to the cell wall but do not escape the cellular control: above a threshold, they are re-absorbed, reduced to less toxic dihydro-alkaloids, derivatized and re-excreted. This recycling process is driven by the novel enzyme sanguinarine reductase. Cloning and site-directed mutagenesis of this enzyme points to a common progenitor within a family of ubiquitios enzymes of plant steroid metabolism, thus highlighting the evolution of a detoxifying mechanism.

IL 7.4–2
Targeting inflammatory pathways for prevention and therapy of cancer

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Chronic infections, obesity, alcohol, tobacco, radiation, environmental pollutants, and high-calorie diet have been recognized as major risk factors for the most common types of cancer. All these risk factors are linked to cancer through inflammation. While acute inflammation that persists for short-term mediates host defense against infections, chronic inflammation that lasts for long-term can predispose the host to various chronic illnesses, including cancer. Linkage between cancer and inflammation is indicated by numerous lines of evidence; first, transcription factors NF-KB and STAT3, two major pathways for inflammation, are activated by most cancer risk factors; second, an inflammatory condition precedes most cancers; third, NF-KB and STAT3 are constitutively active in most cancers; fourth, hypoxia and acidic conditions found in solid tumors activate NF-KB; fifth, chemotherapeutic agents and gamma irradiation activate NF-KB and lead to chemoresistance and radioresistance; sixth, most gene products linked to inflammation, survival, proliferation, invasion, angiogenesis, and metastasis are regulated by NF-KB and STAT3; seventh, suppression of NF-KB and STAT3 inhibits the proliferation and invasion of tumors; and eighth, most chemopreventive agents mediate their effects through inhibition of NF-KB and STAT3 activation pathways. Thus suppression of these proinflammatory pathways may provide opportunities for both prevention and treatment of cancer.

IL 7.4–3
Transcriptional regulation of flavonoid biosynthesis

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Flavonoids (anthocyanins, flavonols and proanthocyanidins) are secondary metabolites presenting valuable properties as antioxidants and colorants for foods, and as human health promoting factors (e.g. nutraceuticals, pharmaceuticals). Flavonoids play important roles in the interactions of plants with their environment; however their role in plant development appears not to be critical. Therefore, the modulation of flavonoids accumulation in different plant tissues/organs is theoretically feasible by breeding or molecular engineering as well as by using various growth conditions. To date, many structural and regulatory genes of the pathway have been discovered, but the identification of some proteins involved in specific branches that are responsible for flavonoid diversity are still to be characterized. In addition, the complex regulatory mechanisms of the pathway by biotic, abiotic or developmental factors remain largely unknown. We will present how the use of the model plant *Arabidopsis thaliana* contributes to the elucidation of some molecular and genetic aspects of flavonoid biosynthesis. The metabolic and regulatory mechanisms involved will be presented and few examples of the possible use of these results for crops improvement will be shown.

IL 7.4–4
Transformation of alkaloids in plant and mammalian cells. Green–red biology

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Alkaloids are nitrogen-containing secondary metabolites in living organisms having a range of structure types, biosynthetic pathways and pharmacological activities. Plants containing quaternary benzo[c]phenanthridine alkaloids have long been used in folk medicine and one of these is sanguinarine (SG). SG is involved in the defense mechanism of the plants that produce it. In the plant, the cell enzyme catalyzing its formation from dihydrosanguinarine (DHSG) is an oxidase. The enzyme catalyzing its reverse conversion to the quaternary cation is sanguinarine reductase. Sanguinarine-containing extracts are employed in a number of phytopreparations for their anti-microbial and anti-inflammatory effects. SG is also linked to toxic effects on humans such as the formation of leukoplakia and the syndrome termed epidemic dropsy. In mammalian cells, SG modulates a number of

molecular targets such as: Na^{+}/K^{+}-ATPase, NF-kappaB, caspase-3, bcl-2 etc. *In vitro*, the effects of SG depend on the presence of the iminium bond, DHSG is biologically inactive. We studied the mechanism of SG and DHSG transformation in mammalian cells: human hepatocytes, HepG2 cell line, porcine enterocytes as well as their pharmacokinetic parameters. In rat, after oral administration, SG is reduced in the GIT and/or liver to dihydrosanguinarine and is eliminated from the organism after 24 hours. Reduction of the quaternary cation was the only proven detoxifying mechanism in rodents. In contrast to plants, reaction is not reverse. Whether this is a reduction of the iminium bond by reduced nucleotides or reduction catalyzed enzymatically, we have not yet established. We are looking for a reductase close to the plant one. A pharmacokinetic study of DHSG showed that DHSG undergoes enterohepatic cycling with a maximum plasma concentration in the first or second hour following application with no oxidation of DHSG to the parent cation. Elucidating the mechanism of reduction/oxidation of the iminium bond in mammals is a key step in clarifying whether or not SG is toxic for mammals.

Acknowledgement: This work was supported by grants MSM6198959216 and GA CR 525/07/0971.

OP 7.4–1
Plant as a factory for breast cancer antigen production

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The characterization of HER2/neu antigen recognized by immune effector cells has opened the perspective of developing therapeutic vaccines in the field of breast cancer. Plants as a source of vaccines and mAb are currently one of the most attractive approaches of modern biotechnology providing the low-tech cheap manufacturing process and animal pathogen-free product. Here we report that *N. benthamiana* leaves agroinjected with virus-based vectors may be used as a source for production HER2/neu specific antigen on surface of TMV called as a herasim. *N. benthamiana* leaves co-agroinjected with TMV- and PVX-based vectors provided accumulation nanoparticles assembled from origin of assembly containing TMV RNA and PVX-encoded TMV coat protein or its C-terminal extension variants. Agroinjection with PVX vectors provided in leaves accumulation of expected CP with linker and HER2/neu peptides revealed in Western tests. Immunization of mice with herasim elicited antibodies, which specifically recognized purified herasim samples and HER2/neu on the surface tumor SKBR-3 cells. It is worth to emphasize that 585 peptide had negligible immunogenicity and did not occur immunoprotective effect on mouse model whereas anti-herasim mouse antiserum gave distinctive positive signal. We suggest that TMV nanoparticles with exposed HER2/neu epitope may be used for developing therapeutic vaccines.

7.5. Biochemical Prospects for Better Food

IL 7.5–1
The main trends in biochemical research helping to improve food and feed

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Biochemical research played important role in elucidation of essential nutritional factors and in this way helped practically to eliminate deficiency diseases through food fortification and offering suitable food and feed products. It helps to understand the metabolic fate of nutrients and the consequences for physiological status and health of humans and animals. Its important role we can find in completely new technologies developed in food and feed production, tremendous improvements in food and feed stability and shelf-life, initiation or even introduction of new approaches. New biochemical techniques and procedures caused revolution in monitoring of food quality and safety, as well as in examination of food and feed origin and adulteration. All these scopes outlined above are still in focus of food and feed biochemists, however, there are a few more targets which can be considered as present main trends of biochemical research in this field. This new generation of issues includes metabolomics, proteomics and genetic engineering. Metabolomics (metabolic profiling) is concerned with identification and quantification of metabolites including small molecules. It offers us many aspects of 'molecular nutrition': analysis of food components, assaying of food quality and authenticity, monitoring of food consumption and physiological monitoring during various diets. These studies will allow examinating well various technological processes, modification of crops, improvement of breeding and feeding livestock or preparation of processed food. Potential of metabolomics is still limited by instrumentation and lack of databases. Proteomics deals with proteins from natural sources and recombinant technologies and monitor changes which occur in proteome (collection of all proteins occurring e.g. in the tissue, cell, organelles or skeletal muscle/meat). Proteome analysis is based on combination of powerful instrumental techniques (mainly two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) and mass spectrometry) with bioinformatics. Proteomics has been already successfully applied in solving various topics in meat and cereal sciences and food technology. Genetic engineering found already application in commercialization of transgenic plants and genetically modified microorganisms used for preparation of various food additives or enzymes.

IL 7.5–2
Increasing the health benefits of wheat

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The consumption of whole grain wheat products is known to have significant health benefits, including reducing the risk of cardiovascular disease and type 2 diabetes. The UE FP6 HEALTH GRAIN programme is focused on understanding the basis for these effects, and increasing the contents of the bioactive components in wheat products. In order to facilitate the latter aim we have carried out an extensive diversity screen, analysing a collection of 150 lines grown on the same site for a range of phytochemicals (alkylresorcinols, phenolic acids, sterols, tocols, folates) and dietary fibre components (β-glucans and arabinoxylans) which are considered to have health benefits. The total concentrations of these components varied significantly, from 1.4-fold for sterols to 3.6-fold for phenolic acids and 4.8-fold for soluble arabinoxylans. Further studies of 26 lines grown on multiple sites/years showed that a substantial proportion of the variation is genetically determined, with additional effects of the environment. Whole genome mapping of the 150 wheat lines is allowing the identification of chromosomal regions associated with high levels of specific phytochemicals and should lead to the development of molecular markers for use in plant breeding. Additional variation in the composition of starch (including the proportion of amylose which is resistance to digestion) and dietary fibre is also being generated by mutagenesis and transgenesis. The programme is therefore expected to lead to benefits to consumers as well as improved understanding of the mechanism of action.

IL 7.5–3
Novel enzyme applications in food and feed improvement

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The use of enzymes for food application are well established in food industry, e.g. cheese making (chymosin), fructose used in soft drinks (amylases/glucose isomerase) and bread fresh-keeping (maltogen amylase). The trends in the food industry with respect to (i) health and wellness, (ii) food safety and (iii) sustainability increase the demands for new product development tools. Enzymes are an obvious choice to fulfill these demands. Furthermore, enzymes can also improve the nutritious value of various animal feed and enzymes is used today in poultry and livestock feed. During the last couple of years Novozymes have launched several new enzymes to the food and feed industry. Two examples are highlighted here. Acrylamide is a potential carcinogenic compound formed in baked and fried starchy foods, such as potato chips, breads and cookies (the Swedish National Food Administration, April 2002). Acrylamide is formed from a maillard reaction between asparagine and a reducing sugar. Novozymes has launced an enzyme (2007) which deamidates asparagine which is able to significant reduce the formation of acrylamide in various foods. Partial hydrogenation of fat is an old process to improve melting point properties of oils. However, trans-fat is unhealthy and is being phased out in many countries. Enzymatic interesterification is a healthy alternative to improve melting point properties of oils.

IL 7.5–4
Positive and negative effects of polyphenol oxidases in food processing

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PPO is an enzyme that catalyzes the oxidation of *o*-diphenols to *o*-quinones; most also catalyze oxygenation of monophenols to *o*-diphenols. The *o*-quinones spontaneously condense to form colored compounds. This color formation is generally perceived as a quality defect, e.g. in bruised or cut fruits and vegetables, grape musts and post-mortem crustaceans. In plants, PPO is encoded in the nucleus but targeted to the chloroplast thylakoid membrane. Yet even tissues that are deficient in chloroplasts, such as grape berries, exhibit PPO activity. PPO contains two active-site copper ions coordinated by four His side chains. It is synthesized as an inactive precursor, and its activation appears to involve proteolytic processing, and perhaps oxidation of a novel

adduct formed between a Cys sulfur and a His side chain. Reducing agents such as ascorbate or sulfite, acidulants and heat treatment are commonly used to inactivate the enzyme. Microwave treatment, γ-irradiation and pulsed electric field processing also inactivate to varying extents. Chemical inhibitors of PPO include chelating agents, sulfhydryl-containing reagents, aromatic carboxlyates lacking (or with alkoxy-substituted) phenolic OH groups, *m*-diphenols (e.g. 4-hexlresorcinol), and other oxygen-substituted planar ring structures (e.g. kojic acid). Cyclodextrins appear to inhibit PPO action by sequestering substrate. The reaction can also be limited by the use of oxygen-barrier packaging films. Color development is sometimes beneficial: endogenous PPO is important in fermentation in black tea leaves, and appears to be involved in betalin synthesis. Exogenous PPO has been used to reduce astringency and bitterness in chocolate nibs and reduce total phenolics in canola meal.

IL 7.5–5
Protection of food lipids by natural antioxidants

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Lipid oxidation, and particularly poly-unsaturated fatty acids' oxidation, is an unavoidable phenomenon which can alter the sensorial and nutritional quality of food products and make them less acceptable to consumers. Preventive actions, like limiting contact with oxygen, metals or light, can limit these reactions, but they are generally not sufficient and the recourse to antioxidants is often necessary. Food antioxidants can either occur as natural constituents of foods or be intentionally added or generated during processing. Their action mechanisms are multiple, particularly depending on the oxidation pathways (i.e. autooxidation, photooxidation or enzymatic oxidation). They can act as chain-breaking antioxidants or as preventive ones by limiting the action of oxidation factors (e.g. metal chelators, oxygen reducing agents, singlet oxygen quenchers). In any case, they are not able to avoid definitely food rancidity, but to delay the onset of oxidation or slow the rate at which it proceeds. If efficient synthetic antioxidants are available for the food market, strategies of valorization of natural molecules are developing, due to the world-wide trend to minimize the use of synthetic additives and to take advantage of the biological interest of natural antioxidants on human health. Three main strategies will be presented and their efficiency on lipids stabilization, food quality improvement and shelf-life extension will be evaluated. (i) The first one consists to enrich edible oils with natural antioxidants (phenolic compounds and tocopherols) by optimizing the traditional industrial process in softer ways respecting the micro-constituents' content of oil seeds (e.g. sunflower, rapeseed) and the environment, as proposed in the Optim'oils® european project (FP 6). (ii) The second one is the addition of antioxidants coming from natural sources (during food processing), with the benefit of possible synergies between the antioxidants. (iii) The third one aims to better value antioxidants naturally generated during thermal food processing, i.e. Maillard reaction products, and to take advantage of their possible interaction with phenolic antioxidants.

OP 7.5–1
Nutrigenomics analysis revealed that the methanol extract of *Tamarindus indica* changes the expression of genes associated with lipid metabolism

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Various parts of *Tamarindus indica L.* (*T. indica*) or locally known as Asam Jawa, have been used for decades as components of foods and in traditional medicine. The *T. indica* pulp fruit extract had been shown to decrease serum cholesterol and triglycerides levels in an experimental model of hypercholesterolaemic hamsters. However, the mechanisms for the lipid lowering action of this plant have not been elucidated. An earlier studies performed in our laboratory showed that apart from the fruit, the methanol extracts of seed, stem and leave of *T. indica* also have high antioxidant activities. A nutrigenomics analysis on the methanol extract from *T. indica* fruit was recently performed in our laboratory to investigate the changes in gene expression in liver cell line, HepG2, in response to treatment with non-toxic dose of methanol extract of *T. indica* fruits. Gene expression microarray studies were performed on Affymetrix Human Genome 1.0 S.T arrays. Data generated were pre-analysed using the NetAffx Analysis Centre before further analysed using The Partek Genomics Suite software. Filtering criteria were set as a fold change ≥1.5. Two-way analysis of variance (ANOVA) was applied to determine differentially expressed sets of genes from control and treated HepG2 cells. The microarray data were validated using reverse transcription-PCR (RT-PCR). Our microarray data generated from three biological replicates showed that 590 genes were up-regulated whereas 656 were down regulated. Amongst the down-regulated genes were those that are involved in cholesterol synthesis and lipoprotein metabolism indicating the fruit's potential as hypolipidaemic and hypocholesterolaemic agents.

7.6 Biofilms: Social Life of Microbes

IL 7.6–1
Biofilms and quorum sensing regulation

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Bacteria are described as the smallest autonomous organism in the environment. Based on the data available on complete genomes, the bacterial genome size varies from 0.27 Mb (*Bacillus anthracis* strain A2012) to 9.7 Mb (*Rhodococcus sp.* RHA1). This genome material allows the binary fission process which is the main way of reproduction of bacteria. This mode of growth limits genetic variations to sporadic mutations. However, the genetic potential of a bacterium is not limited to the genetic materials inherited from its parents. Bacteria have evolved mainly by exchanging genetic material from other bacteria via plasmids and DNA recombinations. This ability confers a large potential to bacteria to adapt to the environment. Moreover, bacterial cells are able to communicate between themselves. This type of interaction offers facilities to coordinate some physiological processes in bacteria such as the quorum sensing. The cell-to-cell communication exports the gene expression at the level of the bacterial population rendering the bacterial world more complex. This complexity is reinforced by the ability of bacterial cells to form a well-structured cell cluster organization, namely biofilms. This dynamic structure ensures a higher potential for bacteria to survive to environmental conditions by combining diverse phenotype responses. Through the biofilm formation, bacteria developed new strategies to adapt and to survive in the environment. Do cells in biofilm communicate between themselves? How and what for? Advances of cell-to-cell communication in bacterial biofilm will be presented.

IL 7.6–2
Biofilms, flocculation and quorum sensing in yeast: from molecular to social interactions

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The budding yeast, *Saccharomyces cerevisiae*, has emerged as an archetype of eukaryotic cell biology. Here we show that *S. cerevisiae* is also a fantastic model for the evolution of cooperative, biofilm-like behavior by revisiting flocculation, a self-adherence phenotype lacking in most laboratory strains. Most feral and industrial yeasts, however, show various degrees of flocculation behavior, ranging from weak to extremely strong. The strongest form of flocculation, where thousands of individual yeast cells clump together in compact 'flocs' of almost one centimeter in diameter, is linked to expression of FLO1. FLO1 encodes a long, rod-shaped lectin-like protein that protrudes from the cell wall and binds mannose residues in the cell walls of adjacent cells. FLO1 expression is under quorum-sensing control, ensuring that FLO1 is not expressed until enough yeast cells are present to form flocs. Flocculating cells enter an altered physiological state, reminiscent of bacterial biofilms (including overexpression of drug exporters, stress genes and repression of genes involved in proliferation). Flocculation protects the FLO1-expressing cells from multiple stresses, including antimicrobials and ethanol. Furthermore, FLO1+ cells avoid exploitation by non-expressing flo1 cells by self/non-self recognition: FLO1+ cells preferentially stick to one another, regardless of genetic relatedness across the rest of the genome. Flocculation, therefore, is driven by one of a few known 'green beard genes', which direct cooperation towards other carriers of the same gene. Moreover, due to the presence of internal coding tandem repeats, FLO1 is highly variable among strains both in expression and in sequence, suggesting that flocculation in *S. cerevisiae* is a dynamic, rapidly-evolving social trait.

IL 7.6–3
Cyclic di-GMP as a second messenger linking cell-cell signalling to biofilm formation in the plant pathogen *Xanthomonas campestris*

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The full virulence of *Xanthomonas campestris* pv. *campestris* (*Xcc*) to plants depends upon cell-cell signalling mediated by the signal molecule DSF (for diffusible signal factor), which has been characterised as *cis*-11-methyl dodecenoic acid. DSF-mediated signalling regulates motility, biofilm formation and the synthesis of particular virulence determinants including the extracellular polysaccharide xanthan. The synthesis and perception of the DSF signal involve products of the *rpf* gene cluster. DSF synthesis is fully dependent on RpfF, whereas a two-component system comprising the complex sensor histidine kinase RpfC and the HD-GYP domain regulator RpfG is implicated in DSF perception. We have previously provided evidence that the HD-GYP domain of RpfG is a cyclic di-GMP phosphodiesterase. Consonant with these observations, we have shown recently that mutation of *rpfG* or *rpfF* leads to an increase in the cellular levels of cyclic di-GMP, whereas addition of DSF reduces cyclic di-GMP levels to wild type in the *rpfF* mutant but not in the *rpfG* mutant. Yeast two-hybrid analysis has indicated that the HD-GYP domain of RpfG can interact with a subset of GGDEF domain (cyclic di-GMP synthase) proteins. FRET analysis indicates that these interactions also occur within *Xcc*. We speculate that DSF signalling influences biofilm formation and dynamics through divergent signal transduction pathways that involve RpfG. This regulator may exert specific regulatory effects through protein–protein interactions with particular GGDEF domain proteins as well as having a broader regulatory influence by the impact on a global pool of cyclic di-GMP.

IL 7.6–4
Biofilm formation of members of the genus *Burkholderia* is dependent on cell-to-cell communication

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Taxonomic studies of the past few years have shown that *Burkholderia cepacia*-like organisms comprise a very heterogenous group of strains, collectively referred to as the *B. cepacia* complex (Bcc). Strains of the Bcc are ubiquitously distributed in nature and have been frequently isolated from water, the plant rhizosphere, the hospital environment, and industrial settings. *B. cepacia* complex strains have an enormous biotechnological potential and have been used for bioremediation of recalcitrant xenobiotics and biocontrol purposes. At the same time, however, Bcc strains have emerged as opportunistic pathogens of humans, particularly those with cystic fibrosis. An important factor that contributes to the great versatility of these bacteria is their ability

to form surface associated consortia, so-called biofilms, on various surfaces. To investigate the factors required for surface colonization random mini-Tn*5* insertion mutants were generated and analyzed both on the genetic and phenotypic level. The identified genes fell into several classes: (i) genes encoding for surface proteins, (ii) genes involved in the biogenesis and maintenance of an integral outer membrane, (iii) genes encoding regulatory factors, and (iv) genes involved in cell-to-cell communication (quorum sensing, QS). To investigate the role of QS in biofilm formation we mapped the QS regulon employing both transcriptomics and proteomics. QS-regulated genes that link cell signaling with the formation of surface-associated consortia, including genes encoding a type I pilus, lectins and a large surface protein, are currently analyzed in better detail.

OP 7.6–1
Galactomannan-based edible film: water vapour barrier

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Edible films and coating are of the highest interest since that have the potential to improve food shelf live and quality at the same time that protect them from deterioration by micro-organism and physical damages. The deterioration of packaged foodstuffs largely depends on the transfers that may occur between the packaged food internal environment and reduce water vapour permeability, oxygen, lipid, and flavour migration between components of the food products, and between food and the surroundings. These films are usually prepared with mixed high molecular weight compounds as proteins, lipids, and polysaccharides. The polysaccharide – protein blends show excellent selective permeability to O_2 and CO_2. Galactomannan (GAL) edible films plasticized by glycerol (GLY) water vapour permeability (WVP) were investigated in several conditions. The WVP increased significantly as the Gal and Gly concentration increased, with a positive effect on WVP values of the film. Combining the two variables the lowest level of WVP (83 g mm/dm^2 kPa) was found when the Gal and Gly concentrations were 0.4% and 1.0%, The model described the process adequately (R2 = 0.92), showing good agreement between experimental and predicted WVP, apparently due to the fact that plasticizing with glycerol reduces polymeric packaging density. On the other hand, the increased interactions between Gal molecules (hydrogen bonding type) increased with Gal concentration, therefore increasing the water vapour transmission rate. The effects of temperature and humidity on WVP were quantified with a Pareto analysis at 95% significant level, showing that in the range of conditions studied, both temperature and humidity were the influential variables. The effect of temperature (T) on WVP was more pronounced than that of relative humidity (RH). The interactive effects between T and RH were also significant, with WVP, increasing with increasing T and RH.

Acknowledgement: Supported by: Capes, CNPq, FUNCAP, α-VALNATURA, RENORBIO.

SYMPOSIUM 8: FROM BIOCHEMISTRY TO MEDICINE

8.1. Molecular Mechanisms of Kidney Diseases

IL 8.1–1
Transcriptional networks in human glomerular disease: markers of podocyte function and failure

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Current glomerular disease classification is based on descriptive classification of structural alterations. However, prognostic information has been limited. An international multicenter study was established and protocols developed to identify and validate molecular diagnostic markers and outcome predictors. Human renal biopsies have been obtained from 24 medical centers spanning four continents. More than 2500 biopsies have undergone manual microdissection of nephron segments and are available for gene expression analysis. Affymetrix HG-U133 based gene expression profiles from nine different renal diseases [focal and segmental glomerulosclerosis (FSGS), minimal change disease, membranous nephropathy (MGN), diabetic nephropathy, lupus nephritis (LN), IgA nephropathy, thin basement membrane disease, and controls] has allowed a network analysis approach to define relatedness of major human diseases on a molecular level. Transcriptional network analysis using automated promoter modeling tools combined with natural language processing was performed. Network analysis identified a shared transcriptome of progressive renal disease. From this shared transcriptome ridge regression analysis enabled glomerular filtration rate (GFR) prediction with a marker panel of 40 mRNAs independent of histopathology ($r = 0.78$, $p < 0.001$). This panel performed equally well in a second series with three independent disease cohorts ($r = 0.69$, $p < 0.001$) and allowed the classification of chronic kidney disease (CKD) stage III–V versus stage I–II with a sensitivity of 0.73 and specificity of 0.82. In an independent cohort this mRNA marker panel was able to correctly predict the CKD stage 36 months after renal biopsy (sensitivity 0.77, specificity 0.75). Transcriptional network analysis of the shared transcriptome observed in microdissected glomeruli of patients with FSGS, MCD and membranous nephropathy identified co-regulated gene sets and define shared promoter modules. This strategies allow to define distinct and share regulatory cascades in glomerular disease and can form the basis of a systems level understanding of human glomerular disease processes.

IL 8.1–2
Hereditary proteinuria syndromes and mechanisms of proteinuria

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Glomerular insults leading to end-stage renal disease (ESRD) are an enormous clinical challenge and socio-economic burden. Over two-thirds of kidney disease cases leading to ESRD initiate as glomerular injuries. Recent identification of gene mutations in some rare glomerular diseases have shown that defects in both GBM proteins, as well as proteins associated with the slit diaphragm can lead to progressive glomerular diseases characterized by malfunction of the glomerular filter. Thus, mutations in the genes for a the GBM specific type IV collagen trimer, α3:α4:α5, cause distorted GBM structure and hematuria (Alport syndrome)[1], while mutations in the laminin beta-2 chain gene cause proteinuria and nephrosis (Pierson syndrome)[2]. The discovery of nephrin as the protein affected in congenital nephrotic syndrome of the Finnish type, demonstrated a major role for the slit diaphragm in the glomerular filter[3]. The important role of the slit diaphragm structure for podocyte foot process function and contribution to the filter functions was substantiated by the discovery of podocin that is mutated in steroid resistant nephrotic syndrome[4], as well as by the discovery of Neph1[5]. Subsequent reports describing mutations in the genes for a-actinin-4[6] and TRPC6 cation channel[7] emphasized the role of the foot processes in maintaining a functional slit diaphragm. Despite the significant progress made in glomerular research during the last decade, the etiology of the bulk of the glomerulopathies, such as diabetic nephropathy, minimal change disease, membranous nephropathy, IgA nephropathy, lupus nephropathy remain to be clarified. This lack of knowledge hampers the development of novel diagnostic methods and identification of novel drug targets.

In order to get more insight into glomerular biology and mechanisms of diseases, we have undertaken a global approach to the vertebrate glomerulus to identify novel proteins important for glomerular development and the filtration function. Analyses of the glomerular transcriptome have yielded the existence of over 300 previously uncharacterized proteins, as well as several known ones that, in the kidney, are highly specific for glomerular cells[8,9]. This systems biology approach has facilitated the establishment of a protein interaction database[10]. Knockouts of several of those genes in mice, as well as knockdowns in zebrafish embryos have yielded genes that, when affected, cause abnormal glomerular development and/or proteinuria (unpublished data). Expression profiles of isolated glomeruli in various diseases leading to nephrosis have been elucidated and they show that despite a similar primary manifestation as proteinuria, they can have different pathways[11] (unpublished observations). It is likely that many of the novel glomerular proteins are involved in the mechanisms of human proteinuria and nephrotic syndromes.

References:

1. Barker DF, *et al*. Identification of mutations in the COL4A5 collagen gene in Alport syndrome. *Science* 1990; **248**: 1224–1 227.
2. Zenker M, Pierson M, Jonveaux P, Reis A. Demonstration of two novel LAMB2 mutations in the original Pierson syndrome family reported 42 years ago. *Am J Med Genet* 2005; **138**: 73–74.
3. Kestila M, *et al*. Positionally cloned gene for a novel glomerular protein–nephrin–Is mutated in congenital nephrotic syndrome. *Mol Cell* 1998; **1**: 575–582.
4. Boute N, *et al*. NPHS2, encoding the glomerular protein podocin, is mutated in autosomal recessive steroid-resistant nephrotic syndrome. *Nat Genet* 2000; **24**: 349–354.
5. Donoviel DB, *et al*. Proteinuria and perinatal lethality in mice lacking NEPHI, a novel protein with homology to NEPHRIN. *Mol Cell Biol* 2001; **21**: 4829–4836.
6. Kaplan JM, *et al*. Mutations in ACTN4, encoding alpha-actinin-4, cause familial focal segmental glomerulosclerosis. *Nat Genet* 2000; **24**: 251–256.
7. Winn MP, *et al*. A mutation in the TRPCG cation channel causes familial focal segmental glomerulosclerosis. *Science* 2005; **308**: 1801–1804.

8. Takemoto M, *et al*. Large-scale identification of genes implicated in kidney glomerulus development and function. *EMBO* J2006; **25**: 1160–1 174.
9. He L, *et al*. Glomerulus-specific mRNA transcripts and proteins identified through kidney expressed sequence tag database analysis. *Kidney lnt* 2007; **71**: 889–900.
10. He L, *et al*. The glomerular transcriptome and a predicted protein-protein interaction network. *J Am Soc Nephrol* 2008; **19**: 260–268.
11. Done SC, *et al*. Nephrin is involved in podocyte maturation but not survival during glomerular development. *Kidney Int* 2008; **73**: 697–704.

IL 8.1–3
The slit diaphragm: a signaling platform to regulate podocyte function. Symposion on the role of podocytes in the glomerular injury

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Diseases of the glomerular filter of the kidney are a leading cause of end-stage renal failure. Recent studies have emphasized the critical role of the slit diaphragm of podocytes for the size-selective filtration barrier of the kidney and revealed novel aspects of the mechanisms leading to proteinuria, both in inherited and acquired diseases. Several critical structural protein components of the slit diaphragm have been identified. Recently, it has been shown that slit diaphragm proteins are signaling proteins. This talk will focus on what is known about the importance of the podocyte for the function of the glomerular filter of the kidney. It will provide a snapshot of our current understanding of the signaling properties of slit diaphragm proteins and project a framework for further studies necessary to delineate the function and dynamics of the slit diaphragm protein complex and the pathogenesis of nephrotic syndrome.

IL 8.1–4
Oxidative stress in glomerular injury

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Reactive oxygen species (ROS) play an important role in the amplification of immune response, regulation of cell adhesion, programmed cell death and different signaling cascades, including MAPK, protein kinase C isoforms and transcription factors AP-1 and NFkappaB. In the kidney, ROS formation in glomerular podocytes may result in the podocyte loss followed by the loss of glomerular filtration rate. Renoprotective effect of angiotensin and aldosterone blockers, statins, and possibly also thiaziolidinediones may be at least partly mediated by the decreased ROS formation. In experimental nephrotic syndrome, puromycine aminonucleoside-induced ROS formation cause podocyte DNA damage resulting in the upregulation of cell cycle inhibitors leading to cell cycle arrest. In adriamycine nephropathy ROS-induced podocyte loss may be attenuated by the specific expresson of the antioxidant protein metallothionein in podocytes. In diabetic nephropathy both hyperglycemia and advanced glycation endproducts (AGEs) through its receptor (RAGE) induce podocyte apoptosis via the activation of MAP kinases (p38) and FOXO1 transcription factor and inhibit antiapoptotic pathways, e.g. PI3K, Akt mediated activation of Bcl-2. Except from that AGE-stimulated podocyte apoptosis is also mediated by the increased production of ROS with subsequent activation of TGFbeta regulated proteins as SMADs and Notch 1. AGE-induced podocyte apoptosis may be attenuated by soluble RAGE. AGEs may also induce cell cycle regulatory protein p27Kip1 with resultant cell cycle arrest and podocyte hypertrophy and AGEs also inhibit podocyte migration by downregulation of neuropilin-1. Decreased migration of podocytes can facilitate the formation of glomerular tuft adhesions to the Bowman's capsule contributing to the focal segmental glomerulosclerosis.

IL 8.1–5
Podocytes and mesangial cells in diabetic nephropathy

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On a molecular level, metabolic pathways activated by hyperglycemia as well as glycated proteins (AGEs) and oxidative stress are key players in the genesis of diabetic kidney disease. A variety of growth factors and cytokines are then induced through complex signal transduction pathways. Transforming growth factor-beta 1 (TGF-b1) has emerged as an important downstream mediator for the development of renal hypertrophy and the accumulation of mesangial extracellular matrix components. Podocyte-derived vascular endothelial growth factor (VEGF), a permeability and angiogenic factor, has been recently implicated in the development of diabetic proteinuria. Current research has focused on the podocyte as a victim of pathophysiological events associated with diabetic nephropathy and a target for the effects of high glucose, glycated proteins, mechanical stretch, and proteinuria itself. Decreased podocyte number and/or density, GBM thickening with altered composition, and foot process effacement are the principal features of diabetic podocytopathy. A reduction in nephrin protein at the slit diaphragm leads to podocyte foot process effacement with further proteinuria. Many of these events are mediated by angiotensin II whose local concentration could be stimulated by high glucose, mechanical stretch, and proteinuria. Angiotensin II in turn stimulates podocyte-derived VEGF, suppresses nephrin expression as well as induces TGF-b1 leading ultimately to an increase in podocyte apoptosis and fostering the development of glomerulosclerosis. Angiotensin II also enhances synthesis of a3(IV) collagen contributing to the increased thickness of the basement membrane in diabetic nephropathy. AGEs are actively taken up by podocytes and induce cell cycle arrest associated by hypertrophy. This effects is mediated by the cell cycle inhibitor p27Kip1. Furthermore, AGEs suppress migration of podocytes through down-regulation of neuropilin-1. AGE-induced inhibition of podocyte migration via down-regulation of neuropilin-1 likely contributes to the development of glomerulosclerosis because a reduced migration of podocytes fosters adherence of uncovered areas of the glomerular basement membrane to Bowman's capsule. Angiotensin II further enhance these effects by inducing upregulation of RAGE, a receptor for AGEs on podocytes. Interestingly, this effect is mediated by the AT2-subtype of the angiotensin II and is not antagonized by sartanes.

8.2. Proteases as Therapeutic Targets

IL 8.2–1
Dipeptidyl peptidase IV (DPP-IV) inhibition as a treatment for type 2 diabetes

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Defective insulin secretion is a hallmark of type 2 diabetes (T2DM), and agents that increase the concentration of circulating insulin have proven beneficial in the treatment of diabetes. Dipeptidyl peptidase 4 (DPP-4) inhibitors are a new oral approach to T2DM that lower glucose via stabilization of the incretin hormone glucagon-like peptide-1 (GLP-1), which has clearly established roles in insulin secretion and inhibition of glucagon production. These agents have been shown to produce clinically meaningful glucose control in a range of patients with T2DM without the many of the liabilities that are associated with other oral therapies, including weight gain, edema, GI intolerability, and hypoglycemia. Accordingly, DPP-4 inhibitors have excellent potential for use in both monotherapy and combination with established agents. Extensive structure-activity-relationship (SAR) studies in many different laboratories have resulted in the identification of a number of clinical candidates. The most advanced of these have recently been approved for the treatment of T2DM, representing the first new oral therapy for treatment of this disease since the introduction of troglitazone in 1998.

IL 8.2–2
Comparative studies on retroviral proteases

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Exogenous retroviruses comprise a large family of enveloped RNA viruses subclassified into seven *genera* and include at least two retroviruses (HIV and HTLV) that cause diseases in humans. In the last stage of their replication, the Gag and Gag-Pro-Pol polyproteins of the virions are processed by the retroviral protease (PR) at a limited number sites, causing ‘maturation’. The PR is a target of chemotherapy, and HIV-1 PR inhibitors are widely used in the treatment of AIDS. The first PR structures were determined 20 years ago, and currently this enzyme is the most represented one in the protein structural databanks. The PR is a homodimeric aspartic protease that recognizes at least seven substrate residues. Even though the retroviral proteases are fairly specific, they do not have consensus substrate sequences. Based on detailed specificity studies and crystallographic determinations, there appear to be a very strong sequence context dependence of their specificity. Currently there are nine protease inhibitors used in AIDS therapy, but due to the development of resistance, new compounds are continuously tested. Important features of the enzyme that may change as a consequence of resistance mutations are the activity and folding capability, substrate specificity, sensitivity to inhibitors and strength of dimerisation. Comparative studies of retroviral proteases suggested a relatively conserved specificity in spite of the great sequence diversity, while HIV-1 PR clinical inhibitors typically only weakly inhibited, if at all, the other enzymes. This apparent contradiction might be due to the fact that the clinical inhibitors are typically rigid molecules, while substrates are more flexible, and capable of adapting to altered substrate binding sites in different proteases. The same phenomenon is observed in drug resistant HIV-1 PR variants. Many of the mutations occurring in drug resistance introduce amino acids that can be found at the equivalent position in other retroviral PRs. Therefore, characterization of the similarities and differences of the specificities of retroviral proteases may help to design broad-spectrum inhibitors against HIV-1 PR.

IL 8.2–3
Protease inhibitors for the treatment of malaria

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Malaria remains one of the most important infectious diseases, and new antimalarial drugs are needed. Among new targets for chemotherapy are proteases. Proteases of all main catalytic classes play key roles in the biology of malaria parasites, including mediation of exit from and entry into host cells and the hydrolysis of hemoglobin. Intraerythrocytic parasites degrade hemoglobin in an acidic food vacuole to acquire amino acids and maintain homeostasis. Among proteases involved in this process are the cysteine proteases falcipain-2, falcipain-3, and dipeptidyl aminopeptidase-1, four plasmepsin aspartic proteases, and the metalloprotease falcilysin. Evidence supporting critical roles for falcipains include inhibitor studies in which specific inhibitors block hemoglobin hydrolysis and gene disruption studies in which parasites with knockout of falcipain-2 had altered hemoglobin hydrolysis and those with knockout of falcipain-3 were not viable. We have determined the roles of unique domains of falcipain-2, potentially identifying novel targets for chemotherapy. In addition, studies of active site-directed inhibitors have identified small molecules with good drug properties and potent inhibition of falcipain-2 and falcipain-3. Optimal compounds also block development of cultured malaria parasites at low nanomolar concentrations and, using a new animal model, cure mice infected with *P. falciparum.* To facilitate drug discovery, structures of falcipain-2 complexed with protein (cystatin, chagasin) and small molecule (E-64) inhibitors, and falcipain-3 complexed with leupeptin have been solved. Improved understanding of the inhibition and structural features of falcipains should facilitate drug discovery directed toward the identification of specific falcipain inhibitors as new antimalarial drugs.

IL 8.2–4
Glutaminyl cyclase, a degenerated aminopeptidase – a new causative target in Alzheimer’s disease therapy

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Post mortem analysis of brain samples from demented and non-demented persons with pathological aging revealed that the plaque load is almost equal in both groups. Moreover, neither BACE-1, Neprylisin, oxidative state nor Ab(1–42) seem to correlate with the neuropsychiatric symptoms of the Alzheimer’s patient group. Correspondingly, mice expressing human wildtype APP do not show deposits or plaque-like histopathology, but develop memory impairment and synaptic loss similar to mutant

APP-expressing mice, which primarily deposit Ab(1–40/42). Similar, newly designed animal models display N-terminal Ab heterogeneity as found in human sporadic AD and memory deficits long before extracellular deposits are detectable. These results support the notion that intraneuronal toxicity of Ab-peptides initiates neurodegeneration. In search of Ab-peptides most prone to form toxic oligomers, we found that Ab-peptides possessing a N-terminal pyroglutamic acid (pGlu) aggregate two orders of magnitude faster as compared to Ab(1–42). In addition, pGlu-peptides are potent seeds of Ab(1–42) oligomerization. Our *in vitro* and *in vivo* studies provide strong evidence for a slow Glutaminyl cyclase (QC, EC 2.3.2.5) catalyzed cyclization of N-terminal glutamic acid, substantiating a crucial role for generation of pGlu-Ab peptides. Characterization of QC catalysis cyclizing both glutaminyl and glutamyl residues revealed similar catalytic proficiency of QCs for the different substrates. Consequently, the formation of pGlu-Ab can be suppressed by QC inhibitors *in vitro* and *in vivo*. This supports a catalyzed formation of pGlu-modified amyloid peptides in neurodegeneration. Our efforts have led to new, Alzheimer mouse models and initiated the discovery of potent and selective compounds as new drugs (Schilling *et al.*, *Nat Med* 2008; **14:** 1106–1111).

IL 8.2–5
The structural basis of serpin function and dysfunction

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Serpins must undergo rapid conformational change in order to inhibit proteases. The energy stored in the metastable native state of the serpin is used to trap the protease in an inactive state. The complexity inherent in this mechanism provides opportunity for regulatory control, but also renders serpins susceptible to dysfunction. Intracellular polymerization of serpins underlies a diverse set of loss-of-function and gain-of-function disorders, including neurodegeneration, thrombosis, emphysema, cirrhosis and angioedema. Previous work has established that polymerization proceeds via a folding intermediate (denoted M*) and involves the expansion of the main β-sheet A from five to six strands. We recently solved the crystal structure of a self-terminating antithrombin dimer that revealed an unexpected 51-residue domain swap, including the intermolecular incorporation of both strands 4 and 5 into β-sheet A. This mechanism provides satisfying explanations to several important features of serpin polymers, including: why partial unfolding is required for polymerization *in vitro*; how polymers propagate; why polymers are hyperstable and yet highly susceptible to proteolysis; and how polymers form larger aggregates. We conducted several biochemical studies that support the general nature of this mechanism, and demonstrate that the previous model of partial strand 4A incorporation is incorrect. It is now possible to envision rational drug design strategies that target the propagation of serpin polymers, their lateral association, or the M* state itself.

OP 8.2–1
Design, structure and activity of potent HIV protease inhibitors based on inorganic polyhedral metallacarboranes

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We recently discovered and characterized a new class of potent HIV protease (HIV PR) inhibitors, substituted metallacarboranes (Cigler *et al.*, 2005). The crystal structure revealed a unique binding mode: two inhibitor molecules occupied hydrophobic pockets in the flap-proximal region of the S3 and S3' subsites of the enzyme active site. In a structure-guided drug design effort, we connected the two parent clusters with a linker and obtained set of compounds based on hydrogen imino bis-8,8-[5-(3-oxa-pentoxyl)-3-cobalt bis(1,2-dicarbollide)]di-ate. We explored inhibition properties of these compounds with various substitutions, determined the HIV PR-inhibitor crystal structure and computationally explored the conformational space of the linker. Our results prove the capacity of linker-substituted dual-cage cobalt bis(dicarbollides) as lead compounds for design of more potent inhibitors of HIV PR.

Reference:

1. Cigler *et al.*, *Proc Natl Acad Sci U S A* 2005; **102:** 15394–15399.

8.3 Biochemistry of Diabetic Complications

IL 8.3–1
Hyperglycemia, oxidative stress and diabetic complications: an overview
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Oxidative stress appears when an imbalance between free radical production and antioxidant defences is generated. This condition is largely present in diabetes, and hyperglycemia plays a major role. Assessment of oxidative stress in diabetic patients, both type 1 and type 2, evidenced the correlation between the pathology and oxidative stress markers. Different studies evidenced an increased production of reactive oxygen species, especially in those with poor glycaemic control. Both levels of oxidative stress markers and antioxidant status is compromised with a concomitant high glucose inhibited expression of antioxidant enzymes. Recent study led to asses oxidative stress in children and adolescents with type 1 diabetes show that diabetic patients had greater oxidative damage to lipids, proteins and DNA. All the oxidative stress markers were significantly raised at onset, decreased during the first 1.5 years of evolution, due to the started insulin therapy, and rose progressively thereafter. In type 2 diabetic patients, endothelial dysfunction, assessed by asymmetric dimethylarginine (ADMA) plasma levels, and oxidative stress levels, assessed by urinary 8-iso-prostaglandin (PG)F(2alpha) levels, were enhanced, while a soluble form of AGE receptor (sRAGE), whose interaction with AGE is able to moderate AGE contribution to vascular complications, is diminished. In recent years attention is growing around effects of acute glucose fluctuations with the high postprandial related glucose levels showing a more specific triggering effect on oxidative stress than chronic sustained hyperglycaemia. Postprandial hyperglycaemia seems to have a direct toxic effect on the vascular endothelium with a central role in cardiovascular disease development in diabetic patients.

IL 8.3–2
Drug-induced changes in biochemical indicators of diabetic vascular disease
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Oxidative stress plays major role in pathogenesis of diabetic vascular disease. Reactive oxygen species participate on the endothelial dysfunction development, the first step preceeding morphological impairment of vessel wall. Activated endothelial cell overexpresses the cell-adhesion molecule receptors and other compounds. It is therefore important whether drugs may protect endothelium against undemanded reactions. Specific effects on oxidative stress have been reported by several drugs. Metformin brings controversies with either increased or decreased oxidative stress. We found oxidative stress induction together with activation of antioxidant system following 3 months of metformin administration. Higher plasma malondialdehyde (MDA) concentration ($p < 0.001$) and erythrocyte superoxide dismutase (SOD) activity ($p < 0.01$) were associated with decreased cell adhesion molecules ($p < 0.001$) and fibrinolysis. Simvastatin activates vascular wall as measured by vonWillebrand factor ($p < 0.05$) and cell adhesion molecules ($p < 0.01$) but also it activates antioxidant system. On contrary, fenofibrate decreases plasma MDA concentration ($p < 0.001$) without significant changes in antioxidant system. Protective effects of hypolipidemic drugs on vascular endothelium were therefore reported. Thiazolidinedions (TZD) reduce oxidative and nitrative stress and increase nitric oxide bioavailability together with PKC pathway correction. Improved vascular function has been observed following TZD administration. Finally, angiotensin converting enzyme inhibitors (ACEI) or receptor blockers (AT1) reduce oxidative stress and improve NO bioavailability as well as they decreased intima-media thickness. In conclusion, several drugs have pleiotropic effects and they may reduce oxidative or nitrative stress in diabetes and thus improve vascular function. Such drugs have anti-atherogenic properties and their administration may protect vascular wall against chronic impairment.

IL 8.3–3
Transient high glucose causes persistent epigenetic changes and altered gene expression during subsequent normoglycemia
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The current goal of diabetes therapy is to reduce time-averaged mean levels of glycemia, measured as HbA1c, to prevent diabetic complications. However, HbA1c only explains < 25% of the variation in risk of developing complications. Because HbA1c does not correlate with glycemic variability when adjusted for mean blood glucose, we hypothesized that transient spikes of hyperglycemia may be an HbA1c – independent risk factor for diabetic complications. We show that transient hyperglycemia induces long-lasting activating epigenetic changes in the promoter of the nuclear factor κB (NF-κB) subunit p65 in aortic endothelial cells both in vitro and in nondiabetic mice, which cause increased p65 gene expression. Both the epigenetic changes and the gene expression changes persist for at least 6 d of subsequent normal glycemia, as do NF-κB – induced increases in proinflammatory gene expression, including monocyte chemoattractant protein 1 and vascular cell adhesion molecule 1. Hyperglycemia-induced epigenetic changes and increased p65 expression are prevented by reducing mitochondrial superoxide production or superoxide-induced α-oxoaldehydes. These results highlight the dramatic and long-lasting effects that short-term hyperglycemic spikes can have on vascular cells and suggest that transient spikes of hyperglycemia may be an HbA1c – independent risk factor for diabetic complications.

IL 8.3–4
Involvement of transcription factor nrf2 and antioxidant response element linked gene expression in countering oxidative stress in diabetes
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Transcription factor NF-E2-related factor-2 (nrf2) regulates gene expression through the antioxidant response element (ARE) promoter signaling system. Nrf2 regulates the transcription of a battery of protective and metabolic enzymes. Activation of the nrf2 system may enhance protection against damage to proteins by oxidation and glycation, enhance proteasomal removal of damaged proteins, down regulate lipogenic enzyme expression to correct dyslipidaemia and counter metabolic dysfunction by increasing pentosephosphate pathway activity. This may potentially reverse activation of multiple pathways of biochemical dys-

function during hyperglycaemia and associated diabetes linked to the development of vascular complications. In initial investigations, we assessed if activation of nrf2 by the dietary bioactive compound, sulforaphane (SFN) prevented metabolic dysfunction induced by hyperglycaemia in human microvascular endothelial cells *in vitro*. Activation of nrf2 by SFN induced nuclear translocation of nrf2 and increased ARE-linked gene expression. For example, 3–5-fold increased expression of transketolase and glutathione reductase. Hyperglycaemia increased the formation of reactive oxygen species (ROS) – an effect linked to mitochondrial dysfunction and prevented by SFN. ROS formation was increased further by knockdown of nrf2 and transketolase expression. This also abolished the counteracting effect of SFN, suggesting mediation by nrf2 and related increase of transketolase expression. SFN also prevented hyperglycemia-induced activation of the hexosamine and protein kinase C pathways, and prevented increased cellular accumulation and excretion of the glycating agent, methylglyoxal. Activation of nrf2 may prevent biochemical dysfunction and related functional responses of endothelial cells induced by hyperglycaemia in which increased expression of transketolase has a pivotal role. Activation of the nrf2 system may emerge as a potent new target of therapeutic intervention.

Reference:

1. Xue *et al*., *Diabetes* 2008; **57:** 2809–2817.

IL 8.3–5
Beyond oxidative stress - is there a cure for vasoregression in the diabetic retina?

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Diabetic retinopathy is characterized by progressive occlusions and increased permeability of retinal microvessels. The latter leads to impaired vision in patients with long-standing diabetes by affecting the macula, particularly in type 2 diabetes, the former causes neovascularizations in response to increasing tissue hypoxia typically in the eyes of type 1 diabetic patients. Vasoregression in diabetic animal models is preceded by pericyte loss which involves apoptotis, but also migration of subtypes of the pericyte population. Pericytes exert a protective role for endothelial cells in retinal capillaries, and pericyte loss may involve the activation of the Angiopoietin-Tie system which regulates vessel maturation during developmental angiogenesis. Angiopoietin-2 (Ang-2) is the natural antagonist of Angiopoietin-1 (Ang-1). Ang-2 is expressed in three cell types of the retina, i.e. the endothelial cell, the Müller cells, and the horizontal cells. Ang-2 is highly upregulated in diabetic animals before the onset of pericyte dropout, and the intravitreal injection and the intraretinal overexpression of Ang-2, both, trigger pericyte ejection from capillaries. Superposition of diabetes to Ang-2 overexpressing mice aggravate pericyte dropout and subsequent vasoregression. Ang-2 transcription is induced by hypoxia, and by hyperglycemia. Chronic hyperglycemia causes the overproduction of reactive oxygen species of mitochondrial sources which leads to inhibition of the glycolytic substrate flux in cells exposed to high glucose, and to the activation of biochemical pathways such as the hexosamine pathway, and to overproduction of alpha-oxoaldehyde-type AGEs. Methylglyoxal is the most abundant intracellular AGE. Methylglyoxal modifies the corepressor mSin3A in microvascular endothelial cells, resulting in recruitment of the enzyme O-GlcNAc transferase to an mSin3A-Sp3 complex. Subsequently, Sp3 modification by *O*-linked *N*-acetylglucosamine decreases its binding to a glucose-responsive GC box in the Ang-2 promoter and increased binding of Sp-1 by which Ang-2 transcription is activated. The same mechanism operates in retinal Müller cells consistent with *in vivo* data from retinae of diabetic rats and mice. The inhibition of metabolic pathways involved in AGE production, and the shift from toxic to non-toxic pathways have been successfull in the prevention of experimental retinopathy. Novel approaches include the combination of antioxidant with prosurvival effects on the retinal vasculature.

OP 8.3–1
Cogenic expression of FAT/CD36 in spontaneously hypertensive rats ameliorates insulin resistance and upregulates protein kinase C

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Insulin resistance and lipid accumulation has been implicated in the pathogenesis of cardiac disease in the metabolic syndrome. To search for mechanism, we examined a role of fatty acid transporter FAT/CD36 on protein kinase (PKC) delta, epsilon expression and triacylglycerols (TAG) content in spontaneously hypertensive rats (SHR). SHR that lack FAT/CD36 and exhibit disorders in lipid metabolism, impaired insulin action and glucose intolerance was compared with the genetically modified SHR-chr4 strain harbouring a segment of chromosome 4 from the strain Brown Norway which possesses FAT/CD36. Two weeks before sacrifice half of the animals were fed high-sucrose diet. PKC delta expression was determined by western blotting in particulate and cytosolic fractions from left ventricles. SHR-chr4 had lower insulin level, free fatty acid concentration in serum and higher relative amount of PKC epsilon in heart compared with SHR fed standard chow. The high-sucrose diet increased the serum insulin level in SHR-chr4 and the free fatty acid concentration in both strains. In heart of SHR-chr4, the high sucrose diet increased the relative amount of PKC delta in the particulate fraction by 51% while had no effect on SHR. In SHR the high-sucrose diet impaired the glucose tolerance during oral glucose tolerance test. Results indicate that expression of FAT/CD36 in the genetically modified SHR-chr4 improved tolerance to high sucrose diet induced insulin resistance and that PKC delta may play a role in this signalling pathway.

Acknowledgement: Supported by grant*:* MSM 0021620858.

8.4. Cancer Stem Cells

IL 8.4–1
Cancer stem cells: genetics, epigenetics and therapy

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The origin of cancer from tissue stem cells for each of the major causes of cancer will be presented and therapy directed to stem cells discussed. (i) Teratocarcinoma serves as the prototype for the development of cancer from germinal stem cells due to a field or niche effect, and for induction of differentiation of cancer stem cells (differentiation therapy). (ii) Exposure to chemicals was thought to induce cancer by de-differentiation. However, study of the cellular reactions of the liver exposed to chemical carcinogens shows that liver cancers arise from maturation arrest of liver stem and progenitor cells. (iii) Virus infections of adult tissues were also thought to induce de-differentiation, but viruses that cause cancer infect tissue stem cells (for example basal epithelial cells) with resultant continued proliferation of immature cells. (iv) Leukemias provide examples of specific gene rearrangements (mutations) that produce constitutive signaling lesions at various stages of differentiation (maturation arrest). Specific reversal of the molecular lesions is effective in restoring maturation. (v) Finally in gastric cancers associated with H. Pylori infection, hypermethylation of the gastric epithelial cells leads to field cancerization which is maintained by long-liver gastric stem cells. The stem cell theory of cancer implies that cancers are made up of the same cell populations as normal tissues: stem cells, transit amplifying cells and mature terminally-differentiated cells. Better ways to identify and characterize cancer stem cells are needed to be able to direct therapy to these cells. Conventional therapies are directed toward the proliferating transit amplifying cells. When these therapies are discontinued, the cancer will often re-grow from the cancer stem cells, which are not proliferating at the time therapy is administered. In order to prevent re-growth new therapies must be developed that are directed to the cancer stem cells.

IL 8.4–2
Epithelial-mesenchymal interaction: a definition of the colon cancer stem cell niche

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It is well described that colorectal cancer initiation and progression strongly depends on genetic alteration and thereby activation of the canonical Wnt signalling pathway. In tumors carrying for instance a mutated APC, a high Wnt signalling is predicted in all cancer cells. Nevertheless, by using a Wnt reporter GFP construct, we found that xenografts of human colorectal cancers display heterogeneous Wnt signalling, which correlates with the heterogeneous nuclear beta-catenin localization. We further show that the CSC marker CD133 and the clonogenic potential correlates with the Wnt activity suggesting that regulation of the Wnt signalling cascade in colorectal carcinomas harbouring APC mutations occurs and is involved in maintaining a functional colon-CSC compartment. This Wnt regulatory mechanism implicates an extrinsic mechanism of regulation, potentially via a niche-like organization. Whether a niche also applies for the organization of the CSCs remains unexplored. However, using a co-culture system, we showed that colonic myofibroblasts prevent differentiation of colon CSCs and regulate their clonogenic potential. We are currently defining the secreted paracrine factors and exploring their potential regulation on Wnt signalling. Moreover we found that CSCs can activate and recruit myofibroblasts in xenografts. Taken together these observations suggest the existence of an epithelial mesenchymal crosstalk that contributes to the extrinsic control of tumor maintenance.

IL 8.4–3
Tumor associated fibroblasts – important players in cancer

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Malignant tumors are widely spread in humans and they form a serious medical, economical and social problem. Despite of the progress in cancer therapy, the prognosis of many patients is not optimistic. Remarkable achievements in stem cell research delineated new horizons of the improvement of cancer therapy because the existence of cancer stem cell has been established. Normal tissue stem cells require highly specialized microenvironment necessary for the maintenance of their stemness. The positive role of tumor stroma in course of vascularisation of tumor bed has already been described. The sum of evidence that cancer stromal fibroblasts exert an important role in cancer progression is enlarged minimally in tumors of breast, pancreas, colon, prostatic gland, skin and oral cavity. When coculture normal keratinocytes with basal/squamous cell carcinoma associated fibroblasts, the phenotype of keratinocytes is heavily altered to resemble cancer cells. Upregulation of genes for regulatory factors/cytokines in tumor stromal fibroblasts seems to be responsible for their biological activity. Nature of these fibroblasts is not well known yet, but principally they can originate in local mesenchyme under the control of cancer cells or from tumor cells undergoing epithelial-mesenchymal transition. The participation of mesenchymal stem cells can be also possible. Summarizing these data, similarly to embryonic development, mesenchymal-epithelial interaction can play an important role in tumor progression and its management may be a promising future anticancer therapeutic tool.

Acknowledgement: Supported by Ministry of Education Youth and Sports of the Czech Republic, projects No. 1M0538, NPVII 2B06106 and 0021620806.

IL 8.4–4
Cancer stem cells in radioresistance

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Recent experiments support that cancer stem cells are a specific subpopulation of all cancer cells, while the vast majority of cancer cells are non-tumourigenic. The question whether cancer stem cells may respond differently to radiotherapy or combined modality treatment compared to the bulk of non-stem tumour cells is of high importance for the field. Recurrent tumours after radiotherapy originate by definition from at least one surviving

cancer stem cell while permanent local tumour control requires inactivation of all cancer stem cells. Local tumour control assays therefore functionally measure survival of the subpopulation of cancer stem cells, and can be considered as a gold standard in this respect. In contrast changes in tumour volume after therapy, i.e. tumour response, are governed by the changes in the mass of tumour cells, i.e. primarily by the non-stem cells. Today the vast majority of preclinical studies in cancer research use volume dependent parameters such as tumour regression or tumour growth delay as experimental endpoints. This carries the risk that new treatments may be optimized for their effect on the bulk of non-stem cancer cells, with no improvement in the curative potential. Experimental and clinical data provide evidence for the importance of cancer stem cell number and density for local tumour control after radiotherapy. Experiments suggest that the response of cancer stem cells and non-tumourigenic cells to radiation and combined treatment may dissociate. It is currently not clear whether this is caused by differences in the intrinsic radiation sensitivity between cancer stem cells and non-tumourigenic cells or whether other factors cause this effect.

IL 8.4–5
Regulation of normal and leukemic human stem cells in immune deficient mice

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The hallmark of hematopoietic stem cells is their motility, self-renewal and developmental potential. Blood forming stem cells are defined in transplantation assays based on their functional ability to migrate to the recipient bone marrow and to durably repopulate it with immature and maturing leukocytes that continuously replenish the blood circulation. Functional, preclinical models for normal and leukemic human stem cells were developed using immune deficient NOD/SCID mice as recipients. These models identified normal human SCID repopulating cells as well as SCID leukemia initiating cells, obtained from some myeloid (AML) and lymphoid (Pre B ALL) leukemias. Engraftment kinetics and repopulation potential of human AML and Pre B ALL stem and progenitor cells in transplanted immune deficient mice can predict clinical outcome. The chemokine SDF-1 is the only powerful chemotactic factor for both human and murine stem cells, which functionally express its major receptor CXCR4. Engraftment and repopulation of immune deficient mice by normal and leukemic human stem cells are dependent on SDF-1/CXCR4 interactions. Homing, retention, release and stem cell mobilization are tightly regulated processes, which involve bone turnover and an interplay between cytokines, chemokines, adhesion molecules and proteolytic enzymes. The roles of osteoclasts, CD44, MT1-MMP and RECK in stem cell regulation will be discussed. Both normal and leukemic human stem and progenitor cells functionally express neurotransmitter receptors, which are involved in regulation of their motility and proliferation. In summary, stem cells are directly and indirectly regulated by dynamic interactions of the nervous and immune systems with the microenvironment.

8.5. Proteomics in Health and Diseases

IL 8.5–1
Clinical chemistry and proteomics

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Diseases are either due to genes and/or the environment; the environment is divided in two: microbes & toxics. Many diseases including toxics and drugs can damage progressively multiple organs without any patient warning or symptoms. Patients can loose roughly up to 40 percent of several organ functions without any degradation of their well being in normal resting condition. Therefore, clinical biomarkers are needed to evaluate the presence and the development of organ damage. Historically, laboratory medicine has moved from metabolomics to proteomics, to transcriptomics and to genomics. The first few measured biomarkers were metabolites such as glucose or urea. The following biomarkers were proteins, especially enzymes. More recently gene transcripts and genes were used as biomarkers. The omic progression has brought new powerful tools especially in proteomics and mass spectrometry. Proteomic and proteomic derived methods offer today to laboratory medicine tools to discover biomarkers in cancer, to unravel blood peptide modifications due to toxics or to screen for drugs and metabolites. Several recent developments will highlight the impact of proteomics and small molecule screening in clinical chemistry and biochemistry.

IL 8.5–2
The coordination of phosphorylation and PTM in heart failure and with cardiac resynchronization therapy

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Cardiac resynchronization therapy (CRT) has become an effective clinical treatment for heart failure (HF) patients leading to improvement in heart function, clinical symptoms and survival. Yet, the molecular mechanisms behind the therapeutic benefit of CRT are not known. Our latest data shows that dyssynchrony-induced heart failure (DHF) and CRT affect the mitochondrial subproteome by altering specific key proteins in cellular redox control and oxidative phosphorylation (OxPhos) pathways, manifested as changes in both protein quantity and PTMs within the mitochondria. As well phosphorylation and other PTM regulate the myofilament proteome which are responsible for the contraction of the heart, itself. The interplay between the mitochondria and myofilament maybe through a coordination of phosphorylation pathways. Cardiac mitochondria and myofilament proteins isolated from the left ventricle of a large mammalian model of produced by 3 weeks of rapid pacing and compared to control unpaced hearts and hearts that underwent three additional weeks of either synchronized or desynchronized pacing. CRT resulted in increase efficient ATP production, reduced ROS production from complex I and increased enzymatic activity of ATP synthase, complex V. These functional improvements have been correlated to a number of novel phosphorylated amino acid residues. In fact, specific phosphorylations have now been correlated or causatively linked to different Oxidative phosphorylation functional aspects including enzymatic activity, supercomplex formation and ROS production. Phosphorylation changes also occur to the myofilament proteins including the identification of two novel sites that change with CRT. The overall, the phosphorylation changes results in improved myofilament ATPase activity and force production. We proposed that the role of phosphorylation is involved in the functional reversal seen with CRT.

IL 8.5–3
Ras nanoclustering and inter cellular transfer: biology, proteomics and exploitation for cancer therapy

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The recent recognition of new types of cell–cell communication pathways challenges classic theories of cell autonomy. Evidence of functional ‘proteome mixing’ among interacting cells, particularly immune cells, supports the notion that no cell is an island, and that even these ‘unsplittable’ units are actually non-autonomous. We present our results on the cell-contact-dependent intercellular transfer of proteins including oncogenic Ras and of microRNA. We show by that human lymphocytes acquire from the cells they scan the inner-membrane protein H-Ras, a G-protein vital for common lymphocyte functions and a prominent participant in human cancer. The acquisition of oncogenic H-RasG12V by natural killer (NK) and T lymphocytes had important biological functions in the adopting lymphocytes: the transferred H-RasG12V induced ERK phosphorylation, increased interferon-γ and tumor necrosis factor-α secretion, enhanced lymphocyte proliferation, and augmented NK-mediated target cell killing. Our findings reveal a novel mode of cell-to-cell communication allowing lymphocytes to extend the confines of their own proteome, which may moreover play an important role in natural tumor immunity. These phenomena suggest exciting new possibilities for proteome research, focusing on system-level proteomics that characterize cell contents and functions in the context of intercellular protein transfer.

IL 8.5–4
Profiling CD antigens on leukaemias with an antibody microarray

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Cluster of differentiation (CD) antigens are defined when two or more independently developed monoclonal antibodies exhibit similar patterns of reactivity with a standard panel of human cells. Monoclonal antibodies to surface CD antigens on leukocytes have been used for flow cytometry, and more recently to construct microarrays that capture live cells. These DotScan microarrays enable the rapid and highly parallel characterization of repertoires of CD antigens whose expression patterns may be correlated with discrete leukaemia subtypes, or used to define biomarker ‘signatures’ for non-haematological diseases. DotScan with fluorescence multiplexing enables profiling of CD antigens for minor subsets of cells, such as colorectal cancer cells and tumour-infiltrating lymphocytes from a surgical sample.

IL 8.5–5
Proteomics of anti-cancer drugs

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Clinical importance of biomarkers as a 'handle' for monitoring therapeutic response is of growing importance. Our study is focused on monitoring molecular mechanisms of response to anti-cancer drugs at earlier time intervals to detect relevant proteins that are responsible for primary changes in signalling networks that subsequently lead to irreversible anti-cancer processes. The effects of typical representatives of conventional chemotherapy at half-time to apoptosis were evaluated using classification of 2-D protein maps by multivariate analyses. Only about 2% of total variances represented differences that enabled grouping of relevant anti-cancer drugs. The proteins specific for the response to individual drugs as well as those overlapping among different types of drugs were identified. This aspect of the study was enhanced using additional proteomic approaches including quantitative mass spectrometry to monitor proteins typical for response to the group of platinum – based anti-cancer drugs.

Acknowledgement: We acknowledge support of grants from the Czech Ministry of School and Education (MSM6198959216 and LC07017) and Institutional Research Concept AV0Z50450515.

OP 8.5–1
Study of urinary proteomes in anderson-fabry disease

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Background: Anderson–Fabry disease (AFD), an X-linked genetic disorder with deficient α-galactosidase A activity, is characterized by kidney disease and kidney failure, particularly in affected hemizygous men. The main aim of this work was to analyze differences between healthy controls and AFD patients and to identify abnormal proteins using MALDI-TOF mass spectrometry (MS) as possible biomarkers of disease.

Material and methods: We studied 2D electrophoreograms of samples from AFD patients and healthy individuals. The proteins were separated by the isoelectric focusing method (IEF). The second dimensional separation was performed using SDS-PAGE. The proteins were then visualized by the Silver Method. Separated proteins were characterized by MALDI-TOF MS.

Results: We found that the urinary spectra of all samples with Fabry disease included identical proteins with molecular weight around 20–40 kDa. There was no significant difference in number of spots on the electrophoreograms from patients with Fabry disease compared with healthy donors. However some proteins in AFD patients′ samples were increased more than three times. Abundant proteins were characterized by MALDI-TOF MS included alpha-1-antitrypsin, alpha-1-microglobulin, prostaglandin H2 D-isomerase, complement – c1q tumor necrosis factor-related protein, and Ig kappa chain V-III. Possible glycosylation of Asn51 and Asn78 could occur in prostaglandin H2 D-isomerase.

Conclusions: AFD urinary proteomics revealed an increased secretion of several proteins. The difference in the amount and position of H2 D-isomerase could be due to different glycosylation in AFD and should be studied as a potential biomarker of the disease.