Structural insights into phosphoprotein chaperoning of nucleoprotein in measles virus

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2017

Guryanov , S , Liljeroos , L J P , Kasaragod , P , Kajander , T A & Butcher , S J 2017 , ' Structural insights into phosphoprotein chaperoning of nucleoprotein in measles virus ' , Instruct Biennial Structural Biology Conference 2017 , Brno , Czech Republic , 24/05/2017 - 26/05/2017 pp. 46 .

http://hdl.handle.net/10138/232485

instruct Integrating Biology

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Structural Biology Conference

Abstract Booklet

International Best Western Hotel, Brno

24th – 26th May, 2017

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Probing protein assemblies and interactions by hybrid mass spectrometry approaches

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Mass Spectrometry based proteomics has played a pivotal role in revealing the plethora of protein interactions that take place inside a cell, wherein proteins form protein assemblies and/or signalling networks. Especially using affinity purification of a tagged proteins followed by mass spectrometric analysis of its binding partners a wealth of data has been gathered revealing the all-embracing protein networks present in cells. Following the charting of all these interactions, a next step will be to gather more in-depth structural and functional information on these individual protein assemblies. This may come from in-depth high-resolution structural models, as well as detailed information on how they function and dynamically evolve during cellular perturbations. Mass spectrometry may also contribute to this next level of protein interaction analysis although it does require partly different and novel approaches. To contribute to this emerging new area in proteomics, our group is developing new methods using native mass spectrometry and cross-linking mass spectrometry with the aim to bridge the gap between interaction proteomics and structural biology. These new innovations and applications of them in interaction proteomics will be central in this presentation.

In the first part of the talk native mass spectrometry and its applications in probing protein assemblies and interactions will be described, focusing on examples wherein the dynamic assembly of a protein complex involved in the circadian timing in cyanobacteria will be highlighted. Herein, by using a combination of native, HD exchange and cross-linking mass spectrometry and cryoEM, we were able to define a novel structural model improving our understanding of the circadian rhythm. Additionally, novel developments in MS instrumentation for native MS will be highlighted, especially a new Orbitrap based instrument that offers high-sensitivity and mass resolution, allowing an in-depth detailed analysis of glycoproteins, viruses and even whole intact ribosomes.

The second part of the talk will highlight our recent work on cross-linking mass spectrometry. Cross-linking combined with mass spectrometry (XL-MS) provides another powerful approach to probe the structure and interaction profile of protein assemblies. Up to now XL-MS has been primarily limited to the characterization of purified protein assemblies. We have set out to develop XL-MS methods aimed at probing protein interactions at the proteome level, using complete cell lysates or whole organelles as starting material. We, therefore, combined several novel innovative methods to address some of the hurdles in this field. These innovations include the use of a low energy CID cleavable cross-linker, novel hybrid peptide fragmentation and acquisition strategies and a dedicated software suite, termed XlinkX. We applied this novel XL-MS strategy to lysates of *E. coli* lysate and human HeLa cell lines, and to mammalian mitochondria and nuclei. In each of these studies we successfully identified thousands of cross-links. Many of the identified cross-links could be validated by mapping them on available high-resolution structures, but the data also provide information on assemblies for which no high-resolution structures are available, and even reveal new protein interaction networks.

Integrative Structural Biology of Telomerase

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Telomerase is an RNA-protein complex that extends the ends of linear chromosomes, and is a highly regulated determinant of cellular aging, stem cell renewal, and tumorigenesis. We are using in integrated structural biology approach, combining NMR spectroscopy, X-ray crystallography, electron microscopy, mass spectrometry, and biochemistry to study the structure and function of telomerase. We previously reported the 3D structure of endogenously assembled *Tetrahymena thermophila* telomerase holoenzyme at 25Å resolution using negative stain electron microscopy. More recently, we determined the cryoelectron microscopy structure of *Tetrahymena* telomerase at ~9Å resolution. A pseudoatomic model of the complex was obtained by fitting solution NMR, X-ray crystal, and model structures of domains of RNA and proteins into the EM map. These include NMR structures of all of the telomerase RNA domains, including the essential pseudoknot, and characterization of two previously uncharacterized proteins, p19 and p45, by NMR and crystallography. In addition to the 7 known holoenzyme proteins, 2 new proteins were identified by mass spectrometry, which form a complex (TEB) with single-stranded telomere DNA-binding protein Teb1, paralogous to heterotrimeric Replication Protein A (RPA). Recent work shows that, remarkably, Teb2 and Teb3 are shared subunits with *Tetrahymena* RPA. The p75-p45-p19 subcomplex was identified as another RPA-related complex, CST (CTC1-STN1-TEN1). These studies provide a model of the telomerase core RNP (TER, TERT, p65) including the path of TER on TERT, reveal extensive subunit interactions of TERT TEN domain, p50, and TEB, and new subunit identities and structures. The structural data has provided a framework for understanding how TER, TERT, and other proteins from ciliate as well as vertebrate telomerase fit and function together and unexpected insight into telomerase interaction at telomeres. The constitutively associated p50-Teb1 appears homologous to the activating function of human TPP1-POT1, which transiently associates with human telomerase. Based on NMR structures of *Tetrahymena*, human and medaka TER domains and the pseudoatomic model of the *Tetrahymena* telomerase core RNP, we have built a model of the human TERT-TER core. We have also proposed a model for assembly of Tetrahymena TER with TERT based on solution NMR studies of the free TER.

Acknowledgments: This work was supported in part by grants from the National Institutes of Health (NIH) (GM048123) and National Science Foundation (NSF) (MCB1022379) to J.F.

Dynamic assembly of RNA polymerase II CTDsome

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The largest subunit of RNA polymerase II (RNAPII) contains a long and flexible C-terminal domain (CTD) that consists of tandem repeats of the heptapeptide consensus $Y_1S_2P_3T_4S_5P_6S_7$. Repetitive nature of the CTD, its dynamic phosphorylation patterns (CTD code), and structural variability make the CTD not only a unique platform to regulate interaction network of RNAP II, but also represent a great challenge for structural biology. To visualize the structural assemblies of CTD, we combined nuclear magnetic resonance (NMR) spectroscopy, X-ray crystallography and small-angle X-ray scattering (SAXS). This hybrid approach allowed us to probe the architecture of the CTD binding platform with processing and transcription factors. We will show the structural bases for (i) reading of the CTD code by several factors involved in transcription elongation and termination, (ii) degeneration of the CTD code, (iii) dynamic exchange of the CTD binders, and (iv) formation of the CTDsome architecture.

On Bugs, Revolution and a Very Sweet Solution

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Trypanosomatids are single cell eukaryotic protozoan parasites that pose a serious health concern afflicting more than 20,000,000 people worldwide. The most notable pathogens belonging to this order are *Trypanosoma brucei*, *Trypanosoma cruzi* and *Leishmania* species which cause African sleeping sickness, Chagas' disease and Leishmaniasis, respectively. The often fatal outcome of infections, lack of effective vaccines, limited selection of available therapeutic drugs, and emerging resistant strains, underline the need to develop novel strategies to combat these pathogens. The Trypanosomatid ribosome has recently been highlighted as a promising therapeutic target due to structural features that are distinct from other eukaryotes. In a serious of structural studies exploiting the recent advances in the field of electron-cryo microscopy (cryo-EM) we revealed the unique architecture of the Trypanosomatid ribosome at extremely high resolutions (2.5-2.8Å) identifying unique elements that play a significant role in ribosome assembly and function as potential drug targets. Additional studies in active Trypanosomatid ribosome complexes also provide atomic resolution snapshots into Trypanosomatid translation and unveil the mechanisms by which anti-ribosomal drugs with promising anti-protozoan properties induce their deleterious effects on the translation apparatus and inhibit parasite growth.

The Origin Recognition Complex – where it all begins

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Binding of the Origin Recognition Complex (ORC) to origins of replication marks the first step in the initiation of replication of the genome in all eukaryotic cells. I will present the structure of the active form of human ORC determined by X-ray crystallography and cryo-electron microscopy. The complex is composed of an ORC1/4/5 motor module lobe in an organization reminiscent of the DNA polymerase clamp loader complexes. A second lobe contains the ORC2/3 subunits. The complex is organized as a double-layered shallow corkscrew, with the AAA+ and AAA+-like domains forming one layer, and the winged-helix domains (WHDs) forming a top layer. CDC6 fits easily between ORC1 and ORC2, completing the ring and the DNA-binding channel, forming an additional ATP hydrolysis site. Analysis of the ATPase activity of the complex provides a basis for understanding ORC activity as well as molecular defects observed in Meier-Gorlin Syndrome mutations.

Towards Sarcomeric Z-disk Structure: by Lego Building Blocks

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The sarcomere is the minimal contractile unit in the cardiac and skeletal muscle, where actin and myosin filaments slide past each other to generate tension. This molecular machinery is supported by a subset of highly organised cytoskeletal proteins that fulfil architectural, mechanical and signalling functions, including the giant proteins titin, obscurin and nebulin as well as the cross-linking proteins α-actinin and myomesin.

The cross-linking of actin and myosin at the boundaries of their filamentous structures is essential for the muscle integrity and function. In the Z-disks – the lateral boundaries of the sarcomere machinery – the protein α-actinin-2 cross-links antiparallel actin filaments from adjacent sarcomeres, and additionally serves as a binding platform for a number of other Z-disk proteins. In striated muscle cells, the Z-disk represents a highly organized three-dimensional assembly containing a large directory of proteins orchestrated in a multi-protein complex centered on its major component α-actinin, with still poorly understood hierarchy and three-dimensional interaction map. On the way to elucidate the molecular structural architecture of the Z-disk, the hierarchy of its assembly and structure-function relationships, we are studying binary and higher order sub-complexes of α-actinin using biophysical, structural and cell biological approaches.

Here we will present recent data on interaction of muscle α-actinin and filamin C with an adaptor proteins ZASP, myotilin and FATZ-1, forming a fuzzy complex with the latter, and discuss findings in view of muscle Z-disk architecture and assembly.

Using structural biology to make novel molecules

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Various peptide macrocycles originating from natural products have been known for a long time to be effective medicines for a number of conditions.
More recently these moleciles have attracted considerable interest as potential therapeutic starting points particularly in disrupting protein protein interfaces.
Our lab has focussed on the enzymes involved in patellamide biosynthesis following the ground breaking work of Schmidt in mapping out the gene cluster.

Patellamides are the exemplar of the cyanobactin class of macrocyclic peptides and contain multiple chemical modifications. We have characterised all the
enzymes in the pathway at both structural and mechanistic level. We have used this information to engineer the enzymes to be useful for in vitro synthesis.

We have been able to make peptide non-peptide hybrid macrocycles and a small library of macrocycles which we tested for biological activity. I will report our progress on
combining solid phase synthetic chemistry to generate libraries coupled to enzymes. I will discuss very recent work on new classes of modifying enzymes that are not in the patellamide pathway but have significant potential.

The molecular toolbox for building axonemal microtubules

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Eukaryotic cilia and flagella are complex cell organelles and play important roles in various cell, such as propeller and antenna. Although they consist of hundreds of different proteins, those proteins are precisely organized by self-assembly mechanisms. For example, outer and inner dyneins are precisely arranged along the microtubule with 24-nm or 96-nm repeats, respectively. By studying cilia/flagella, we will be able to learn how to use "toolbox" to build intricate machine by bottom-up route with sub-nanometer precision.

To address this question, we combine genetics and structural biology. We have developed a novel method for identifying the 3D locations of proteins by combining genetic manipulation of model organisms to introduce biotin-tag to specific genes and cryo-electron tomography (cryo-ET). Using this method, we studied the FAP59/172 complex. The two proteins form a complex, and their absence disrupts 96-nm repeats in axonemes. Cryo-electron tomograms revealed that the FAP59/172 complex takes a 96-nm-long extended conformation along axonemal microtubules. Elongation of the complex resulted in extension of the repeats and duplication of specific axonemal components. We conclude that the FAP59/172 complex is the molecular ruler that defines 96-nm repeats in cilia/flagella.

We have also recently identified the new class of microtubule associated proteins, which bind to the lumen of axonemal microtubules. These proteins are called MIPs (microtubule inner proteins) and the structures and their functions became clear by combining genetics, cryo-ET, and high-speed AFM.

Identification, characterization and structural analysis of the N-terminal domain of human ryanodine receptor 2

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Ryanodine receptors are so far known largest ion channels. They are located in the membrane of sarcoplasmatic/endoplasmatic reticulum. Their main physiological role in organism is a transfer of calcium cations into the cytoplasm which leads to a cascade of reactions resulting in muscle contraction [1]. In mammals there were identified three isoforms of this channel, which are predominantly expressed in skeletal muscle (RyR1), myocardium (RyR2) and different tissues (RyR3). Dysfunction of RyR channel causes serious muscle diseases – myopathies (RyR1 dysfunction) [2], tachycardias and arrhythmias (RyR2 dysfunction) [3] and Alshaimer's disease (RyR3 dysfunction) [4].

In our laboratory we predominantly carry on structural studies of RyR2 isoform from human (hRyR2) by combination of "*in silico*" and "*in vitro"* approaches. Availing bioinformatics analysis we have identified 14 domains in the hRyR2 channel [5,6], some of which were cloned, expressed and purified in *E.coli* bacterial expression system [5]. The fragment involving first two and the core of the third N-terminal domain was crystallized [7] and its structure has been determined by X-ray and SAXS analysis [8]. At present, we have cloned, expressed, purified, and partially characterized the prolonged hRyR2 N-terminal fragment which includes a dantrolene-binding site. The SAXS structure of this fragment has been also determined. This helps us to understand the contribution of third N-terminal domain in stability of hRyR2 channel. "*In silico"* analysis of selected residues, mutation of which are responsible for serious hearth diseases, helps us to understand their role in the structure and function of hRyR2 channel.

Acknowledgement: This work has been financially supported by grants VEGA no. 2/0140/16 and Grant Agency for Research and Development APVV-0628-10.

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Transporting cargos over long distances: insight from dynein/dynactin structures

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Cells depend on components being moved to the correct place at the correct time. My group is interested in cytoplasmic dynein-1 (dynein-1), a motor which delivers many different cargos via the microtubule network. When dynein-1 is mutated it leads to neurodegeneration and it is susceptible to hijack by viruses which use it to travel into the cell. We have determined X-ray crystal structures of the dynein motor before and after it binds and hydrolyses ATP. We subsequently used single molecule fluorescence assays to show how a cofactor, dynactin, activates the full length 1.4MDa dynein complex to move long distances along microtubules. A high resolution (4.0Å) cryo-electron microscopy (cryo-EM) structure of dynactin explained how this 23 subunit complex is assembled. We also used a combination of cryo-EM and X-ray crystallography to show how dynactin binds to dynein. The two complexes are only brought together in the presence of an adaptor protein, Bicaudal-D2, that links them to the cargo they will carry. This suggests the large and intricate dynein/dynactin transport machine only assembles when a cargo is ready to move.

Solving the structures of small molecules with the Volta phase plate

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Recently, we developed the Volta phase plate (VPP) for transmission electron microscopy. It greatly improves image contrast and holds promise for further performance gains in cryo-electron microscopy (cryo-EM).

Currently, we are investigating the advantages of the VPP for cryo-EM single particle analysis. The initial results are very encouraging.

We expect that the VPP will enable cryo-EM studies of "difficult" macromolecular complexes in terms of small size, heterogeneity and flexibility.

Haploid Genetics to study Pathogen entry and Genetic Interactions

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Basic research carried out over the last thirty years in the field of molecular biology has revolutionized our understanding of cell biology. However, that even with today's knowledge it remains largely impossible to predict key players in networks related to human disease. Therefore, our goal is to advance genetics in human cells in order to obtain accurate and complete overviews of genes that play a role in phenotypes of interest and to map genetic networks that enhance or suppress disease-related cellular states. Using random mutagenesis in haploid human cells we apply a sensitive approach to directly couple genomic mutations to protein measurements in individual cells.

This scalable, sequencing-based procedure elucidates the genetic landscapes that control protein states, identifying genes that cause very narrow phenotypic effects and genes that lead to broad phenotypic consequences. Besides this, we also study genes that are needed for the entry of pathogens and the principles of genetic interactions in human cells.

"Decoding protein-RNA recognition in gene regulation by integrated structural biology"

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We study molecular mechanisms of multidomain proteins and the role of conformational dynamics during molecular recognition of RNA and proteins by these factors. Solution NMR-spectroscopy and X-ray crystallography is combined with complementary techniques, such as Small Angle X-ray and/or Neutron Scattering [1,2] or FRET to unravel conformational dynamics and population shifts in protein-protein and protein-RNA molecular recognition.

3' splice site recognition involves binding of the U2AF heterodimer to the intron RNA. We have previously reported that the tandem RNA binding domains (RRM1,2) of the large U2AF65 subunit adopt closed/inactive and open/active arrangements free and when bound to Py-tract RNA ligands, respectively [2,3]. Recently, we found that a dynamic interaction of the linker connecting the tandem RRM domains plays an auto-inhibitory role to discriminate against weak (low affinity) Py tracts. Furthermore, by combining FRET and NMR experiments, we demonstrate that the small U2AF35 subunit induces a population shift of the conformational equilibrium of U2AF65 RRM1,2 towards the RNA-accessible open state, providing an unexpected molecular explanations for U2AF function [4].

We have elucidated structural mechanisms of protein-protein interactions involving dynamic binding and avidity effects in the recognition of spliceosomal Sm proteins by OCRE [5] and Tudor domains will be discussed.

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Purification and structural studies of a Tremella fuciformis mushroom lectin

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Lectins are carbohydrate-binding proteins of non-immune origine widely distributed in living organisms. They play a role in different biological processes, serve as storage proteins, are fundamental during fungi and plant morphogenesis and development and take part in their defense processes [1]. Due to their carbohydrate specific binding, some lectins are able to recognize, in a reversible way, the sugar moieties present on the surface of erythrocytes (N-acetylgalactosamine, D-galactosamine), causing a phenomena called hemagglutination. Furthermore some lectins have been found to possess antitumoral properties [2]. Specifically they recognize the Tn-antigenic determinant (Galβ1-3GalNAcα) on the malignant cells surface causing apoptosis, cytotoxicity, inhibition of tumor growth and preventing the proliferation of tumor cells. Considering the fact that this kind of residues are masked on healthy cells, the highly specific carbohydrate-lectin interaction can be exploited to target malignant cells. The Tn-antigen is the most specific human cancer-associated structure, expressed in about 90% of human carcinomas. Although the function and biological properties of several lectins have been determined, there are still many lectins that remain to be structurally and functionally characterized. As reported in the literature, some Tremella fuciformis proteins have been investigated for their potential therapeutical properties [3] and in the light of this, we have examined the crude extract proteins of this fungus to assess the
presence of lectins.
A lectin of 22 KDa was isolated and purified from the dried fruiting bodies and used for testing several crystal screening conditions. Crystals were grown in 0,1 M TRIS pH 8.5, 1,5 potassium phosphate dibasic and preliminary data sets were collected at the ESRF of Grenoble. The space group is P21 and the cell parameters are a= 61,6 Å, b= 61,8 Å, c= 67,8 Å with β= 106,87 °. The highest resolution of these crystals is 1,5 Å and the total number of reflections collected were 740651. Dynamic Light Scattering (DLS) analysis reveals that TFL is a monomer under normal conditions. The distribution plot shows a size distribution of 2,9 nm ± 0,2 nm, with a polydispersity index (PDI) of 0,4 ± 0,1. Thermal protein stability was examined by means of differential scanning calorimetry, while chemical and pH-induced unfolding was investigated using fluorescence spectroscopy. Isothermal titration calorimetry yielded preliminary data on sugar binding, justifying a more detailed study to be undertaken in the future. It has also been observed that Tremella fuciformis lectin shows no cytotoxicity on malignant and healthy cells and its antitumoral properties are currently being investigated.

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Molecular chaperones in action

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Scarcity of high-resolution structural data has impeded an understanding of the recognition and anti-aggregation mechanisms of molecular chaperones. We recently reported the first ever structures of molecular chaperones in complex with unfolded proteins. We used advanced NMR spectroscopy techniques and isotope labeling approaches to determine the solution structure of the 50 kDa Alkaline Phosphatase (PhoA) captured in the unfolded state by three molecules of the Trigger Factor (TF) chaperone (~50 kDa), forming a ~200 kDa complex in solution [1]. We determined the high-resolution structure of each one of the TF molecules in complex with the corresponding unfolded PhoA region and reported how a chaperone dynamically engages its substrate. Very recently, we reported the structure of SecB, a chaperone that exhibits strong antifolding activity, in complex with PhoA and maltose binding protein (MBP) captured in their unfolded states [2]. The structural data revealed a unique complex architecture that explains the activity on the chaperone. Taken together, the data show how the different architectures of chaperones result in distinct binding modes with client proteins that ultimately define the activity of the chaperone.

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Recent improvements in the BioSAXS setup at synchrotron SOLEIL

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SWING is the SAXS/WAXS beamline at Synchrotron SOLEIL, dedicated to structural biology and soft condensed matter. SWING has pioneered SEC-SAXS set-ups, now available at all BioSAXS beamlines in modern synchrotrons. Agilent HPLC devices directly coupled to the in-vacuum BioSAXS measurement cell are available for online purification since 2007 [1]. The HPLC equipment has since been doubled to obtain two independent circuits of purification. A complete elution profile can be obtained in 12 min with enough resolution to separate BSA dimer from monomer. The SEC-SAXS procedure results in an optimized buffer subtraction and a range of concentration with a single injection. A series of up to 54 injections for SEC-SAXS can be programmed and parameterized independently. The twin column system allows recording data with two different buffer conditions without waiting for the next column equilibration. An efficient sample injection automated protocol has been developed, making possible to alternate automated direct injection and SEC-SAXS experiments using a unique interface and without any manual intervention. We specifically report on two recent improvements:

1/ Combination of the SEC-SAXS system with MALS and RI is now available. A Peltier-thermostated Wyatt HeleosII MALS device including 18 detectors, one of which devoted to DLS, and a Optilab T-rEX dRI detector, can be plugged in the elution circuit at user request. These two devices are expected to provide online biophysical data which are complementary to SAXS, and could be particularly useful for macromolecular complexes with components of different nature.

2/ In complement to the still available data reduction application Foxtrot, an automated workflow, called AutoBioSAXS, now runs permanently during both direct injection and SEC-SAXS experiments. Based on the Foxtrot libraries, this workflow automatically reduces the 2D experimental images to 1D curves for both buffer and sample frames immediately after the files are created. It then selects which buffer frames should be averaged, performs subtraction of the buffer averaged curve, and selects the subtracted curves that should be further averaged. The workflow then uses the Atsas suite [2] to analyze the final curve and publishes the results into the IspyB database [3]. The information is then remotely accessible via a web interface accessible from the Soleil User Interface.

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Biochemical reconstitution of (parts of) chromosome segregation

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During cell division, each daughter cell receives from its mother an exact, full copy of the genome. For this to happen, the sister chromatids in the mother cell must bi-orient on the mitotic spindle, a self-organizing structure made of microtubules, microtubule motors, and microtubule-associated proteins. Sister chromatid separation at the metaphase-toanaphase transition then leads to equal segregation of the genome to the two daughters. Chromosome attachment to spindle microtubules takes place at complex protein structures named kinetochores, which contain multiple copies of as many as ~30 individual core subunits, as well as a vast array of regulatory subunits. Our approach to the study of kinetochores has been largely relying on biochemical reconstitution of its subunits, focusing on the human kinetochore as our preferential model system.

A highlight of this effort was the reconstitution of a 21-subunit kinetochore particle capable of bridging the DNA-rooted part of the kinetochore (a specialized nucleosome structure containing the histone H3 variant CENP-A, which is the kinetochore's hallmark) with microtubules (Weir *et al.* Nature 2016). These particles are now being harnessed in ur laboratory to approach fundamental question of kinetochore regulation, including the mechanism of force sensing that controls the ability of kinetochores to stabilize bioriented microtubule attachments over incomplete or incorrect attachments and to regulate the spindle assembly checkpoint.

Over the years, the kinetochore reconstitution approach in our laboratory has also supported a rich structural biology program targeting individual kinetochore subunits or complexes. Highlights of this work included the determination at high-resolution of structures of several components of the KMN network, a 10-subunit complex that controls the complex functions of the microtubule-binding moiety of the kinetochore (Ciferri *et al.* Cell 2008; Petrovic *et al.* Mol. Cell 2014; Petrovic *et al.* Cell 2016; Huis in 't Veld *et al.* eLife 2017).

With the recent reconstitution of the chromatin-proximal components of the kinetochore (the so-called constitutive centromere associated network, or CCAN) in our laboratory, and with the introduction of negative-stain and cryo electron microscopy (EM) methods, we are now able to tackle the structure of near complete kinetochore complexes. With their initial structural investigation, we are for the first time in a position to address the core kinetochore structure and reveal its plan of assembly. The implications of this work will be discussed.

Structure and function of contractile injection systems

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The process of protein and DNA translocation across lipid membranes is central to the function of any organism. Bacteria have evolved several distinct pathways for secreting DNA and proteins into the external milieu and sometimes directly into the cytoplasm of neighboring cells. The Type VI Secretion System (T6SS) represents one such pathway that translocates a range of substrates that vary in size and properties. Interestingly, there is no unique sequence motif or any other signature that targets these proteins for secretion. T6SS is orthologous to a contractile tail of a bacteriophage, which is used by the phage for translocation of proteins and DNA in the opposite direction — from the capsid located outside of the cell into the cell's cytoplasm. Functionally and structurally, T6SS and phage tail resemble a stretched spring (or sheath) wound around a non-contractile tube that carries a spike-shaped protein at its tip. Upon interaction with a target cell in the case of phage or certain environmental signals in the case of T6SS, the sheath contracts and drives the tube through the cell envelope. The contraction is accomplished by a massive structural rearrangement of the sheath in which a mesh-like topology of the sheath is preserved and the subunits move as rigid bodies. I will discuss our current level of understanding of this remarkable structural transformation.

Trends in scattering and imaging in structural biology

Professor Dave Stuart
University of Oxford

Currently 130,000 macromolecular structures are available through the protein data bank and with the revolution in electron microscopy surely the sky's the limit? I will try to give a flavour of where scattering and imaging methods currently stand, where the bottlenecks are, where we might be in a few years and how structural biology, integrated by default, will broaden its scope to survey the cellular context in molecular detail. It is possible now to conceive this extension, what remains more difficult to predict are the next steps, to realise the 2005 vision of Dino Moras of 'watching molecules dancing in the cell', so I will consider briefly where we are with capturing dynamics with these scattering/imaging methods that have been honed to deliver snapshots. Examples will be mainly of viruses and their interaction with cells

Instruct Centre-France 1

Alberto Podjarny and Bruno Klaholz

The Instruct Centre-France 1 which coordinates the French Infrastructure for Integrated Structural Biology (FRISBI) (IGBMC/CBI, Strasbourg) provides a cutting-edge technological environment for integrative structural biology approaches, from the molecular to the cellular level, with an emphasis on protein production linked with preparation of samples for EM and crystallography.

Flagship platform: Cryo electron microscopy (Titan Krios, Polara, FIB-SEM, Tecnai F20, super-resolution microscopy)

Protein production and biophysical characterization

Protein engineering and production are essential tools for structural, biophysical and functional studies as well as for biotechnology and medical applications. The Strasbourg centre offers a state-of-the-art infrastructure for the production of biomolecules and biomolecular complexes in *E. coli*, yeast, insect cells (baculovirus expression system) as well as in mammalian cells using an attenuated vaccinia virus vector. The Strasbourg platform has a unique expertise on production of recombinant multi-protein complexes as well as on purification of endogenous complexes using the TAP-tag technology in yeast.The biophysical platform monitors protein properties and function, including protein stability, protein complex formation, oligomerization, protein interactions and shape determination by SAXS.

Integrated Structure determination

The Strasbourg centre provides core-technologies for structure determination by crystallography, cryo-EM and tomography. Strasbourg is a centre of expertise for crystallographic analysis of protein-protein and protein-nucleic acid complexes (nano and micro-automated screening and HTP crystal production, data collection and structure determination) and cryo-electron microscopy/tomography using cutting-edge instrumentation (Titan Krios & Polara electron microscopes with direct electron detectors and FIB-SEM).Since the opening of the infrastructure, the Instruct centre France 1 accepted more than 40 Instruct projects corresponding to 78 visits for protein production, biophysical characterisation, crystallization, and structural analysis by cryo electron microscopy and X-ray crystallography.

CERM/CIRMMP- Italian Instruct Center Instruct Biennial Meeting

The Instruct Centre-CERM/CIRMMP-Italy which coordinates the Italian Infrastructure for Integrated Structural Biology provides a cutting-edge technological environment for integrative structural biology approaches, CERM has developed a unique strategy for in-cell NMR, based on protein expression and labelling in human cells

Flagship platform: The NMR platform includes a number of spectrometers, operating at 950 MHz, 900 MHz, 800 MHz, WB 800 MHz, 2x 700 MHz, WB 700 MHz, 2x600 MHz, 500 MHz, and 400 MHz spectrometers. Each instrument is equipped with several probes to meet all conceivable experimental conditions.

Users are provided with access to the NMR instrumentation and expertise to perform the most comprehensive array of NMR experiments needed for the structure and dynamic characterization of biological macromolecules and their complexes. These services will allow users to attain fundamental molecular level information on cellular pathways which represent targets of interest in human health and diseases.
The NMR platform offers unique research capabilities in the field of high-resolution NMR of proteins, with a special expertise in metalloproteins, for:
i) solution structure determination of biomolecules and of their complexes, ii) investigation of protein-drug interactions, iii) methodological advancements exploiting specific properties of large and/or paramagnetic biomolecules and iv) study of immobilized macromolecules.
The NMR platform offers in-cell NMR expertise for the investigation of biological macromolecules in their physiological context, for both in bacterial and human cell studies. Users have access also to Solid State NMR spectroscopy measurements. CERM/CIRMMP has experience with several different kinds of biosolids: microcrystalline proteins, amyloids and fibrils, and sedimented solutes. CERM/CIRMMP has a long tradition in the development of new NMR methods for the determination of paramagnetic effects in the solid state to access structural information.

Electron Microscopy at Grenoble, France

Instruct– France Grenoble

The EM platform is at the Institute of Structural Biology (IBS) in Grenoble. The platform provides user access via European Instruct and French FRISBI infrastructures and is certified ISO9001. It has two components: the first is the structural study of isolated particles by negative staining and cryo-electron microscopy coupled to image analysis; the second, cellular EM, focuses on the structural and morphological studies of cellular samples (bacteria, cells, tissues). Over the past 18 months, the entire electron microscopy platform has welcomed 80 users from 30 research teams in 12 different national organizations (10 academic and 2 industrial). Currently 20-25% of the Polara

Instruct-NL

Reinout Raijmakers
Utrecht University

Instruct-NL is both the Dutch national Instruct Centre and a community encompassing all main areas of structural biology research within the Netherlands. Instruct-NL consists of several partners and covers a wide range of structural biology techniques. The Bijvoet Center of Utrecht University contributes biomolecular NMR spectroscopy, native mass spectrometry and proteomics, computational structural biology and protein crystallography. The Protein Facility of the Netherlands Cancer Institute provides access to biophysical characterization of proteins and the Netherlands Centre for Electron Nanoscopy (NeCEN) at Leiden University offers access to two FEI Titan Krios microscopes for cyro-electron microscopy

Instruct Centre-UK for Integrated Structural Biology

Instruct - UK

The Instruct-UK Centre brings together user facilities from the University of Oxford and Diamond Light Source to address challenging problems that require an integrated approach to Structural Biology. The Centre offers technology and support from sample preparation to structural analysis with a particular focus on protein production in mammalian cells. Access to specialised crystallization methods for integral membrane proteins are available at the Membrane Protein Laboratory which is closely linked to the beamlines at Diamond. In addition, cryoEM, biophysical analysis and mass spectrometry are all provided by groups at the University of Oxford.

Virus Purification

Centre for Virus and Macromolecular Complex Production (ICVIR)

Our platform provides large amounts of pure viruses and macromolecular complexes for structural studies using ultracentrifugation and chromatographic methods. The platform is equipped with seven ultra and four superspeed centrifuges as well as gradient making and fractionation instrumentation. Monolithic chromatography suitable for large biomolecules is also available for purification. We also develop new purification methods using field-flow fractionation and resins in co-operation with the manufacturers. Our technologies can be used in different combinations to purify large macromolecular complexes, such as viruses.

Portugal at Instruct

Maria Arménia Carrondo, ITQB-UNL, Oeiras, Portugal
Carlos Cordeiro, FC-UL, Lisbon, Portugal

Portugal has set up the **Portuguese Centre for Integrated Structural Biology, PCISBIO,** which was approved as an Affiliate Centre of Instruct. This Centre integrates research groups from six Portuguese Universities and one private non-profit Institution and also the Portuguese networks on NMR and MS. Since 2012, the Portuguese participation at Instruct has been financially supported by the Portuguese funding agency, Fundação para a Ciência e a Tecnologia, and from 2018 onwards ITQB-UNL will represent Portugal and the support will be endorsed by those Institutions, namely ITQB-UNL, FCT-UNL, FC-UL, UC, UA, UP, UM and IBET.

Portugal is one of the founding members of Instruct and has also participated as a founding member in the proposal for Instruct-ERIC.

Portuguese expertise in Structural Biology spans over methodologies such as **mass spectrometry** and **proteomics, metabolomics, spectroscopy, NMR** and **X-ray crystallography**, as well as **cell- and bioimaging systems**. Additionally, in the BioPilot Plant of IBET, expertise is available in large-scale biomass production
Portuguese researchers have greatly benefited from Instruct through all the activities provided and available to country members. Highlights of relevant results of this participation in the last years will be presented.

Instruct-IL: Weizmann Inst. & Tel Aviv University

Instruct – ISPC, Israel

Flagship platforms: Crystallization & structure determination: The ISPC employs 3 crystallization robots, including an LCP-equipped Mosquito for crystallization of membrane proteins and two 1,000 plate Formulatrix visualization robots (at 4° and 19°C) using the CRIMS visualization system (developed at the EMBL, Grenoble, in Jose Marquez's group).

From gene to protein Proteins are produced for structural, biochemical and clinical studies. Target genes are cloned using the Restriction Free (RF and TPCR) methodologies. Protein expression is performed with the following systems: E. coli , Yeast, Baculovirus and HEK293 cells. Proteins are typically purified via a standard protocol which includes: affinity capture, ion exchange and size exclusion.

CEITEC/BIOCEV - Czech Republic Instruct centres

Instruct – Czech Republic

Cryo- EM

The Core Facility of Cryo-electron Microscopy and Tomography provides access to high-end electron microscopes including FEI Titan Krios and supports its users in data collection, analysis, and consultation of experimental design for single particle analysis and electron tomography applications. The facility possesses a unique small SEM dual-beam system, which allows preparation of thin cellular cross-sections from vitrified cellular material for *in situ* cryo-electron tomography.

Figure 1: TEM microscope FEI Titan Krios equipped with a direct detector and an energy filter with a 4k CCD camera

Solution NMR

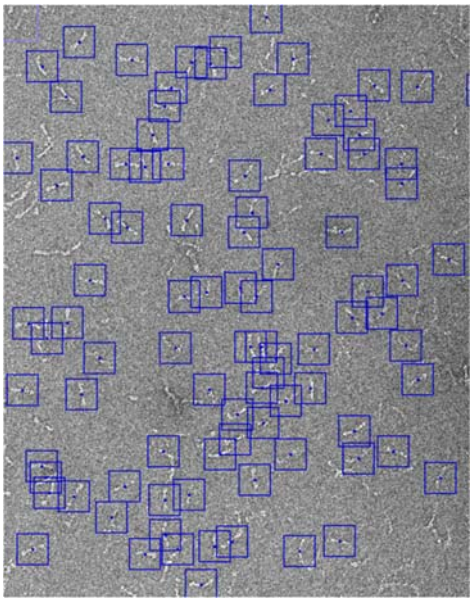
The Core Facility of High Field NMR Spectroscopy provides access to NMR spectrometers in the range of proton frequencies from 500 MHz to 950 MHz. The equipment is suited mainly to the studies of structure, dynamics and interactions of biomolecules, i.e. proteins, nucleic acids and carbohydrates. However, the instrumentation is flexible enough to cover also various research needs in material science, organic and inorganic chemistry, biochemistry, biology and biophysics

Figure 2: 950 MHz NMR spectrometer Bruker Avance III HD for high resolution spectroscopy in liquids

Personalized support for Image processing project

Instruct – Centre for Image Processing, Spain

The Instruct Image Processing Center provide personalized support to Instruct projects requiring specialized processing of either electron or X-ray microscopy images. Currently our main offer is focused in the field of single particles analysis using Xmipp and Scipion softwares. Xmipp is a comprehensive suite of image processing algorithms with a strong emphasis in the analysis of single particles, although it is extending towards electron and X-ray tomography. Scipion, in turn, provides a multi-package workflow-oriented platform bridging several software suites, like EMAN, Spider, Relion, Bsoft, Frealign and ctffind. More information can be found at: http://xmipp.cnb.csic.es and http://scipion.cnb.csic.es/docs.



The I2PC provides remote access to its computational environment. Currently our main offer is focused in the field of single particles analysis using Xmipp and Scipion softwares

Nanobody Discovery

Nanobodies4Instruct – Belgium Instruct Centre

Nanobodies are the small (15 kDa) and stable single-domain fragments harbouring the full antigen-binding capacity of camelid heavy chain antibodies. Nanobodies are exquisite chaperones for crystallizing membrane proteins, multiprotein assemblies, transient conformational states and intrinsically disordered proteins. Domain-specific Nanobodies have been used in single-particle electron microscopy (EM) to track these domains in particle projections. Nanobodies can be functionally expressed as intrabodies in eukaryotic cells and can be used to track their targets inside a living cell.
Small amounts (<1mg) of quality controlled protein are required to produce Nanobodies that recognize conformational epitopes on the native proteins. By learning more about the nature of each project, the team will advise in designing better antigens and work out optimal immunization schemes, panning strategies and screening methods.

Towards the crystal structure of the *S. aureus* ACP-FabI complex

Sandra Eltschkner, Rudolf Virchow Center for Experimental Biomedicine, University of Wuerzburg

In parallel to the clinical application of antibiotics many pathogens developed resistances against these compounds, which rapidly spread in bacteria. Among them are, for example, methicillin-resistant *Staphylococcus aureus* (MRSA), which can cause serious infections. To overcome this problem, new and effective antibiotics are urgently needed.
The bacterial fatty acid synthesis pathway II (FAS II) is an important metabolic pathway in bacteria since it is essential for the biosynthesis of cell membrane components which are essential for the survival of bacteria and their susceptibility to several natural compounds and antibiotics. Therefore, the FAS-II enzymes serve as promising targets for the development of novel antibacterial compounds.
In contrast to the huge type I fatty acid synthase multienzyme complex of mammals and fungi (FAS I), FAS II in bacteria and plants utilises one distinct enzyme for each step in the elongation cycle. Between these enzymes the growing acyl chain is transported by the acyl carrier protein (ACP) which only forms short-lived complexes with its interaction partners of the FAS-II cycle.
The *trans*-2-enoyl-ACP reductase FabI serves as a promising drug target since it catalyses the last and rate-limiting step in the elongation cycle of fatty acids in FAS II. Elucidating the nature of interaction between ACP and the FabI enzymes will create new possibilities for the design of compunds which abolish these specific contacts and thereby substrate delivery by ACP to the distinct enzymes of the FAS II.
To capture and characterise the short-lived and therefore little stable complex between ACP and the FabI enzyme, several approaches were tested.
Analytical gel filtration experiments were conducted to monitor complex formation of acyl-modified saACP with different FabI proteins. The complex fractions extracted from the column were screened for crystal growth, as well as co-cristallisation setups of acyl-saACP with saFabI. These approaches resulted in either non- or low-diffracting crystals.
To obtain a more stable complex between saACP and saFabI and gain better ordered crystals, several fusion constructs were created to bring both components into close proximity. Still, the diffraction only reaches very low resolution and crystal quality needs to be improved.

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Structure of human natural killer cell receptor NKR-P1

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Natural killer cells (NK cells) belong to innate immunity lymphocytes. They were discovered in 1970s and comprise 5-10% of lymphocytes circulating in blood. Their role in the immune system is to discover and kill cancer cells, stressed cells and cells infected by viruses. NK cells have a number of receptors on their surface, which are used for contact with other cells and for initiation of the cytotoxic response.

Human NKR-P1 (natural killer cell receptor protein 1; CD161; gene *klrb1*) is an inhibitory receptor on the surface of natural killer cells. It also occurs on other cells, having different roles there. NKR-P1 is a transmembrane glycoprotein with extracellular C-type lectin-like domain. Its binding partner on a target cell is LLT1 (lectin-like transcript 1; gene *clec2d*) with the same fold of the extracellular domain.

3D structure of NKR-P1 has been recently determined using X-ray crystallography. The protein produced in HEK293S GnTI$^-$ cells was crystallized in Oxford in the frame of an Instruct project. Diffraction data were collected at the Diamond light source. Two crystal structures of NKR-P1 were solved: the structure with full $GlcNAc_2Man_5$ glycosylation and the structure of NKR-P1 deglycosylated after the first GlcNAc.

Deglycosylated NKR-P1 forms crystals with diffraction limit 1.9 Å and with large asymmetric unit containing 4 dimers. Glycosylated NKR-P1 forms crystals with diffraction limit 1.8 Å and with smaller asymmetric unit containing only 1 dimer. The way of the dimerization is the same in the glycosylated and deglycosylated case. This is important, because the observed NKR-P1 dimerization mode is very different from the dimerization mode expected for the protein based on structures of other C-type lectin-like receptors/dimers.

This study was supported by BIOCEV (ERDF CZ.1.05/1.1.00/02.0109), Czech Science Foundation (15-15181S), Ministry of Education, Youth and Sports of the Czech Republic (LG14009), Charles University (UNCE 204025/2012, SVV 260079/2014, GAUK 161216), Foundation "Nadání Josefa, Marie a Zdeňky Hlávkových", COST Action (CA15126 MOBIEU), and BioStruct-X (EC FP7 project 283570). The authors also acknowledge the support and the use of resources of Instruct, a Landmark ESFRI project through the R&D pilot scheme APPID 56 and 286.

Structural insights into phosphoprotein chaperoning of nucleoprotein in measles virus

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Measles virus is an important, highly contagious, human pathogen. The nucleoprotein N binds only to viral genomic RNA and forms the helical ribonucleocapsid that serves as a template for viral replication. We address how N is regulated by another protein, the phosphoprotein, P, to prevent newly synthesized N from binding to cellular RNA. Here, we pulled down an $N^0_{1\text{-}408}$ fragment lacking most of its C-terminal tail domain by several affinity-tagged, N-terminal, P fragments to map the N^0-binding region of P to the first 48 amino acids. We showed biochemically and using P mutants the importance of the hydrophobic interactions for the binding. We fused an N^0 binding peptide, $P_{1\text{-}48}$, to the C-terminus of an $N^0_{21\text{-}408}$ fragment lacking both the N-terminal peptide and the C-terminal tail of N protein to reconstitute and crystallize the N^0-P complex. We solved the X-ray structure of the resulting N^0-P chimeric protein at 2.7 Å resolution. The structure reveals the molecular details of the conserved N^0-P interface and explains how P chaperones N^0 preventing both self-assembly of N^0 and its binding to RNA. We compare the structure of an N^0-P complex to atomic model of helical ribonucleocapsid. We thus propose a model how P may help to start viral RNA synthesis. Our results provide a new insight into mechanisms of paramyxovirus replication. New data on the mechanisms of phosphoprotein chaperone action allows better understanding of the virus genome replication and nucleocapsid assembly. We describe a conserved structural interface for the N-P interaction which could be a target for drug development not only to treat measles but also potentially other paramyxovirus diseases.

Understanding Allosterically Controlled Chaperonin Machines

Ilia Korobko, Dept. of Structural Biology, Weizmann Institute of Science, Rehovot, Israel.

Chaperones prevent or reverse aggregation of other proteins and help them reach their correct and active form. Some chaperones are heat-shock proteins (hsp) but most chaperones are expressed constitutively and are essential for life under normal conditions. One family of chaperones that can be found in all kingdoms of life is the chaperonins (hsp60). Chaperonins are large ATP-fueled molecular machines, which assist folding of both newly synthesized and denatured proteins.

Homo-oligomeric group I chaperonins can be found in prokaryotic life forms and in certain eukaryotic organelles (1). These chaperonins are constructed of two back-to-back stacked homo-oligomeric seven-membered rings with a cavity at each end where protein folding can take place. An example for a group I chaperonin is GroEL from E. coli, which is responsible for the folding of 1-5% of the bacteria's proteins.

Group II chaperonins are hetero-oligomers that are found in the cytosol of eukaryotic cells and in archaea. Group II chaperonins consist of two back-to-back stacked eight- or nine-membered rings that are composed of several different types of subunits.

The eukaryotic group II chaperonin CCT (also known as TRiC) is built from two identical rings that are composed of 8 different subunits. The arrangement of the subunits was established only recently but the exact function of every different subunit and the reaction cycle of CCT remain unclear.

It has been shown that chaperonin subunits function in a highly coordinated (allosterically controlled) fashion. GroEL, for example, displays intra-ring positive cooperativity and inter-ring negative cooperativity during its functional cycle, with respect to ATP, and in the absence of substrate protein. These cooperativities can be well represented by the MWC and KNF mathematical models, respectively (2).

The more complex and less well-characterized CCT also displays positive intra-ring and negative inter-ring cooperativities, but models for their mechanisms and function have not yet been developed.

In my project, the transient kinetics of ATP hydrolysis by WT CCT and several mutants were studied (3). The reaction was found to have at least four phases: two burst phases, a lag phase and a steady-state phase. The traces were well fitted to the following three-exponential equation with a linear term:

$$y = A_1\left(1 - e^{-k_1 t}\right) + A_2\left(1 - e^{-k_2 t}\right) + mt + A_3\left(e^{-k_3 t} - 1\right)$$

The values of the 7 variables in the equation change monotonically with the change in ATP and potassium concentrations.

A kinetic model that can account for the observations was developed and verified in a computer simulation. The kinetic model fits well with all our observations. For example, the decrease in the values of the two rate constants of the burst phase with increasing ATP

or potassium concentrations is explained by a conformational transition that occurs prior to substrate binding.
For a deeper understanding of the communication between the subunits we are continuing to study the transient kinetics of CCT as a function of other reaction altering factors such as ADP, AMP-PNP, PLP2 and mutations in CCT.

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From protein sequence to NMR structure in a matter of days using CHAINS/Rosetta

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We describe a fully automated two-step approach to NMR resonance assignments and *de novo* structure determination of large proteins without deuteration. First, the CHAINS algorithm uses a combination of two 4D spectra – a TOCSY and a NOESY, providing residue type and distance information, respectively – to perform resonance assignments of aliphatic sidechain atoms in addition to the backbone amide groups, thus allowing near-complete coverage of ^{1}H probes along the protein sequence. The automated assignments are then imported to iterative NOE cross-peak assignment and structure calculations in Rosetta, to generate high-resolution NMR structures of proteins. The novel approach goes beyond the current state-of-the-art methods in NMR structure determination in three aspects: minimum number of NMR experiments, automation in data analysis, and size of proteins.

The Centre of Molecular Structure (CMS), Institute of Biotechnology CAS, v.v.i.

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http://www.ibt.cas.cz/core-facility/CMS/

The CMS is a technology platform providing access to specialised technologies and services to researchers in the field of "Life sciences", both from the Czech Republic and from abroad. It is a constituent of CIISB (the Czech Infrastructure for Integrative Structural Biology) and a component of Instruct-CZ.

The CMS is made-up of four Core Facilities (presented on the poster): CF-Biophysical Techniques (CF-BT), CF-Structural Mass Spectrometry (CF-SMS), CF-Crystallisation of Proteins and Nucleic acids (CF-CPN), CF-Diffraction Techniques (CF-DT).

A new Method for crystallization of weak-affinity proteinprotein Complexes

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Protein crystallization remains the major bottleneck in X-ray crystallography. While X-ray structures of high-affinity protein-protein interactions (PPIs) are abundant in PDB, only a handful of structures are available for low-affinity or weak PPIs. Present methods for crystallization of such complexes rely on trapping them with chemical cross-links or non-native disulfide bridges. However, these methods are not always successful and also could promote crystallization of these PPIs in non-native conformation. We developed a novel approach to facilitate crystallization of weak PPIs through introduction of a few affinity-enhancing mutations that do not change the binding mode of the complex. Our strategy relies on a unique computational methodology for design of a few mutations that significantly enhance affinity between the binding partners (1-3). In our protocol, we first construct an approximate structure of the PPI using structural information available on homologous complexes. This structure serves as an input to the in silico saturated mutagenesis protocol that calculates the changes in free energy of binding due to all possible mutations in the PPI binding interface. Finally, we introduce a few affinityenhancing mutations into one or both protein chains to enhance affinity ~100-fold, thereby facilitating crystallization. Our strategy has been already applied to crystalize and solve the first structure of a complex between the inactive state of Ras, Ras-GDP, and its effector (4). Such a weak PPI, with affinity of ~100 μM, was not amendable to crystallization in the wild-type form. We are presently testing the efficacy of our approach in crystallization of various weak PPIs including ubiquitin/ubiquitin binding proteins and kinase/substrate peptides. Solving the structures of weak-affinity PPIs is crucial for understanding how such complexes drive various cellular processes such as signal transduction, post-translational modification, and protein quality control.

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Structural investigation of XIAP-BIR1 in solution and in living cells

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X-chromosome linked Inhibitor of Apoptosis Protein (XIAP) is a cytosolic multifunctional metalloprotein, expressed in all human cells except peripheral blood leukocytes. The main function of XIAP is to block programmed cell death by caspase inhibition [1-4], however literature reports its involvement even in copper homeostasis [5-8] and in NF-κB activation [9]. XIAP is frequently overexpressed in tumors, in which it potentiates cell survival and resistance to chemotherapeutics, and thus it has become an important target for the development of cancer treatments [10]. The partial structure of BIR1 (residues 20-99) has been determined by X-ray crystallography [9], nevertheless the unstructured regions of such domain are highly conserved and could have unexplored roles. In this study, the structural properties of the entire BIR1 domain (residues 1-105) have been characterized in solution and the oxidation state of BIR1 along with and its potential relation with copper have been investigated directly in living cells with atomic resolution NMR experiments. Our results provide an elucidating description of this domain in its physiological environment.

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The role of CTD tyrosine phosphorylation in recruitment of the elongation factor Spt6

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The recruitment of distinct processing factors to RNA polymerase II (RNAPII) is governed by dynamic post-translational modifications upon its highly flexible CTD domain (1) composed of repetitive sequence Y1-S2-P3-T4-S5-P6-S7 (2). This led to the formulation of the so-called "CTD-code" (3). How the CTD of RNAPII recruits, (de)activates, or displaces relevant processing factors, remains still poorly understood. Genome-wide mapping of CTD phosphorylation patterns revealed that Y1 and S2 phosphorylation levels are raising simultaneously during early elongation. Increased Y1 phosphorylation releases factors associated with RNAPII at the beginning of genes, and impairs assembly of the termination factors. In addition, phosphorylated Y1 mark is recognized by a tandem SH2 (tSH2) domain of elongation factor Spt6, consistent with Spt6 occupancy within Tyr1-phosphorylated region of genes *in vivo* (4).

Via a combination of structural methods, we have acquired and confirmed by biochemical assays data that are explaining the role of the individual SH2 domains. Additionally, we will show the physico-chemical driving forces of recognition Y1-phosphorylated CTD by the tSH2 domain of the elongation factor Spt6. Finally, we will show structure of the CTD complexed with Spt6.

Acknowledgement: The grant was supported by grants: (zeptej se Richarda na vsechny granty, kt. mas podekovat.)

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Structural basis for DNA recognition by human RECQ4 DNA helicase

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RecQ helicases are ubiquitous enzymes that are considered to be multifunctional genome "protectors" maintaining chromosomal stability. Among the members of the family, human RECQ4 plays a role at the intersection of DNA replication, recombination and DNA damage response. Here, we aim to characterize the DNA-binding properties of RECQ4 to understand its function in the above mechanisms.
RECQ4 contains an N-terminus region with high affinity for branched DNA substrates. Our NMR analyses show that two of these regions are intrinsically disordered both in free and DNA-bound forms. However, through a combination of NMR and biophysical techniques, we have studied those fuzzy complexes (i.e. concept of disorder extended into the bound state) and we have identified specific residues implicated in binding of diverse DNA structures, such as single-stranded, double-stranded, Y-form, Holliday junction, or G-quadruplex, in a salt-dependent manner. The electrostatic component is of prime importance to the interactions, yet the presence of a junction further stabilizes the complexes. Future structural studies aim to understand in detail the DNA-binding mode and the underlying functional plasticity.

The neuronal S100B protein targeting amyloid-β aggregation in Alzheimer's disease

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Insoluble β-amyloid peptide (Aβ) deposits formed in the synaptic cleft and neuroinflammation are consistent features in Alzheimer's disease (AD) and strong candidates for the initiation of the neurodegeneration process. S100B is one of the most abundant pro-inflammatory proteins which is up regulated in AD and is found associated with senile plaques. S100B is a small dimeric protein whose structure and functional regulatory interactions with other proteins are modulated by calcium-binding through EF-hand motifs and by zinc-and copper-binding to the dimer interface. These facts and our recent observation that S100 proteins have intrinsic β-aggregation propensity [1,2] have prompted us to investigate the impact of S100B on Aβ fibrillation. Here we report the co-aggregation phenomena involving S100B and Aβ and the development of nanobodies targeting S100B. Our studies that combine biochemical, biophysical and structural approaches indicate that S100B is a new key modulator of Aβ42 aggregation. We found that S100B forms a complex with monomeric Aβ as shown by NMR and ITC, delaying the formation of ThT-binding Aβ oligomers, a finding corroborated by TEM imaging. Analysis of Aβ aggregation kinetics and subsequent data fitting elicited quantitatively the mechanisms involved. In parallel, we will report the characterization of a library of 20 nanobodies targeting S100B developed in Nanobodies4Instruct with the aim to generate biological tools to regulate the interaction between S100B and other proteins, such as Aβ42. With this approach, we expect to generate knowledge that will translate into the potential use of S100B as a new druggable target to prevent or ameliorate inflammation in neurodegeneration.

Acknowledgments: This work was supported by Fundação para a Ciência e Tecnologia (FCT/MCTES, Portugal) through PhD fellowship SFRH/BD/101171/2014 (to JSC) and research grant PTDC/NEU-NMC/2138/2014 (to CMG); by Bial Foundation research grant 343/14 (to CMG) and by the EU-Instruct ESFRI project for the support and the use of shared resources in Structural Biology (fellowship to JSC).

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New Structure-activity Paradigms for Amyloids from Pathogenic Microbes

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Microbial functional amyloids are structured protein aggregates serving specific and highly diverse functions, including biofilm structuring and interactions with the host. Despite their role as key virulence factors and antimicrobial drug targets, amyloids are mostly known for their involvement in fatal human aggregation diseases, and their structures have been studied in depth only in eukaryotes. To bridge this informational gap, we leverage methodological advances in X-ray microcrystallography to shed light on this unheeded aspect within amyloid research and microbial physiology. We found unique amyloid-like structures, including, to our surprise, a structure of a full-length bacterial cross-alpha amyloid-like fibril which is unprecedented in >100 structures of eukaryotic cross-beta amyloids solved to date. The fibrils, of the PSMα3 peptide secreted by *Staphylococcus aureus*, are toxic to human cells, clarifying their involvement in pathogenicity (Tayeb-Fligelman *et. al., Science* 355(6327): 831-833; 2017). Given our results we predict that the structural and functional repertoire of amyloids is far more diverse than previously anticipated, providing a rich source of targets for antimicrobial drug discovery.

Interaction partners of RNA polymerase from *Bacillus subtilis*

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RNA polymerase from Gram-positive bacterium *Bacillus subtilis* is the essential multisubunit protein complex responsible for DNA transcription. The RNAp core (consists of β, β', ω and two α subunits) is responsible for transcription elongation. It can form complexes with several different interaction partners. The RNAp core with a sigma factor (18 identified in *B. subtilis* so far) forms the RNAp holoenzyme responsible for transcription initiation. Besides various sigma factors, the RNAp core can interact with a variety of partners based on the stage of transcription. Two of the important interaction partners are HelD [1, 2] and δ subunit [3]. HelD itself is an ATP-dependent helicase-like protein. It can stimulate transcription by enhancing transcriptional cycling and elongation. Its effect can be further amplified by subunit δ. Based on sequence homology HelD belongs to the superfamily of DNA and RNA helicases. Unfortunately, due to the low sequence similarity with possible homologues with known structure, details of HelD structure and function cannot be easily deduced from the knowledge about helicases from *E. coli*. Approximate localization of HelD on RNAp was determined, however, details of function, structure and interaction with NAs remain to be solved. We have expressed, purified and characterized close and distant homologs of *B. subitlis* HelD to characterize their biophysical and biochemical properties and to investigate possible NA binding by a combination of techniques including SPR and SAXS.

The project is supported by projects CZ.1.05/1.1.00/02.0109 BIOCEV, and CZ.02.1.01/0.0/0.0/16_013/0001776 CIISB4HEALTH provided by ERDF and MEYS, and CSF (15-05228S).
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Biochemical and structural studies on urease inhibition, a nickel-dependent virulence factor

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Urease is a Ni(II)-dependent enzyme that catalyzes the hydrolysis of urea in ammonia and CO2, determining an overall pH increase and causing negative effects for human health as well as agriculture.[1] Hence, a tight control of its activity is required and several classes of inhibitors were studied in the last decades.[1]
In this work, we present a kinetic and structural characterization of *Sporosarcina pasteurii* urease (SPU) inhibition by five chemical species: i) fluoride, ii) sulphite, iii) 1,4-benzoquinone (BQ), iv) catechol (CAT) and v) N-butylthiophosphotriamide (NBPT). Both fluoride and sulphite show a pH-dependent inhibition on SPU.[2,3] Fluoride acts with a predominant uncompetitive mechanism,[2] while sulphite is a competitive inhibitor.[3] Crystal structures of SPU bound to fluoride and sulphite reveal that both ions directly bind to the two Ni(II) ions in the enzymatic active site.[2,3] Unlike the previous cases, BQ and CAT act as time-dependent urease inhibitors.[4,5] BQ inhibition mechanism has a typical exponential decay profile,[4] while CAT shows a more complex behaviour.[5] Crystal structures of SPU bound to BQ and CAT reveal that they covalently bind to a conserved cysteine residue located on a flexible flap controlling the access to the active site cavity. Finally, latest structural results obtained at the highest resolution achieved so far for urease structures (1.27 Å) shed light on the inhibition of SPU by NBPT, a commercial product extensively used in agriculture.[6] Altogether, this data will be useful to develop novel and more efficient urease inhibitors necessary to modulate its activity and to counterbalance its negative effects.

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A new macrocyclase

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Ribosomally synthesized and post- translational modified peptides (RiPPs) are currently of major interest in the field of drug development. This is due to their wide-ranging bioactivities, which promise the development of new drug classes, much needed to fight cancer and multi-drug resistant bacteria. Cyclic RiPPs exhibit beneficial properties such as increased proteolytic resistance and structural rigidity increasing their appeal in the field of drug development. Due to the challenging synthesis of cyclic RiPPs, it is pivotal to establish novel routes to access those compounds and derivatives, in order to investigate their bioactivities in further detail. An auspicious approach is to harness of enzymes involved in the biosynthesis of those natural products. However, a major bottleneck is the head- to- tail macrocyclization of those peptides, which is – in nature - catalysed by so called macrocyclases. To date only one macrocyclase has been structurally characterized. Here we present the crystal structure of a novel promiscuous, yet efficient, macrocyclase. The X-ray crystal structures not only shed light on substrate binding and mode of action, but also suggest novel biotechnological applications.

Towards the identification of HMGB1 inhibitors: structural and functional studies

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HMGB1 is an incredibly versatile drug target as it is involved in a plethora of signalling pathways triggering and sustaining the inflammation response associated to several pathological conditions, ranging from chronic inflammatory disorders to autoimmune diseases and cancer, to mention a few. Though, the importance of HMGB1 inhibition is accompanied by a concrete difficulty in precisely and directly targeting this protein by small molecules due to the lack of a well-defined binding pocket, to the peculiar amphoteric and flexible structure and the existence of several oxidation and posttranslational states.
Starting from preliminary studies that brought us discovering two small molecules that directly bind to HMGB1, namely Glycyrrhizin and Aspirin, although suffering from low potency and efficiency, we set up a multidisciplinary protocol aiming at identifying new and more potent drug-like direct inhibitors.
We first virtually screened a large ZINC database of compounds, shortlisted a set of plausible ligands and tested their ability to inhibit HMGB1 cytokine activity in cell migration assays. Among these a very promising hit emerged, *5,5'-Methylenedi-2,3-cresotic acid (MCA)* that displayed an inhibitory effect of HMGB1 chemotaxis activity in the submicromolar range.
The comprehension of the structural and dynamical features of MCA in complex with HMGB1 by means of both experimental (NMR and spectroscopic) and computational (Molecular Dynamics simulations) techniques, lead us exploring further the MCA core performing structure-activity relationship studies. By playing with small scaffold modifications, we tested a set of MCA derivatives by NMR (waterLOGSY, STD and HSQC NMR titration) and cell migration experiments and found, among the others, the Pamoic Acid as direct binder with a Kd=200 □M, better than any other HMGB1 inhibitor so far.
Most importantly, by thoroughly characterize the protein-ligand complexes at the atomicdetailed level we pinpoint and address the structural and dynamical features that small molecules require to bind and inhibit our target protein, clearly helping in the optimization and rational design of new HMGB1 inhibitors.

Investigation of the iron(II) release mechanism from human ferritin as a function of pH.

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Human ferritins are 24-mer nanocage structures that self-assemble from 4-helix bundle subunits. The resulting structure has octahedral symmetry. The eight C3 channels are formed by three symmetry-related motifs, specifically helix α4-loop-α3, from as many chains. Furthermore, they have been identified as the entry points of iron(II) ions. Instead, the iron release mechanism in biomineralized ferritins is less characterized.
Our kinetic measurements showed a distinct pH dependence of iron release. Lowering the pH to 4.0 resulted in significantly faster discharge of iron, accompanied by an increase of the total amount of released ions. Our MD simulations provide a detailed atomic-level view of the mechanism of iron(II) release at pH 4, which occurs through the C3 channels.
Within each individual channel, two nearby rings formed by symmetry-related Asp and Glu sidechains define the binding site to which iron ions are rapidly attracted from the internal cavity by the electrostatic gradient. Before the iron ion actually reaches the C3 site, the Asp sidechains move apart thereby allowing it to get inside the channel, coordinated by three Glu sidechains. Subsequently, the iron ion switches from three to two glutamate sidechains coordination. These two glutamates are positioned below two histidine residues, close enough to generate a hydrogen bond with them (Figure 1).

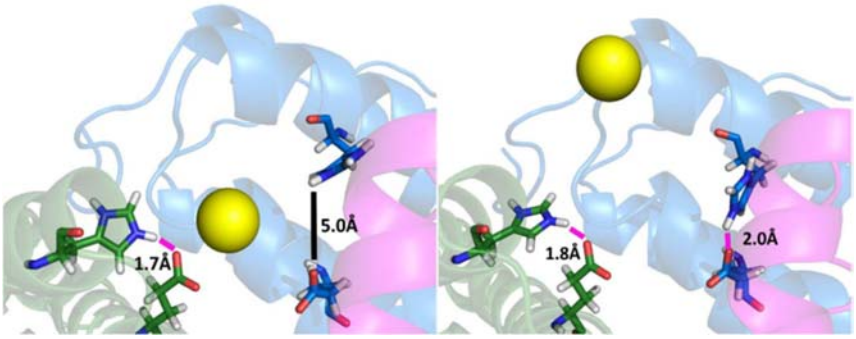

Fig. 1 Iron release after the formation of two Glu-His hydrogen bonds.

About 5 ns before the release of the iron ion, the lifetime of the hydrogen bonds becomes significantly longer, resulting in two strictly related effects. First, a partial compensation of the negative electrostatic charge of the carboxylates that coordinate the iron ions. Second, the reinforcement of the correlation between protein dynamics and the movement of the Glu sidechains, which in turn causes an increase of the distance between the Glu sidechains. These two synergic events weaken the interaction between the metal ion and the carboxylate oxygen atoms (Figure 1). Eventually, the iron ion escapes the C3 binding site and diffuses into the bulk solution.

Quasi-atomic model of Rift Valley fever virus using localized reconstruction and flexible fitting of X-ray structures

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Rift Valley fever virus (RVFV) is a medically and economically significant BSL3 pathogen. It is transmitted by mosquitoes and causes diseases in humans ranging from mild flu-like symptoms to severe hemorrhagic fever. The virion is enveloped and relatively large, 110 nm in diameter. The two viral glycoproteins Gc (class II fusion) and Gn (receptor binding) make heterodimers that further form hexamers and pentamers, arranged on a T=12 icosahedral lattice. Inherent flexibility of the virion has limited earlier cryo-EM reconstructions to 22 A (Huiskonen et al. 2009 J Virol).

Here, we improved sample preparation and collected a larger dataset using a direct electron detector, resulting in a significant improvement in resolution. However, virion flexibility still limited the attainable resolution to 13 A. We then further developed and applied the localized reconstruction method we have developed earlier (Ilca et al 2015 Nat Commun) on the individual pentamers and hexamers. This helped to improve the resolution to 7 A. Now for the first time we can perform molecular dynamics flexible fitting of the Gc (Dessau & Modis 2013 PNAS) and other X-ray structures into the EM density in an unambiguous manner.

X-ray structure fitting allowed us to create a quasi-atomic model of the virion surface. We are now carrying out similar analysis on virions in complex with antibodies and DC-SIGN receptor fragments. Furthermore, we have been able to complement these studies (that were carried out using fixed virions) by cryo-ET and subtomogram averaging of native, unfixed virions. Similarly, these methods yielded sub-nanometer resolution reconstructions of the Gc-Gn dimers. These studies open up new avenues in understanding Rift Valley fever virus entry and neutralization.

Structural studies on RcnR, a Ni(II) and Co(II) sensing transcription factor

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Escherichia coli RcnR (resistance to cobalt and nickel regulator, *Ec*RcnR), is a metal-responsive transcriptional regulator that represses the expression of the Ni(II) and Co(II) exporter proteins RcnAB by binding to their promoter site in the apo-form. Protein-DNA release occurs when either Ni(II) or Co(II) binds to *Ec*RcnR. *Ec*RcnR also binds the non-cognate metal ions Cu(I) and Zn(II), which have no effect on protein-DNA interaction. Prior work has shown that Ni(II) and Co(II) are found in distinct sites: while both Ni(II) and Co(II) are bound to the N-terminal Cys35 and His64 residues, Co(II) is additionally bound to His3 (1). On the other hand, Cu(I) and Zn(II) have a solvent-exposed binding site and further coordinate protein ligands that do not include the N-terminus amine (2). A molecular model of apo-*Ec*RcnR revealed the presence of Glu34 and Glu63 in the vicinity of the Ni(II) and Co(II) binding site (3). The roles of Glu34 and Glu63 in Ni(II) and Co(II) binding and selectivity were further demonstrated using site-directed mutagenesis, X-ray absorption spectroscopy (XAS) and functional assays (4).
The aim of this work has been to shed light on the coordination geometry of Ni(II) and Co(II) at the *Ec*RcnR binding site by using paramagnetic NMR spectroscopy. The use of such a solution technique enabled to obtain structural information under near-physiological conditions, in order to either corroborate or review previous results.

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Structural studies of natural killer cell receptor complexes: affinity vs. avidity

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Natural killer (NK) cells possess a unique ability to recognize and induce death of tumour and virus-infected cells without a prior antigen sensitization. Their function is regulated by a fine balance of signals induced by multiple activating and inhibitory cell surface receptors and their interaction with the ligands present on the target cell. Human natural killer receptor protein 1 (NKR-P1; gene klrb1) and its physiological binding partner lectin-like transcript 1 (LLT1; gene clec2d) are representatives of the NK cell receptor C-type lectin-like family.

Human embryonic kidney 293 cell line deficient in N-acetylglucosaminyltransferase I (HEK293S GnTI$^-$) is well known tool for expression of proteins with homogeneous and deglycosylatable N-glycosylation, a feature crucial especially for protein crystallography. We have adapted HEK293S GnTI$^-$ cell line to growth in suspension and optimized its transient as well as stable transfection.

We demonstrate this on the production of soluble LLT1 naturally present on natural killer (NK) and T-lymphocytes, but upregulated in glioblastoma cells, one of the most lethal tumours, where it acts as a mediator of immune escape. Prepared soluble domain of LLT1 with homogeneous glycosylation was readily crystallized and following optimization of crystal conditions this protein preparation ultimately led to the first structure determination of this receptor.

To improve the productivity even further, we have optimized transposon-based doxycycline inducible mammalian cell expression system piggyBac within HEK293S GnTI$^-$ cell line generating stably transfected cell pools with a tenfold yield improvement. This approach was successfully used for production of NKR-P1 and ultimately led to crystallization and structure solution of NKR-P1 alone as well as LLT1:NKR-P1 complex. Structure of this complex suggests potential mode of receptor:ligand multimerization on the cell surface explaining how efficient ligand recognition in this low affinity complex might be achieved through its increased avidity within immunological synapse.

Acknowledgement: This study was supported by BIOCEV (ERDF CZ.1.05/1.1.00/02.0109), Czech Science Foundation (15-15181S), Ministry of Education, Youth and Sports of the Czech Republic (LG14009), Charles University (UNCE 204025/2012, SVV 260079/2014, GAUK 161216), Foundation "Nadání Josefa, Marie a Zdeňky Hlávkových", COST Action (CA15126 MOBIEU), and BioStruct-X (EC FP7 project 283570). The authors acknowledge support and use of resources of Instruct, a Landmark ESFRI project through the R&D pilot scheme APPID 56 and 286.

Zebrafish GDNF and its co-receptor GFRα1 activate the human RET receptor

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Glial cell line-derived neurotrophic factor (GDNF) is a ligand that through complex formation with co-receptor GDNF family receptor alpha-1 (GFRα1) and receptor tyrosine kinase "RET" activates several signaling pathways crucial in the development and maintenance of neurons of the central and peripheral nervous systems. In the current study we investigated whether non-mammalian orthologs of these three proteins have conserved their function and would be able to activate the human counterparts. We used the baculovirus expression system to produce zebrafish RET, GFRα1 and GDNF, and *Drosophila melanogaster* RET and two isoforms of co-receptor GDNF receptor-like. A high-level insect cell expression of RET proteins was reported. As expected, the purified zebrafish GFRα1 and GDNF bound to each other with nanomolar affinity. Also, the first measurements of the affinity of the complex to RET in solution were reported. Surprisingly, we also discovered that both zebrafish GDNF and zebrafish GFRα1 robustly activated human RET signaling and promoted the survival of cultured mouse dopaminergic neurons with comparable efficiency to mammalian GDNF, unlike *E. coli*produced human proteins. Our results contradict previous studies suggesting that nonmammalian GFRα1 and GDNF cannot bind and activate mammalian RET and *vice versa*.

Novel adhesins of *Clostridium difficile* for covalent attachment

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Clostridium difficile is a Gram-positive pathogen that mostly infects elderly and hospitalised patients who have been treated with antibiotics for a long time. Since 2000, highly virulent strains have emerged which cause more severe cases of colitis associated with increasing mortality rates. Due to the higher virulence and resistances to antibiotics, treatment of *C. difficile* infections becomes increasingly difficult. The adhesion of pathogens to host tissues is one of the most important steps in establishing an infection, and has been identified as a new target for novel antimicrobial agents. Until recently, bacterial adhesion has been understood to involve multivalent, non-covalent interactions, which are difficult to prevent by small molecules.

Recently, novel adhesins called Thioester-Isopeptide-Ester domain (TIE) proteins have been found that are likely to bind covalently to their targets, for instance on host cell surfaces. Adhesin domains containing intramolecular thioester bonds mediate this binding. *C. difficile* expresses at least three different TIE proteins, which differ in size and domain architecture. Structural studies of thioester domains of these TIE proteins using X-ray crystallography are conducted to get an insight into the determinants of target specificity, and NMR spectroscopy is used to study reactions of small nucleophiles with the thioester bond. Additionally, epithelial cell binding experiments are used for target identification.

Structural characterization of chemical harpoon proteins from Gram-positive pathogens

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Commensal and pathogenic bacteria rely on surface-associated adhesins for host colonization and pathogenesis. Until recently, adhesins were known only to interact with host targets through non-covalent interactions. We have identified a large class of surface proteins composed of domains containing autocatalytic, intra-molecular cross-links between amino acid sidechains. These TIE (thioester, isopeptide, and ester) domain proteins are exceedingly prevalent and diverse in Gram-positive bacteria, including highly relevant human pathogens *Streptococcus pyogenes*, vancomycin-resistant enterococci, *Bacillus anthracis*, *Clostridium difficile*, and *Streptococcus pneumoniae*. The most remarkable feature of TIE proteins is the occurrence of reactive thioester bonds in their distal adhesin domains. We liken these to "chemical harpoons" that covalently anchor bacteria to cognate host receptors, which we have demonstrated for the surface protein SfbI from *S. pyogenes*.

TIE proteins may have evolved to mediate rapid, mechanically persistent binding of bacteria to host tissues, but their thioester domains remain largely uncharacterized. We present a new class of thioester domains with a novel fold from *B. anthracis* and vancomycin-resistant *Enterococcus faecium*. Despite very low sequence similarity, these thioester domains share the same fold and topology, their thioester bonds tying together N- and C-terminal subdomains. Access to the thioester is restricted to a single pore at the domain tip. Differences in surface amino acids surrounding this pore suggest structural determinants for target recognition. We also present a first structure of a full-length TIE protein from *B. anthracis* (BaTIE), which in addition to its thioester domain contains three isopeptide domains arranged in a linear array projecting approximately 20 nm from the bacterial surface. BaTIE to our knowledge represents the first full-length surface protein structure solved from Gram-positive bacteria.

Memory and Disorder

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All cognitive functions result from physical and chemical processes in the brain, as it is the case for instance for memory and learning.
Understanding the basis of memory from a cellular and molecular stand point represent an important challenge, but is also crucial for the cognizance and eventually treatment of various neurological diseases. In the brain, neurons are the main stakeholders of all cognitive functions.
They communicate between each others continuously thanks to connections called synapses. Depending on brain activities, these synapses constantly evolve, and these modifications define the "synaptic plasticity", a central phenomenon in memorization and learning processes. This plasticity involve a large range of molecules and in particular numerous proteins. Among them, the protein NMDA receptor, located on synapses, is one of the key protagonists and play a preponderant role in memory, but also in numerous neurological disorders (schizophrenia, depression, Alzheimers and Parkinsons diseases).
This project aims at better understanding how the NMDA protein is involved in memory and synaptic plasticity. To do so, various biophysical techniques (Nuclear Magnetic Resonance and Cryo-Electron Microscopy) will be used in order to better grasp which part of the protein is essential for its functions, but also to understand how its interaction with other proteins within synapses can affect the synaptic plasticity which is central in memory establishment.
To do so we will first study the interaction between NMDA receptor and the Calmodulin-dependent Kinase II by cyo-EM, since this interaction plays a central role in LTP. We are still at the early stage of this project, but deciphering the structure of this complex will give us key clues regarding this intricate molecular process that is LTP. Thus, from a long-term perspective, the understanding and description of the mechanisms governing memory and learning will give access to new targets for drug development in the treatment of related neurological disorders.

The N-terminal domain plays a crucial role in the structure of a full-length human mitochondrial Lon protease

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The Lon protein is a protease belonging to the superfamily of ATPases Associated with diverse cellular Activities (AAA+). Its main function is controlling the protein quality and the maintenance of proteostasis by degrading misfolded and damaged proteins, which occur in response to numerous stress conditions. Lon protease has been also shown to participate in regulation of levels of transcription factors that control pathogenesis, development and stress response. Furthermore, it seems to play an important role in aging, and it is supposed to be involved in mtDNA replication, translation, or repair [1]. We focus our interest on the structure of human mitochondrial Lon (hLon) protease whose altered expression levels are linked to some severe diseases, such as epilepsy, myopathy, or lateral sclerosis. At the moment, it is assumed that Lon subunits assemble into oligomeric structures whose conformations are supposed to differ at ATP, ADP, and protein substrate binding. Nevertheless, the mechanism of Lon action is not well known.

Here, we present the first 3D structure of full-length hLon using cryo-electron microscopy [2]. hLon has a unique three-dimensional structure, in which the proteolytic and ATP-binding domains (AP-domain) form a hexameric chamber, while the N-terminal domain is arranged as a trimer of dimers. These two domains are linked by a narrow trimeric channel composed likely of coil-coiled helices. In the presence of AMP-PNP (EMD-3275), the AP-domain has a closed-ring conformation and its N-terminal entry gate appears closed, but in ADP binding (EMD-3274), it switches to a lock-washer conformation and its N-terminal gate opens accompanied by a rearrangement of the N-terminal domain. We have also found that both the enzymatic activities and the 3D structure of a hLon mutant lacking the first 156 amino acids are severely disturbed, showing that hLon's N-terminal domains are crucial for the overall structure of the hLon, maintaining a conformation allowing its proper functioning.

Acknowledgement: The work was supported by the grants: Slovak Research and Development Agency (APVV-0375-15) and by the Slovak Grant Agency (VEGA 2/0113/14) and 13-32339P and P302/12/G157 from the Czech Science Foundation, the Prvouk/1LF/1 and UNCE204022 grants from Charles University in Prague, the OPVK CZ.1.07/2.3.00/30.0030 and by the grant CZ.2.16/3.1.00/24010 (OPPK program).

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Increasing human NKR-P1 affinity by rational mutagenesis

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Natural killer (NK) cells possess a unique ability to recognize and induce death of tumour and virus-infected cells without a prior antigen sensitization. Their function is regulated by a fine balance of signals induced by multiple activating and inhibitory cell surface receptors and their interaction with the ligands present on the target cell [1]. Human natural killer receptor protein 1 (NKR-P1; gene *klrb1*) and its physiological binding partner lectin-like transcript 1 (LLT1; gene *clec2d*) are representatives of the NK cell receptor C-type lectin-like family [2].

The inhibition of NK cell immune response facilitated by the interaction between NKR-P1 expressed on NK cells and LLT1 on the target cell is regarded as a primary function of these receptors. Previous research showed that this signalization pathway is exploited by glioblastoma tumour cells, which dampen the NK cell immune response by overexpression of LLT1 [3]; furthermore, LLT1 was recently suggested as a biomarker for B-cell non-Hodgkin's lymphoma and implicated in other cancerous manifestations [4]. However, besides the NK cells, LLT1 is also present on circulating T and B cells and on antigen presenting cells; and NKR-P1 is also expressed by NKT and T cells. Interestingly NKR-P1 is considered as a marker for all Th17 [5] and some Tc17 cells [6], and could play a role in the homing and transendothelial migration of these lymphocytes into immunologically privileged sites [5]. Therefore, it is not surprising that relationship between NKR-P1 and LLT1 positive cells is more and more being implicated with diseases connected with immune malfunctions – e.g. multiple sclerosis [7], rheumatoid arthritis [8] or Crohne's disease [9].

Here we present the results of our efforts to design and identify a human NKR-P1 mutant with high affinity towards its physiological ligand. The most promising mutations based on crystal structure of the complex and designed *in silico* were expressed in stably transfected HEK293 cells and their affinity towards LLT1 was examined by SPR. These efforts should lead to design of novel immunotherapeutics and promote the connection between structural biology and translational medicine.

Acknowledgement*:* This study was supported by BIOCEV (ERDF CZ.1.05/1.1.00/02.0109), Czech Science Foundation (15-15181S), Ministry of Education, Youth and Sports of the Czech Republic (LG14009), Charles University (UNCE 204025/2012, SVV 260079/2014, GAUK 161216), Foundation "Nadání Josefa, Marie a Zdeňky Hlávkových", COST Action (CA15126 MOBIEU), and BioStruct-X (EC FP7 project 283570). The authors also acknowledge the support and the use of resources of Instruct, a Landmark ESFRI project through the R&D pilot scheme APPID 56 and 286.

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Preparation and Structural Characterization of Human NK Cell Activating Immunocomplex NKp80:AICL

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Natural killer cells (NK cells) play a key role in recognition and elimination of infected, stressed or malignantly transformed cells. Recognition is promoted by surface NK cell receptors. NKp80 and its myeloid-specific activating ligand AICL are both C-type lectin-like receptors with C-type lectin-like domain (CTLD) [1]. Immunocomplex NKp80:AICL promotes lysis of malignant myeloid cells, mediates crosstalk between NK cells and monocytes, is engaged in cytokine release and contributes to initiation of immune response during inflammation.

This complex can be used for therapeutic NK cell activation and immunotherapy based on natural NK cell cytotoxicity. Activation of NK cells through AICL:HER2-scFv fusion construct recognizing HER2 tumor antigen, while displaying AICL, as NK cell activating ligand, on tumor surface, was described recently [2]. Structural description of NKp80:AICL interaction interface might be helpful for design of similar novel immunotherapeutics.

We used mammalian expression system of modified human embryonic kidney cell line 293 (HEK293) to produce glycosylated NKp80 ectodomain. With piggyBac system we were able to create stable cell lines expressing soluble extracellular parts of NKp80 in inducible way. AICL ectodomain contains odd cysteine which is not conserved, compared to other CTLD receptors. After mutation of this cysteine to serine (C87S mutation) the yield as well as stability of prepared protein are greatly enhanced compared to wild-type construct.

Thanks to these approaches, we are able to produce both proteins in sufficient amount to initiate structural studies using analytical ultracentrifuge, dynamic light scattering and finally crystallization of this immunocomplex.

Acknowledgement: This study was supported by Czech Science Foundation (15-15181S), Charles University (UNCE 204025/2012, SVV 260079/2014), COST Action (CA15126 MOBIEU), and BioStruct-X (EC FP7 project 283570). The authors also acknowledge the support and the use of resources of Instruct, a Landmark ESFRI project through the R&D pilot scheme APPID 56 and 286.

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Probing the interaction between bacterial virulence factors and host proteins

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The Seattle Structural Genomics Center for Infectious Disease (ssgcid.org) was established in 2007 to provide structure determination services to the scientific community. With a world-wide network of over 250 collaborators, we have deposited close to a thousand structures in the PDB and are distributing clones and protein samples free of charge. So far, we have focused on determining the structures of single protein targets by X-ray crystallography and NMR. The pilot project presented here aims to evaluate cross-linking followed by mass-spectrometry (CX-MS) as a potential technology for expanding our capabilities to interrogate protein-protein complexes *in situ*, as well as to identify interaction partners for experimental structure determination of complexes *in vitro* by X-ray crystallography or CryoEM. Our model system explores the interaction between *Bartonella* effector proteins and their targets in human cells.

Bartonella species are facultative intracellular bacterial pathogens that cause a range of human illnesses (including Carrion's disease, trench fever and cat scratch disease). They are attractive models for studying bacterial persistent infection due to their widespread occurrence in multiple mammalian reservoirs. The type IV secretion system (T4SS) plays an essential role in the pathogenicity of *Bartonella* by translocating a cocktail of effector proteins (Beps) into host cells, thereby triggering cellular processes that allow the pathogen to establish persistent infection. For example, BepA likely promotes formation of tumors by inhibiting apoptosis of endothelial cells, whereas BepC is involved in invasome formation. While they mediate diverse functions, the Beps share a modular structure usually composed of a "filamentation induced by cAMP" (FIC) domain followed by an "intracellular delivery" (BID) domain. FIC-domain enzymes are found in all kingdoms of life and catalyze post-translational modifications of various target proteins to modulate their function, while the BID domain (together with the C-terminal tail) provides a secretion signal that is required for translocation of Beps into host cells through the T4SS.

Using a high-throughput approach, we determined the crystal structures of FIC and BID domains from seven different Bep clades. In order to identify the host proteins that interact with individual Beps, we overexpressed a tandem affinity purification (TAP) tagged single Bep as 'bait' in human cells and analyzed the pulled-down complex by mass spectrometry. The purified complex was then cross-linked prior to MS analysis to locate protein-protein interaction sites on the Bep structure. Results of these experiments will be presented.

NKp30 N-glycosylation type influences its oligomerization and B7-H6 ligand binding

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Immune system can recognize tumour cells and subsequently to eliminate them. Special sort of lymphocytes – natural killer (NK) cells can provide this function by causing apoptosis of tumour cells using Fas ligand binding or granzymes. These processes are activated when signals from their inhibitory receptors are decreased and on the other hand, signals from activating receptors are increased. NKp30 is an activation receptor of NK cells with one Ig-like extracellular domain. Out of its ligands, two of them are stimulating NK cells through NKp30 when bound to membrane: B7-H6, a membrane protein with two Ig-like domains, which is present on the surface of some tumour cells; and cell nuclear protein BAG6 present on tumour cell exosomes [1].

The structure of the B7-H6 ligand bound to NKp30 produced in *E. coli* has already been solved [2]. It is also known that the glycosylation and length of C-terminal chain of NKp30 extracellular domain as well as its oligomerization status influence its ability to bind ligands [3]. However, the structural basis of these effects is not known.

For our studies the extracellular domains of NKp30 and B7-H6 have been cloned into the pTW5sec vector with C-terminal histidine tag. To study the effect of C-terminal region of extracellular domain of NKp30, shorter and longer constructs have been cloned. Both proteins have been produced in human HEK293S GnTI$^-$ cell line possessing homogeneous N-glycosylation profile as well as in HEK293T cell line with wild-type human N-glycosylation, purified by affinity and size exclusion chromatography. Glycosylation of NKp30 was confirmed by mass spectrometry and formation of its oligomers was observed by analytical ultracentrifugation, transmission electron microscopy and size exclusion chromatography with multi angle laser light scattering. Impact of glycosylation and C-terminal length of NKp30 construct on B7-H6 ligand binding was monitored using analytical ultracentrifugation, surface plasmon resonance and isothermal titration calorimetry.

Acknowledgement: This study was supported by BIOCEV (ERDF CZ.1.05/1.1.00/02.0109), Czech Science Foundation (15-15181S), Ministry of Education, Youth and Sports of the Czech Republic (LG14009), Charles University (UNCE 204025/2012, SVV 260079/2014, GAUK 927916), COST Action (CA15126, MOBIEU), and BioStruct-X (EC FP7 project 283570). The authors also acknowledge the support and the use of resources of Instruct, a Landmark ESFRI project through the R&D pilot scheme APPID 56 and 286.

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Advances in our understanding of lipopolysaccharide biosynthesis

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Bacterial surfaces are coated with a wide-variety of complex polysaccharides, crucial to the survival of bacteria upon infection of a host organism, including lipopolysaccharide (LPS). LPS consists of a serotype-specific O-antigen, attached via a core-oligosaccharide to lipid A in the gram-negative outer membrane. LPS biosynthetic enzymes not only represent potential therapeutic targets, but LPS biosynthesis also shows parallels with N-linked glycan formation. However, the difficulties associated with studying integral membrane proteins have precluded their extensive study. Numerous mechanisms have been proposed to explain the control of O-antigen chain length, facilitated by the O-antigen polymerase Wzy and its polysaccharide co-polymerase Wzz, including chaperone- and timer-based models. However, there is a lack of evidence supporting any interaction between Wzy and Wzz (Woodward. L. et al., 2016; Tocilj, A. et al., 2008). Here we describe biochemical evidence supporting a direct interaction between Wzz and Wzy, in the form of tandem-affinity purifications, coupled with western blotting, BN-PAGE and mass spectrometry. Characterisation of this interaction by cryo-EM is currently ongoing.

A number of gram-negative bacteria have been found to produce O-antigens containing modified heptoses, the biosynthesis of which remains relatively uncharacterised. Here we describe the structures, determined by x-ray crystallography, of the GDP-D-glycero-D-*manno*-heptose C6 dehydratase DmhA required for 6-deoxyheptose biosynthesis in *Yersinia pseudotuberculosis* and DdahB and MlghB, C3 and C5/C3 epimerases involved in the biosynthesis of 6-*O*-methyl-L-*gluco*-heptose and 6-deoxy-D-*altro*- heptose in *Campylobacter jejuni* strains NCTC 11168 and 81-176, respectively (Butty, F. et al., 2009; McCallum M. et al., 2012; McCallum M. et al., 2013). These will improve our understanding of the biosynthesis of these unique sugars.

Podocyte damage: Are nanoparticles safe?

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Podocytes are highly specialized epithelial cells of glomerulus typical by their complex cellular organization arising from their very specific localization - they surround the glomerular capillaries facing the Bowman's capsule and the primary urine. Podocytes have a large central cell body from which primary processes are projected towards the glomerular capillaries and eventually divided into numerous foot processes which rest on the glomerular basement membrane. They constitute essential part of glomerular filter as their form filtration slits about 32 nm wide preventing the loss of serum proteins into urine. Already small changes of podocyte actin cytoskeleton lead to effacement and disappearance of podocyte actin-rich foot processes that is a hallmark of early podocyte damage that can result in their detachment and shedding into the urine. Unfortunately, as podocytes do not proliferate their loss is irreversible.

The nanotechnology-based drug-delivery system targeted specifically towards various diseases has several advantages over conventional therapies. However, nanoparticles may accumulate in the kidneys as was confirmed by several studies. Elucidation of nanoparticle interaction with podocytes, mechanism of distribution and accumulation, estimation of their toxic effects, changes in signaling pathways as well as changes of podocyte cytoskeleton due to nanoparticle uptake are necessary for the development of safe and functional nanomedicine.

To effectively investigate these effects, various types of microscopy (light, fluorescent, confocal, electron, etc.), PCR and western blot assay, and other molecular biology techniques have become essential tools.

Acknowledgement: The work was supported by the SASPRO Programme project No. 0084-01-02 and by the VEGA grant No. 2/0113/15.

Xdskappa: processing home source data with synchrotron tool

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Laboratory instruments for X-ray diffraction measurements are becoming more powerful with new technologies such as the liquid anode sources or HPAD detectors. It allows efficient diffraction experiments, including data for native SAD phasing, on an increasing number of protein samples in house, without the need of a synchrotron source. The crucial part of the diffraction measurement is data processing. It is a very much standardized process for synchrotron data and users are used to processing data with tools such as XDS [1]. However, in house data collection has a few specific parameters compared to synchrotron: the measurement consists of several runs with various geometry settings and the geometries are non-orthogonal. Even though XDS can process such individual runs, settings are non-trivial and behind the scope of a general user. Therefore, *xdskappa* was introduced to simplify processing of in house data using XDS. *Xdskappa* automatically generates input for XDS and runs XDS on multiple datasets simultaneously. Moreover, a possibility of manual fine tuning remains. *Xdskappa* is distributed under the GNU General Public License 3 [2].

Acknowledgement: This work was supported by project BIOCEV (CZ.1.05/1.1.00/02.0109) from the ERDF, by the ERDF fund (CZ.02.1.01/0.0/0.0/16_013/0001776), by MEYS CR (LM2015043 CIISB and LQ1604 NPU II), and by the Grant Agency of the Czech Technical University in Prague, grant No. SGS16/246/OHK4/3T/14.

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Substrate binding mechanism of bilirubin oxidase

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Bilirubin oxidase from plant pathogen *Myrothecium verrucaria* (BOD; EC 1.3.3.5) is a member of the blue multi-copper oxidase family (MCOs). BOD acts on various aromatic and some inorganic substrates, nevertheless its physiological role in nature remains unknown.

BOD is used in laboratory diagnostics for determination of quantity of bilirubin in serum for clinical investigation of liver. Its stability in a broad pH range, high thermal tolerance, usage of molecular oxygen as a second substrate, and oxidation without production of H_2O_2 make BOD attractive for applications in biotechnology such as decolourisation of synthetic dyes, pulp delignification or detoxification, or for experimental biofuel technology [1].

BOD contains two active sites – the oxidation site containing one Cu ion (T1), the primary acceptor of electron provided by substrate, and the reduction site composed of three copper ions (trinuclear copper cluster - TNC) responsible for the dioxygen reduction to two water molecules. Electrons are transferred from T1 to TNC via an intramolecular highly conserved Cys-His bridge [2].

The mechanism of dioxygen reduction is well understood as it was studied intensively through the whole MCOs family. On the other hand, the position and mechanism of substrate binding in BOD as well as the electron transfer pathway from the substrate to T1 were not described yet. Two structures of BOD have been published (PDB code: 2XLL [3], 3ABG [4]), nevertheless, none of them containing any ligand in the oxidation site. This work presents the first structures of BOD complexes binding ligands in the oxidation site. These structures and measurements of WT and mutants' activity against several types of substrates clarify the position of the substrate binding site and point out the amino acids involved in the electron transfer route from substrate to the T1 copper ion.

Acknowledgement: This work is supported by the project BIOCEV (CZ.1.05/1.1.00/02.0109) and the project Structural dynamics of biomolecular systems (CZ.02.1.01/0.0/0.0/15_003/0000447) from the ERDF, institutional support of IBT CAS, v. v. i. RVO: 86652036) and by the Grant Agency of the Czech Technical University in Prague, grant No. SGS16/246/OHK4/3T/14.

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Binding requirements for recognition of human immune receptor CD160 by herpesvirus entry mediator HVEM, the HVEM crystal structure and the molecular architecture of co-signaling molecules BTLA, gD and LIGHT

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The tumor necrosis factor (TNF) receptor superfamily member herpesvirus entry mediator (HVEM) (TNFRSF14) regulates T-cell immune responses by activating both inflammatory and inhibitory signaling pathways. HVEM acts as both a receptor for the canonical TNF-related ligands, LIGHT and lymphotoxin-α, and as a ligand for the immunoglobulin superfamily proteins BTLA (B and T lymphocyte attenuator) and CD160, a feature distinguishing HVEM from other immune regulatory molecules. The ability of HVEM to interact with multiple ligands in distinct configurations creates a functionally diverse set of intrinsic and bidirectional signaling pathways that control both inflammatory and inhibitory responses. The HVEM system is integrated into the larger TNFR network through extensive shared ligand and receptor usage. Experimental mouse models and human diseases indicate that dysregulation of HVEM network may contribute to autoimmune pathogenesis, making it an attractive target for drug intervention.
Natural killer (NK) cells possess a unique ability to detect and destroy the tumor and virus-infected cells without a prior antigen sensitization. Their function is regulated by a fine balance of signals induced by multiple activating and inhibitory cell surface receptors and their interaction with the ligands present on the target cell. In turn, many pathogens have evolved countermeasures to avoid detection and clearance by natural killer cells, however, regulatory mechanisms limiting cytokine activation of NK cells that reduce non-specific tissue damage remain poorly defined. CD160 is a 27 kDa glycoprotein which was initially identified with the monoclonal antibody BY55. Its expression is tightly associated with peripheral blood NK cells and CD8 T lymphocytes with cytolytic effector activity. The cDNA sequence of CD160 predicts a cysteine-rich, glycosylphosphatidylinositol-(GPI)-anchored protein of 181 amino acids with a single Ig-like domain weakly homologous to KIR2DL4 molecule. It was found that TNF receptor HVEM preferentially engages CD160 trimer to costimulate activation, while a viral ortholog of HVEM specifically binds to BTLA to suppress this signaling. Thus, regulation of CD160 bidirectional binding may represent a common mechanism of immune regulation targeted by multiple pathogens, which by extension is a potential target for therapeutic manipulation. We have found that CD160 is expressed at the cell surface as a tightly disulfide-linked multimer. The isolation, expression and purification of human recombinant CD160 trimer as well as monomer was performed in baculovirus-mediated (BV) expression in *Spodoptera frugiperda* (Sf9) and (Hi5) insect

cells. The level of expression was monitored by western blot analysis. Metal-ion- and Streptavidin-affinity and cation exchange chromatography were followed by size-exclusion to purify the monomeric and trimeric CD160.

The homology model of CD160 antigen domain shows cysteine-rich region that was found to be responsible for CD160 tight-timer formation even under reduced conditions. CD160 trimer forms stable complex with HVEM, while monomeric form refused to bind its cognate ligand with the same affinity. We have attempted crystallization of HVEM and CD160 molecules in singles, as well as in complex. Protein crystals were grown by using PEG ion and Morpheus screens and successfully tested for diffraction. The obtained crystal structures were deposited under accession code PDB ID 5T2Q and 5T2R.

IN is Marie Curie Fellow financed by Programme SASPRO, co-funded by European Union and the Slovak Academy of Sciences under the contract No. 0003/01/02. The authors gratefully acknowledge the contribution of the Slovak Research and Development Agency under the project APVV-14-0839 and the contribution of the Scientific Grant Agency of the Slovak Republic under the grant 2/0103/15.

Crystal structure of *Listeria monocytogenes* Ca2+-ATPase guided by single-molecule FRET

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P-type ATPases are primary active transporters pumping ions and lipids across cell membranes against steep concentration gradients. The transport mechanism is defined by a cycle of conformational changes initiated by phosphorylation of a conserved aspartate. Most of the conformations in the transport cycle are well characterized, primarily by crystal structures of the sarco/endoplasmic reticulum Ca2+-ATPase (SERCA), but the structure of the substrate-occluded E2P state will complete the cycle. Single-molecule FRET (smFRET) has the unique ability to resolve different conformations and thus directly determine the conformational ensemble occupied by a protein. This is crucial to crystallography as homogeneity is the main predictor of crystallizability. We use the pathogenic *Listeria monocytogenes* Ca2+-ATPase (LMCA1) as a model system to reveal conditions stabilizing a particular conformational state. smFRET studies reveal a novel state of LMCA1 in the presence of Ca2+ and ATP that potentially reflects the Ca2+- occluded E2P state. Initial crystallization setups lead to a crystal structure of LMCA1 in a Ca2+-free closed E2P state diffracting to 2.95 Å, which is the first high-resolution structure of any bacterial Ca2+-ATPase. It reveals important details of a single Ca2+ binding site and the pH-sensitivity of bacterial Ca2+-ATPases. Optimization of the crystallization conditions through smFRET data, to achieve the Ca2+-occluded E2P state, is still in process, and a structure will further contribute to development of potential drugs for targeting the Gram-positive bacterial pathogen *Listeria monocytogenes.* The combination of smFRET data and crystal structures sheds light on the transport mechanism of a large class of important transport proteins, and these results indicate that smFRET can guide crystallization to obtain atomic resolution structures.

Phenotypically strong and lethal mutations in *Met* gene of *Drosophila* are reflected in the structure of 3D homology models of Met protein.

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The action of steroid and thyroid hormones, retinoids, vitamins D, benzoates, some of the prostaglandins and few other small lipophilic ligands is mediated via members of nuclear receptor (NR) superfamily. In most of the cases NR proteins homodimerize or heterodimerize while this dimerization is frequently stabilized upon ligand and DNA binding. For DNA binding nuclear receptors recognize sets of conserved hormone responsive elements (HREs) in the regulatory regions of the genes where they regulate transcription. During *Drosophila* metamorphosis which is crucial decisive switch between allometric larval growth and novel morphogenetic differentiation is regulated by concerted action of insect steroid hormone ecdysone and its receptor EcR (or complex of EcR/Usp) and the modulatory action of sesquiterpenic juvenile hormone (JH). During the genetic screen as a key component in the mechanism of JH action was identified protein Methoprene tolerant (Met), encoded by *Met* gene, which belongs to the bHLH-PAS family. To gain more insights into molecular action of the Met protein we have generated series of homology models for wild type bHLH and both PAS domains, and for few other motifs. Subsequently we made structure models bearing EMS- or X-ray-induced mutations the molecular breakpoints of which we analysed in our laboratory.

Compact structure of PAS B domain of Met consists of αA helix (Pro382 to Gly390) followed by ß-sheet ßA (Glu403 to Leu409), short loop and antiparallel ßB sheet (Ile414 to Asp416). Then αB helix (Asp418 to Ala425) continues to αC helix (Lys429 to Val432) followed by αD helix delimited by Pro437 and Met441. The last helix, αE, runs from Ile450 up to Asp458. The PAS B then continues with ßC sheet (Ser446 to Ser472), ßD sheet (Leu480 to Val488), and is completed by final ßE sheet ranging from Val495 to Thr503.

Analysis of *Met*$^{-}$ mutations predicted several interesting structural alterations that provide clues for explanation of lethal or strong JH-resistance phenotype. In *Met*^{1} allele characterized by G166D substitution, the lesion affect PAS A domain by significant truncation and distance shift of the second α-helix, which in turn conformationally affect and truncates the length of both the 3rd and 4th α-helices, and causes the complete absence of an additional two ß-sheets.

The *Met*^{31} allele due to E487K substitution in the putative mid β-sheet between two α-helices forming NR boxes behind PAS B domain has more fatal consequences at the 3D level than would be expected from prediction of its secondary structure. The E487K substitution prevents folding of the mid β-sheet with potential N-terminal extension to the configuration of PAS B domain. Moreover, more C-terminally this mutation affects delimitation of at least two β-sheets and associated coiled-coils and loops.

Interestingly, the sequence polymorphism T103P, we found during deep sequencing campaign, represents relatively strong non-conservative change in the coil structure placed

between first and second α-helix of bHLH domain with similar consequences as in the case of *Met*^{1} allele. It results not only in shortened second α-helix within bHLH domain but also in a long distance changes affecting PAS A domain.

Photochemistry of Quinazolinone-Derived Schiff's Bases

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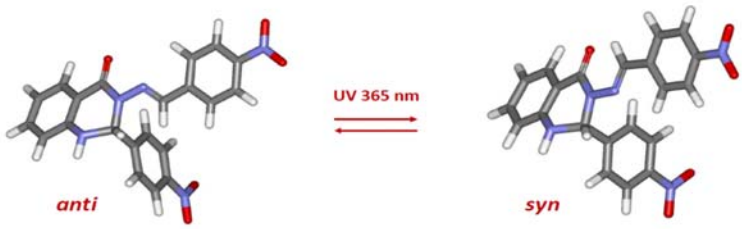
Photoisomerization acts as the first step in a number of light-induced biological processes, of which the vision process is among the most known. Many photochemically-induced biological events, including those involved in vision, are associated with Schiff's bases. Presented analysis deals with isomerization process of two new photochemically active quinazolin-4-one-derived Schiff's bases. The analysis was performed using various spectroscopic methods including EPR, NMR and UV-VIS. The experimental data were rationalized by DFT calculations. UV irradiation at 365nm led to isomerization around the N-N bond in the -C(=O)-N-N=C-(H) array of atoms with a formation of the *syn* isomer:



The signals arising from the newly formed *syn* isomer were detected in ^{1}H NMR spectra at 600 MHz. UV-VIS spectra also revealed significant effects of the substituents in the Schiff's bases upon the absorption bands. EPR spectroscopy confirmed generation of the reactive intermediates resulting from the interaction of activated molecular oxygen with solvent molecules. The activation of molecular oxygen was caused by electron transfer from the excited states of the Schiff's bases. DFT calculations enabled description of conjugation of the nitrogen lone pairs in the isomers affecting the bond order in the azomethine group. Moreover, the presented theoretical and experimental data showed that the degree of conversion and the rotational barriers are affected by substitution at the aromatic rings. Further details describing the structure and properties of the studied Schiff's bases will be discussed.

Single molecule FRET of a Ca^{2+}-transporting P-type ATPase: Identification of rate-limiting step, direct observation of a novel intermediate and guided crystallization.

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P-type ATPases are ubiquitous primary transporters that pump cations across cell membranes through the formation and breakdown of a phosphoenzyme intermediate. Structural investigations have revealed a transport mechanism defined by large conformational changes in the cytoplasmic domains of the protein that allosterically reorient transmembrane helices to expose ion binding sites to alternate sides of the membrane. We have employed single-molecule fluorescence resonance energy transfer (smFRET) to directly image functional conformational changes in the *Listeria monocytogenes* Ca^{2+}-ATPase (LMCA1). Our findings delineate reversible and essentially irreversible steps in the transport process wherein Ca^{2+} efflux by LMCA1 is rate limited by phosphoenzyme formation. Using the smFRET approach we also identify key intermediates with no known crystal structures that shed new light on ATPase-coupled transport mechanism in this essential transporter family. We have solved the crystal structure of LMCA1 in an outward open conformation, and are currently using smFRET to guide crystallization of the structurally undescribed intermediate

OneDep: Unified wwPDB System for Deposition, Biocuration, and Validation of Macromolecular Structures in the PDB

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The worldwide PDB (wwPDB) is the international consortium which manages the Protein Data Bank (PDB) - the single global repository for three-dimensional structures of biological macromolecules and their complexes. Over the past decade, the size and complexity of macromolecules and their complexes with small molecules deposited to the PDB have increased significantly. The PDB archive now holds more than 125,000 experimentally determined structures of biological macromolecules, which are all publicly accessible without restriction. These structures provide essential information to a diverse user community worldwide. There are more than 590 millions data file downloads from the PDB archive and more than 1 million unique IP addresses access the archive every year.

In an effort to meet evolving archiving requirements of the scientific community over the coming decades, the wwPDB partners have launched and continue to improve a global unified system for deposition, biocuration, and validation of macromolecular structures - OneDep. It replaces legacy pipelines across PDB, EMDB, and BMRB deposition sites and will be able to interface with other archival resources. OneDep focuses on data quality and completeness across all three archives, while supporting growth in the number and complexity of depositions.

In this poster, we describe the design, functional operation, and supporting infrastructure of the OneDep system, and provide performance assessments.

PDBe: Bringing structure to biology and beyond

Matthew Conroy
Protein Data Bank in Europe

Protein Data Bank in Europe (PDBe) [1] a founder member of the wwPDB, has a mission to bring structure to biology. Data in the PDB archive is utilised by a diverse range of users including biochemists, geneticists, medicinal chemists, physicians, and even artists and school children. The complex nature of 3D structural data means that those with a limited background or training in structural biology do not always find it easy to exploit the rich information content of the structural archives to help them answer their research questions. It is for archive keepers such as PDBe to address this challenge and develop new ways of making structural information more easily accessible, relevant, and up-to-date.

Recently, PDBe implemented several new features to facilitate easy access to macromolecular structure data. These are inherently visual and, in collaboration with CEITEC in the Czech Republic, we have incorporated an easy to use, in-browser 3D visualizer on all structure webpages. With minimal data transfer needed, this works on all devices, including smartphones. In addition to models, electron density (for crystal structures) and electric potential maps (for cryoEM models) can be viewed where possible. 3D visualisation is interactively linked to sequence and topology views, a helpful teaching tool to link sequence and structure. All views can be overlaid with annotations of domain architecture and validation.

Structure data is rendered a much richer and powerful pedagogical aid if linked to accurate up-to-date annotations. The SIFTS resource (pdbe.org/sifts) [2] provides updated residue-level mappings to UniProt and annotations from a variety of other databases on a weekly basis. In conjunction with Europe PMC [3], PDBe is able to display figures and legends from open-access publications describing PDB entries and datamine literature for instances of PDB codes.

PDBe pages show the availability of, and indicate the results from, other resources useful to understanding macromolecular structure using a collection of PDB components. These include data from the PDB redo team at NKI, Amsterdam; Rajini from Dr. Madan Babu's lab at LMB, Cambridge; and a component providing biological relevance of assembly annotation in the PDB from Dr. Emanuel Levy's lab.

Via a RESTful API, data can be served programmatically and this is used in educational services such as Proteopedia, where a 'preferred' assembly of a structure is selected, and JalView where structure selection is used to select an appropriate structure and annotation is displayed on sequence data.

Resources are being developed to support the school science curriculum in collaboration with Newcastle University. We are also introducing structures to school students studying art, and this venture bringing together art and science, has already produced some stunning artworks. We hope that this collaboration will inspire the next generation of scientists and molecular graphic artists, and also enable the current generation of scientists to view their data through fresh eyes.

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In vivo cross-linking mass spectrometry visualizes the structural proteome of intact mitochondria and the architecture of respiratory supercomplexes

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Mitochondria host a variety of crucial metabolic pathways, such as fatty acid beta oxidation, the Krebs cycle and the respiratory chain. Many steps within these pathways are governed by large protein complexes and higher-order assemblies thereof, the so-called supercomplexes. Most notably, supercomplexes are thought to be formed within the mitochondrial respiratory chain, which consists of four multi-subunit protein assemblies (CI–CIV). Recently, a CI-CIII-CIV supercomplex could be visualized in molecular detail by single particle cryo electron microscopy (cryoEM) after detergent-based extraction. However, evidence for the respiratory supercomplex formation in intact mitochondria is sparse and knowledge about their structural arrangement *in vivo* is entirely lacking.
Closing in upon the *in vivo* organization of mitochondrial supercomplexes and protein interaction networks, we here probe intact mouse heart mitochondria by cross-linking mass spectrometry (XL-MS). Our analysis covers large parts of the mitochondrial proteome, yielding 3,322 unique lysine–lysine connections among 359 mitochondrial proteins from all mitochondrial compartments. The identified cross-links provide insights into different aspects of the structural proteome of intact mitochondria. First, they reflect native protein complex architectures, as we demonstrate by comparison with published high-resolution structures. Second, they illuminate the topology of structurally uncharacterized protein complexes, such as the recently discovered mitochondrial contact site and cristae organizing system (MICOS). Third, they reveal multiple contacts among individual protein complexes, confirming the formation of higher-order assemblies including respiratory supercomplexes. A subset of our cross-links corroborates the CI-CIII-CIV supercomplex architecture seen with cryoEM, however, we also find several crosslinks pointing toward alternative supercomplex arrangements, which we visualize by cross-linkbased interaction space analyses. Moreover, we identify several supercomplex cross-links involving CII, which has previously not been considered a stable part of the respiratory supercomplexes. Finally, we demonstrate the specificity of the detected supercomplex cross-links by performing a comparative XL-MS analysis of mitochondria after biochemical disruption of the respiratory chain. This treatment indeed abolishes the vast majority of the native-state supercomplex cross-links, showing that the mitochondrial protein contacts identified by *in vivo* XLMS distinctly reflect the respective biological condition.
In all, our results provide the so far largest survey of mitochondrial protein interactions. Most importantly, they give evidence for the *in vivo* formation of respiratory supercomplexes, which likely include all four respiratory complexes and exhibit diverse binding stoichiometries and interfaces.å

Probing USP7 mechanism of action through p53

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USP7 is one of the most important DUBs, involved in processes ranging from DNA damage to neuronal development. Among many targets, it can deubiquitinate p53, thereby initiating apoptosis. However, USP7 activity regulation and mode of action, although extensively studied, remain elusive. Previous structural studies have elucidated the target recognition domain, the catalytic domain as well as Ubl domains that are all required for full activity in cells. Most importantly, USP7 needs its C-terminal tail for full activity, but how this self-activation is regulated remained unclear.
Making use of chemical tools we could generate synthetic p53-ubiquitin molecules, that allowed us to generate USP7-substrate complexes and probe the activation mechanism. Using biochemical and biophysical methods we show how the recognition of targets actually increases the self-activation of USP7. Furthermore, we show that this self-activation is a two-step process where the presence of ubiquitin plays a pivotal role. Combined with structural data and NMR methods we could identify crucial regions for this, substrate-induced, self-activation.

Neutron protein crystallography: New developments and recent application examples

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With the advent of new instruments (e. g. Imagine at HFIR, MANDI at SNS and BIODIFF at FRMII) and well established instruments (iBIX at JPARC and LADI at ILL) neutron protein crystallography has seen a resurrection from the past pioneering work by Schoenborn. New sample environment options at the instruments and a growing user community have greatly enhanced the outcome of the existing neutron diffractometers. Measurements at 100 K in a nitrogen gas stream (cryostream) are now routinely possible at most neutron diffractometers. Efforts to increase the flux at the sample position and to reduce the background at the detector enable to measure smaller and smaller protein crystals. Yet, measuring crystals with volumes below 0.1 mm^3 is still a big challenge and usually works only in exceptional cases. The main scientific questions addressed are: Hydrogen bonding to ligands/substrates, protonation states of amino acids in intermediate states of the catalytic process and determining the correct structure of metallo-proteins which are subjected to reduction due to the radiation damage caused by x-rays. But also the water shell on the outer surface of the protein can be studied, whereby water molecules of different flexibility can be observed.
As an example, for a neutron diffractometer, the instrument BIODIFF is introduced: It is a joint project of the Jülich Centre for Neutron Science (JCNS) and the FRM II. BIODIFF is designed as a monochromatic instrument with a narrow wavelength spread of less than 3 %. To cover a large solid angle the main detector of BIODIFF consists of a neutron imaging plate in a cylindrical geometry with online read-out capability. An optical CCD-camera pointing at the sample position is used to quickly align the sample (or a Cadmium replica of it) with respect to the neutron beam. The main advantage of BIODIFF is the possibility to adapt the wavelength to the size of the unit cell of the sample crystal while operating with a clean monochromatic beam that keeps the background level low.
In this contribution, a review of most recent application examples of neutron protein crystallography is given. New developments are discussed which may lead to a widening of the application scope of this method. Especially the need for large protein (typically > 0.5 mm^3 in volume) crystals is addressed.

The Unique domain forms a fuzzy intramolecular complex in Src family kinases

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c-Src is the foremost representative of the Src Family Kinases, a group of related tyrosine kinases that control key cellular processes. Because their role as signaling hubs, lack of a tight regulation is related with a number of tumoral processes.
Combining SAXS and NMR, here[1] we show that the N-terminal regulatory region of c-Src including the SH4, Unique and SH3 domains adopts a compact, yet highly dynamic, structure that can be described as an intramolecular fuzzy complex. The folded SH3 domain acts as a rigid scaffold for the intrinsically disordered Unique and SH4 domains, in which multiple residues interacting with the folded partner are scattered.
Most of the long-range interactions within the Unique domain are also observed in constructs lacking the structured SH3, indicating a considerable degree of preorganization of the disordered Unique domain.
We show that these distant contacts defining conformational prearrangement are mediated by functionally relevant phenylalanine residues. We also demonstrate that the interactions between the disordered domains with the SH3 domain are affected as well by removal of these important residues.
Using sequence alignment and coevolution analysis, we find that not only Src homologues but also members of the Src family of kinases (SFK) share well-conserved sequence features involving aromatic residues in their Unique domains.
This observation contrasts with the supposed lack of sequence homology implied by the name of these domains and suggests that the other members of SFK also have a regulatory region involving their Unique domains. We argue that the Unique domain of each SFK is sensitive to specific input signals, encoded by each specific sequence, but the entire family shares a common mechanism for connecting the disordered and structured domains.
In a more general sense, we introduce an example on how the functional properties of intrinsically disordered regions are encoded in their sequence by means of sparse key

elements embedded in a dynamic mesh. Hopefully, this bring one step closer to decipher how disordered regions are able to channel information.

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The role of Lon-mediated proteolysis in the dynamics of mitochondrial nucleic-acid complexes

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Mitochondrial nucleoids are the intricate nucleo-protein complexes formed of mitochondrial DNA and two sets of proteins, DNA-binding core-proteins involved in mtDNA organization, maintenance and transcription, and a range of peripheral factors acting as components of various signalling pathways. The molecular interactions of nucleoid components with the organellar network and cellular metabolism are still unclear and continue to be studied widely. Our study focuses on mitochondrial ATP-dependent protease Lon which is the essential part of protein quality control mechanism and a component of mitochondrial nucleoids. Recently, Lon was shown to regulate TFAM, the most abundant mtDNA structural factor in human mitochondria. Based on such information, we have examined Lon's inter-connection with proteins involved in mtDNA metabolism by comparing the *in vitro* digestion profiles of the *Saccharomyces cerevisiae* TFAM functional homologue Abf2, the yeast mtDNA maintenance protein Mgm101, and two human mitochondrial proteins, Twinkle helicase and the large ribosomal subunit protein MrpL32. The Mgm101 degradation was also shown *in vivo* in yeast mitochondria and a closer characteristic of Lon's biochemical activities under various conditions was employed. In our case, the association of studied proteins with nucleic acids proves to be an important determinant of Lon's substrate recognition and activity. Such regulatory mechanism might facilitate dynamic changes to the mitochondrial nucleoids and ribosomes, which are crucial for conducting the mitochondrial functions and maintaining the mitochondrial homeostasis.

Acknowledgements: This work was supported by the grants of Slovak Research and Development Agency (APVV-0375-15) and Slovak Grant Agency (VEGA 2(0113/14).

A fully automatic method for measuring local resolution in electron microscopy maps.

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Resolution has been discussed since the beginning of electron microscopy. The most used resolution metric is the Fourier Shell Correlation (FSC), which is a measurement of self-consistency between two half volumes by means of the normalized cross correlation at different frequencies. However, the quality of a 3D-reconstruction is anisotropic, and the mentioned methods are invalid for computing local resolutions.

In this proceeding a new and fully automatic method, named MonoRes, for determining the local resolution of a 3D electron microscopy map is presented. The root of this algorithm is an extension of the concept of analytic signal, called monogenic signal. Thus a frequency sweep is applied to the original map. At each frequency we determine whether the local energy at a voxel is significantly different from the local energy expected for a noise voxel. The local resolution is defined as the inverse of the first frequency for which the voxel of the macromolecule fails to be significantly above noise.The results have shown that the algorithm is highly accurate in all tests and being computationally fast in comparison with existing methods in the field. Moreover, MonoRes can perform local filtering of the original map based on the calculated local resolution.

The HIV-1 pre-integration complexes: structures, functions and dynamics

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After retroviral infection of a target cell, during the early phase of replication, the HIV-1 genomic viral RNA is reverse transcribed by the viral reverse transcriptase to generate the double-stranded viral DNA that interact with viral and cellular proteins to form the pre-integration complex (PIC). Viral integrase (IN) is a key component of the PIC and is involved in several steps of replication notably in reverse transcription, nuclear import, chromatin targeting and integration. Viral components such as IN cannot perform these functions on their own and need to recruit host cell proteins to efficiently carry out the different processes. IN is a flexible protein, property allowing its interaction with multiple partners and enabling its multiple functions in viral replication. The molecular mechanisms and dynamics of these processes remain largely unknown. Purification of proteins that participate in these large transient complexes is impeded by low amounts, heterogeneity, instability and poor solubility. To circumvent these difficulties we develop methodologies that enable the production of stable complexes for structural and functional studies [1] as well as system for the production of multi-protein complexes from mammalian cells enabling assembly of entire complexes within cells. Using these strategies we reconstruct in vitro stable and soluble complexes around IN. We solved cryo-EM structures of the IN/LEDGF/DNA [2] and IN/LEDGF/INI1/DNA [3] complexes. Other IN complexes as well as IN post-translational modifications have been characterized and are under study.

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A single tool to overcome the two major chokepoints of protein Crystallography

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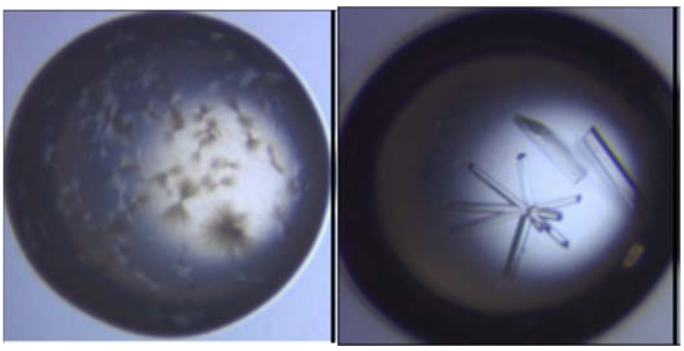
Protein crystallography, the major technique for protein structure determination, is limited by two major bottlenecks: (a) obtaining protein crystals and (b) solving the phase problem. Since 2000, our team has developed luminescent lanthanide complexes as auxiliary for structure determination of macromolecules, in particular, exploiting the high-phasing power of lanthanide elements [1-7].

Our previous works highlighted the supramolecular complexes-protein interactions and the required technical specifications to design compatible agents with high throughput crystallization platforms. This unprecedented approach leads to a new terbium complex, named Tb-Xo4. This lanthanide complexe added to the protein solution before crystallization shows exceptional nucleant properties.

Up to now this effect was tested on 12 proteins (including 4 unknowns) with different molecular weights and different oligomeric states. It was observed that, in addition to new crystallization conditions, Tb-Xo4 also improves the quality of the crystals obtained (see picture) and is a high phasing power lanthanide complex, fully compatible with conventional phasing methods.

We will present our last results using this new agent for protein crystallography which, in addition to nucleant and phasing properties, also allows an easier detection and centering of the protein crystals thanks to their luminescence. We believe that this all-in-one lanthanide complex could offer a convenient alternative to the tedious and time-consuming work of seleno-methionine labelling or of heavy-atom incorporation.



Crystallization drops obtained for the same protein without (left) and with Tb-Xo4 (right).

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Human tyrosine hydroxylase – structural study of regulatory mechanism.

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Tyrosine hydroxylase (hTH1) catalyzes hydroxylation of L-DOPA and it is a rate-limiting enzyme in biosynthesis of important neurotransmitters and hormones: dopamine, noradrenaline and adrenaline. Its activity is regulated by phosphorylation of its regulatory domain and by the interaction with 14-3-3 protein. So far, the full length hTH1 structure was not solved and the activation mechanism stays unclear.

We used single particle cryo-electron microscopy to determine structure of full length hTH1, focusing on the intra-domain orientation of regulatory domains towards catalytic domain tetramer. And NMR spectroscopy was applied in order to monitor structural changes within the regulatory domain of hTH1 (RD-hTH1, residue 1-169) caused by phosphorylation of serine residues within intrinsically disordered N-terminal region and by binding of 14-3-3 protein.

Acknowledgments: The project is financed from the SoMoPro II programme. The research leading to this invention has acquired a financial grant from the People Programme (Marie Curie action) of the Seventh Framework Programme of EU according to the REA Grant Agreement No. 291782. The research is further co-financed by the South-Moravian Region. The article/paper reflects only the author's views and the Union is not liable for any use that may be made of the information contained therein. In addition, this work was also supported by Czech Science Foundation (I 1999-N28).

Structural investigation of viral genome release mechanism

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Last century witnessed an accelerating trend in the emergence or resurgence of human pathogens. Virus-caused diseases rank among the deadliest and have a high potential to reach pandemic status. While immunization proved to be an effective strategy against some virus-caused diseases, many viral pathogens exhibit high mutation and recombination rates allowing them to rapidly evade vaccines and treatments. Effective anti-viral therapies will no doubt require a combination of approaches, targeting simultaneously multiple stages of viral life-cycle. To close the substantial gaps in our understanding of viral life and pathogen-host interaction, this project aims to **elucidate viral genome organization and the mechanism of its release into host cells** for two virus models, employing a combination of biochemical and biophysical approaches allowing a description of this process at an atomic-level of detail. We use the minute virus of mice (MVM), strain I, from the *Parvoviridae* family, and the human rhinovirus A serotype 2 (HRV2) from the *Picornaviridae* family, as model species of non-enveloped viruses carrying single-stranded DNA or RNA genomes, respectively. In the MVM particle, non-structural protein NS1 is covalently linked to the 5' end of the genomic DNA and is exposed together with 24 nucleotides at the virion surface. As the genome does not follow the icosahedral symmetry of the capsid, we are performing an asymmetric single-particle reconstruction of the native virus from cryo-electron microscopy (EM) data to describe the structure of native MVM virions with the 5' end of the genome passing through the capsid. This approach could additionally provide a picture of the genome organization inside the MVM particles. To study viral genome release, we produced HRV2 uncoating intermediates by inducing genomic RNA cross-link formation by UV irradiation of the particles in the presence of a cross-linking agent, followed by a partial genome expulsion by heating. To facilitate the identification of the exiting genome in cryo-EM micrographs, we are developing a method for enzymatically attaching heavy-metal labels on the exposed RNA ends. Again, asymmetric high-resolution cryo-EM reconstruction will allow to locate the capsid pore with the exiting genome, describe the structure of the nucleic acid upon release, and characterize the interactions allowing RNA sliding through the pore. These structures describing viral genome reorganization during uncoating could provide a foundation for rational design of viral genome release inhibitors, furthering the development of new anti-viral therapeutic strategies.

Structural and functional studies of the MBD3 subunit of NuRD

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The Nucleosome remodeling and histone deacetylase complex (NuRD) is one of the major chromatin remodeling entities. This multiprotein assembly, present in all eukaryotic organisms, is involved in transcription regulation through chromatin compaction and de-compaction. This complex is composed of at least 8 subunits including the Methyl Cytosine Binding Domain protein 3 (MBD3) and only few information is known about the stochiometry of the complex and the structure of some subunits.

MBD3 subunit is quite unique among the MBD family. Indeed, MBD proteins have the ability to bind methylated Cytosine-Guanine (mCpG) dinucleotides and act as translators between DNA methylation and histone modifications and thus interpret the DNA methylation patterns. MBD3 turns out to be an exception in this family and shows a loss in the capacity to bind methylated DNA. Therefore the methylation level of the DNA target (hydroxymethylated or non-methylated) is still unclear and the functional role of MBD3 and its structure remain unknown.

Our current project focuses on an integrated structural biology analysis of MBD3, involved in DNA recognition. The objective of this approach is the structural analysis of the DNA-bound MBD3 protein, which will provide information about the specificity of interaction areas and the mechanism of molecular recognition of nonmethylated DNA.

We set up the purification of different constructs of MBD3 and realized different complexes to study them with biophysical approach such as gelfiltration, DLS or EMSA gels. This allows us to highlight importance of a disordered region of MBD3 in DNA interaction. This protein characterization paves the way to structural analyse by crystallography.

Iterative model-based density improvement enhances atomic model refinement in cryo-EM maps

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Refined atomic models based on high-resolution density maps are the ultimate result of the cryo-EM structure determination process. Current cryo-EM model refinement procedures use globally sharpened and filtered maps as constant map targets to generate atomic models. Here, we introduce a general model refinement procedure that iteratively improves cryo-EM density maps based on prior knowledge of atomic models such that the resulting map faithfully restores local structural features. Thus, the procedure requires an atomic model to optimize contrast of experimental cryo-EM densities by local amplitude scaling (LocScale). We tested the integrated refinement procedure on four cryo-EM structures of TRPV1, β-galactosidase, γ-secretase and RNA polymerase III. We demonstrate that LocScale density modification improves atomic models and reveals previously undiscovered structural details. The presented approach enhances the interpretability of single-particle cryo-EM density maps and provides an implementation reminiscent of iterative density improvement as it is routinely employed in the refinement of X-ray crystallographic models.

IBiSS, a versatile and interactive tool for integrated sequence and 3D structure analysis of large macromolecular complexes

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Motivation:
In the past few years, an increasing number of crystal and cryo electron microscopy (cryo-EM) structures of large macromolecular complexes, such as the ribosome or the RNA polymerase, have become available from various species. These multi-subunit complexes can be difficult to analyze at the level of amino acid sequence in combination with the 3D structural organization of the complex. Therefore, novel tools for simultaneous analysis of structure and sequence information of complex assemblies are required to better understand the basis of molecular mechanisms and their functional implications.

Results:
Here, we present a web-based tool, Integrative Biology of Sequences and Structures (IBiSS), which is designed for interactively displaying 3D structures and selected sequences of subunits from large macromolecular complexes thus allowing simultaneous structure-sequence analysis such as conserved residues involved in catalysis or protein-protein interfaces. This tool comprises a Graphic User Interface and uses a rapid-access internal database, containing the relevant pre-aligned multiple sequences across all species available and 3D structural information. These annotations are automatically retrieved and updated from UniProt and crystallographic and cryo-EM data available in the Protein Data Bank (PDB) and Electron Microscopy Data Bank (EMDB).

Integrated Structural Biology of Unstructured Proteins: Microtubule Associated Protein 2c

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Intrinsically disordered proteins (IDPs) do not adopt a well defined 3D structure, but are physiologically active and involved in various physiological and pathological processes. Microtubule associated proteins (MAPs) represent a class of IDPs important for development and function of neurons. Among MAPs, Tau, present mostly in axons, has been studied most extensively because it is associated with the Alzheimer's disease. Its homolog, microtubule associated protein 2c (MAP2c), is localized in dendrites and is supposed to play a role in the early development of brain. Functions of both Tau and MAP2c are regulated by posttranslational modifications and protein-protein interactions. We employed a range of biophysical method to characterize structural features, dynamics, phosphorylation, and interactions of MAP2c. We found striking differences between MAP2c and Tau, suggesting that both MAP2c and Tau respond to the same signal (phosphorylation by cAMP-dependent protein kinase) but have different downstream effects, indicating a signaling branch point for controling microtubule stability.

Acknowledgement: This work has been supported by the Czech Science Foundation (grants GA15-14974S and GF15-34684L) and by the Ministry of Education, Youth and Sports of the Czech Republic under the project CEITEC 2020 (LQ1601).

THE TAZ2 (CBP) TAD (C/EBPB) INTERACTION

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Cyclic-AMP-response element-binding protein (CREB)-binding protein, (CBP), and its paralog p300 are histone acetyl transferases playing a critical role in embryonic development, cell growth control, division as well as homeostasis. Via its Taz2 domain, they interact with multiple transcription factors to regulate gene expression. These transcription factors include CCAAT-enhancer-binding proteins, the C/EBP family. Through transactivation domain (TAD), C/EBPs recruit the co-activators (p300, CBP) that open up chromatin structure and mediate its phosphorylation through the recruitment of specific kinases. C/EBPβ binds to the closed chromatin and acts as a pioneering factor for initiating tissue-specific gene expression at several promoters. Here, we present the detailed structural characterization of the C/EBPβ interaction with CBP.

Acknowledgement*:* This work is supported by the Charles University Grant Agency (grant No. 227020).

Crystal structure of the Cpf1 endonuclease R-loop complex after target DNA cleavage

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Cpf1 is a single RNA-guided endonuclease of class 2 type V CRISPR-Cas system, emerging as a powerful genome editing tool 1,2. To provide insight into its DNA targeting mechanism, we have determined the crystal structure of *Francisella novicida* Cpf1 (FnCpf1) in complex with the triple strand R-loop formed after target DNA cleavage. The structure reveals a unique machinery for target DNA unwinding to form a crRNA-DNA hybrid and a displaced DNA strand inside FnCpf1. The protospacer adjacent motif (PAM) is recognised by the PAM interacting (PI) domain. In this domain, the conserved K667, K671 and K677 are arranged in a dentate manner in a loop-lysine helix-loop motif (LKL). The helix is inserted at a 45° angle to the dsDNA longitudinal axis. Unzipping of the dsDNA in a cleft arranged by acidic and hydrophobic residues facilitates the hybridization of the target DNA strand with crRNA. K667 initiates unwinding by pushing away the guanine after the PAM sequence of the dsDNA. The PAM ssDNA is funnelled towards the nuclease site, which is located 70 Å away, through a hydrophobic protein cavity with basic patches that interact with the phosphate backbone. In this catalytically active conformation the PI and the helix-loop-helix (HLH) motif in the REC1 domain adopt a “rail shape” and “flap-on” conformations, channelling the PAM strand into the cavity. A steric barrier between the RuvC-II and REC1 domains forms a “septum” that separates the displaced PAM strand and the crRNA-DNA hybrid, avoiding reannealing of the DNA. Mutations in key residues reveal a novel mechanism to determine the DNA product length, thereby linking the PAM and DNA nuclease sites. Our study reveals a singular working model of RNA-guided DNA cleavage by Cpf1, opening up new avenues for engineering this genome modification system 2-5.

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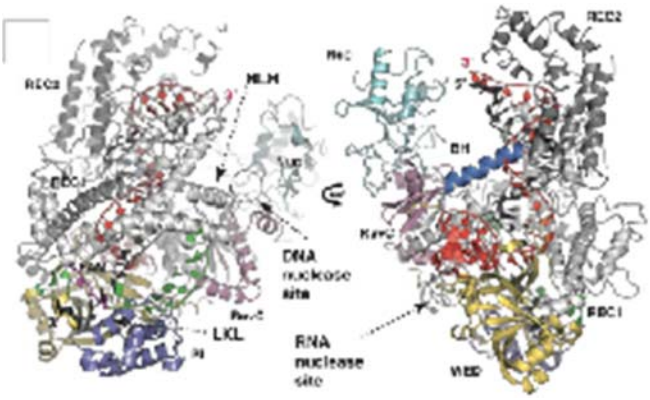

Figure 1.- Ribbon diagram of the Cpf1-crRNA-target DNA ternary complex showing the R-loop formed after catalysis by the endonuclease. The 200 kDa triple complex is coloured according to the different domains in the polypeptide

Structural and Functional Insights into Bacterial Thiosulfate Dehydrogenases

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The oxidative condensation of two thiosulfate anions to tetrathionate constitutes a well-documented and significant part of the natural sulfur cycle, however little is known about the enzymes catalyzing this reaction. In the purple sulfur bacterium *Allochromatium vinosum*, the reaction is catalyzed by the periplasmic diheme *c*-type cytochrome thiosulfate dehydrogenase (TsdA). Here, we report the crystal structure of the "as-isolated" form of *A. vinosum* TsdA and those of several redox states of the enzyme. The protein contains two typical class I *c*-type cytochrome domains wrapped around two covalently bound hemes axially coordinated by His53/Cys96 and His164/Lys208. These domains are very similar suggesting a gene duplication event during evolution. A ligand switch from Lys208 to Met209 is observed upon reduction of the enzyme. TsdALys208Asn or Lys208Gly variants exhibit similar substrate affinities as the wild-type protein but much lower specific activities. Cys96 is an essential residue for catalysis with the specific activity of the enzyme being completely abolished in several TsdA Cys96 variants. Based on this data, we draw important conclusions about the enzyme and propose a possible reaction mechanism for TsdA (1).

In addition, we have solved the three-dimensional structure of *Marichromatium purpuratum* TsdA fused with TsdB protein providing insights into internal electron transfer. TsdB is the electron acceptor of TsdA. In the oxidized state, this tetraheme cytochrome c contains three hemes with axial His/Met ligation, while heme 3 exhibits the His/Cys coordination typical for TsdA active sites. Interestingly, thiosulfate is covalently bound to Cys330 on heme 3. Both, AvTsdA and the MpTsdBA fusion react efficiently *in vitro* with high potential iron sulfur protein from *A. vinosum* (Em +350 mV). HiPIP not only acts as direct electron donor to the reaction center in anoxygenic phototrophs but can also be involved in aerobic respiratory chains (2).

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Ultrasound as potential "INSTRUCTor" of protein crystallisation

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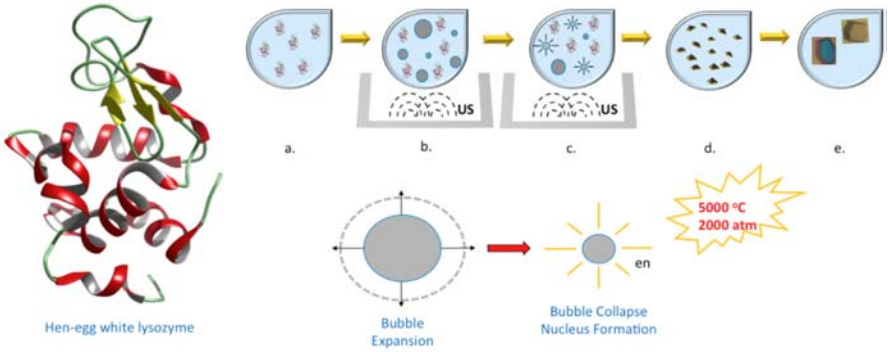
Research on structural biology has maintained its foundational character throughout the years, delivering scientific breakthrough and setting new lines of investigation in proteins 3D structure, while advancing technological achievements. The key actor remains X-ray protein crystallography as reflected on the number of protein structures deposited annually with the Protein Data Bank, followed by NMR, EM and SAXS. Knowledge of the detailed protein structure at high resolution, still requires growing crystals, a laborious and expensive task, not at all trivial. Cost-effective resources have been employed involving a selection of crystallization screens for random or rational testing of a broad range of conditions, automated equipment handling miniaturized sample volumes and imaging systems for crystal detection. The quest also for factors that would affect the rate of protein precipitation is still on. Previous studies have shown that use of external fields such as ultrasound1-2 could be beneficial promoting the nucleation stage accelerating protein crystallization. Our work focuses on the investigation of ultrasonic irradiation on protein samples and its effect on crystal growth using hen egg white lysozyme (HEWL). The oligomeric state and the hydrodynamic radius of the protein solutions were thoroughly investigated employing dynamic light scattering prior to crystallization and the samples were irradiated using different time frames. For this purpose multi angle DLS coupled with a water bath tank (Branson 1520, Bransonic) and our in house robotic crystallization facility (OryxNano, Douglas Instruments, UK) were employed. The results showed that ultrasound acted as a nucleation promoter affecting the kinetics of nucleation and crystal growth.



Acknowledgements: This work has been supported by ARCADE Grant agreement FP7-REGPOT-2009-1-No 245866.

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Crystal structure of the Cpf1 endonuclease R-loop

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Cpf1 is a single RNA-guided endonuclease of class 2 type V CRISPR-Cas system, emerging as a powerful genome editing tool [1,2]. To provide insight into its DNA targeting mechanism, we have determined the crystal structure of *Francisella novicida* Cpf1 (FnCpf1) in complex with the triple strand R-loop formed after target DNA cleavage. The structure reveals a unique machinery for target DNA unwinding to form a crRNA-DNA hybrid and a displaced DNA strand inside FnCpf1. The protospacer adjacent motif (PAM) is recognised by the PAM interacting (PI) domain. In this domain, the conserved K667, K671 and K677 are arranged in a dentate manner in a loop-lysine helix-loop motif (LKL). The helix is inserted at a 45o angle to the dsDNA longitudinal axis. Unzipping of the dsDNA in a cleft arranged by acidic and hydrophobic residues facilitates the hybridization of the target DNA strand with crRNA. K667 initiates unwinding by pushing away the guanine after the PAM sequence of the dsDNA. The PAM ssDNA is funnelled towards the nuclease site, which is located 70 Å away, through a hydrophobic protein cavity with basic patches that interact with the phosphate backbone. In this catalytically active conformation the PI and the helix-loop-helix (HLH) motif in the REC1 domain adopt a “rail shape” and “flap-on” conformations, channelling the PAM strand into the cavity. A steric barrier between the RuvC-II and REC1 domains forms a “septum” that separates the displaced PAM strand and the crRNA-DNA hybrid, avoiding re- annealing of the DNA. Mutations in key residues reveal a novel mechanism to determine the DNA product length, thereby linking the PAM and DNA nuclease sites. Our study reveals a singular working model of RNA-guided DNA cleavage by Cpf1, opening up new avenues for engineering this genome modification system [2-5].

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Structural Study of RNA - protein interaction causing mis-splicing of CFTR exon 9

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The human genome contains a relatively low number of protein-coding genes. However, the comlexity of our organism is being achieved by the diversification of the genes through various mechanisms, such as alternative splicing of precursor mRNAs. The later is a complicated, tightly regulated process, corruption of which may lead to disruption of gene expression and eventually disease. Here, we study the regulatory RNA-protein interaction surrounding the aberrantly spliced cystic fibrosis transmembrane conductance regulator (CFTR) exon 9. Skipping of exon 9 leads to the production of a non-functional chloride channel which is associated with severe forms of cystic fibrosis. This unfavourable splicing event depends primarily on DNA variations in the polymorphic (TG)mTn locus upstream of exon 9. On the pre-mRNA level, this locus generates an extended UG-rich binding site for TDP-43 (43 kDa TAR DNA binding protein) which - in concert with recruitment of hnRNP A1 (heterogeneous nuclear ribonucleoprotein A1) – prevents, according to our hypothesis, recognition of the 3' splice site (3'ss) of exon 9 by the splicing machinery. Thus, exon 9 is excluded from the spliced mRNA resulting in a non-functional protein product. While TDP-43 is the dominant inhibitor of exon 9 inclusion, hnRNP A1 is also essential for aberrant splicing but its role is much less clear.

The protein hnRNPA1 is composed of two RNA recognition motifs (RRM1 and RRM2) followed by a glycine-rich domain. We characterize the interaction between hnRNPA1 and the CFTR exon 9 3'ss RNA using NMR spectroscopy, binding studies and in vivo splicing assays. The later revealed that a 15 nt sequence at the intron8-exon9 junction which comprises a previously unknown exonic splicing silencer (ESS) element. Using NMR spectroscopy, we show that two copies of hnRNPA1 bind to this 15 nt ESS element. In an attempt to further characterize the binding mode, we undertook RNA-binding measurments by ITC using either both RRMs or each one separately. The affinities are in the submicromolar range and stoichiometries obtained indicate that both the individual RRMs and RRM1+2 form 2:1 complexes with the ESS RNA. RRM1+2 binds to a 15 nt model RNA of the CFTR 3'ss in an intermediate exchange regime on NMR timescale, restricting us from obtaining structural information about the RNA-protein interaction. In contrast, RRM2 alone binds to a shorter 8 nt model RNA in a fast exchange regime on the NMR timescale enabling us to determine the structure by NMR spectroscopy. Future structural studies, using both traditional NMR workflows and paramagnetic relaxation enhancement, aim to understand in detail the RNA-binding mode and the structure of the hnRNP A1 – CFTR ESS RNA interaction. These results will shed light on the molecular basis of aberrant CFTR exon 9 splicing.

Structural studies of Acad9 and mitochondrial complex I assembly factors to investigate their role in neurodegeneration

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European Synchrotron Radiation Facility

Alzheimer's disease (AD) is a fatal neurodegenerative disorder characterized by amyloid-β (Aβ) plaques and whose causality remains unclear. There is evidence that Aβ enters into the mitochondria in brains of AD patients and impairs the mitochondrial respiratory complex I (CI) system, leading to defective neurotransmission, synaptic damage and cognitive impairment. The assembly of CI is a complicated process facilitated by specific assembly factors. However, how CI malfunctioning affects neuronal integrity remains a major open question.

Acyl-CoA dehydrogenase 9 (ACAD9) was first identified as a member of the acyl-CoA dehydrogenase family involved in faLy acid beta-oxidation, but more recently, it has been reported to play a critical role as an assembly factor of CI. ACAD9 associates with NDUFAF1 and ECSIT in a co-dependent fashion to form the so called mitochondrial complex I assembly (MCIA) complex. Interestingly, ACAD9 mutations result in CI deficiencies while the long-chain faLy acid oxida7on remains unperturbed. Furthermore, ECSIT has been iden7fied as an interacting node between Aβ enzymes and chaperones involved in mitochondrial energetics.

Our project pursues the investigation of the 3D architecture and atomic structure of ACAD9 in complex with the MCIA assembly factors and elucidate the functional link between CI misassembly and Aβ toxicity. The biochemical and biophysical characterization of Acad9 will provide essential information to determine its domain organization and solution conformation. In vivo and in vitro protein interaction studies with the other MCIA factors will help us to unveil the stoichiometry, binding affinities and interaction interfaces of the MCIA complex. Finally, the structure determina7on of ACAD9 and in complex with NDUFAF1 and ECSIT will shed light into the molecular mechanisms underlying complex I biogenesis and its interplay with Aβ dynamics.

V/A-ATPase as a novel drug target against the human intracellular parasite *Chlamydia trachomatis*

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Chlamydia trachomatis (*C.t.*) is an obligate intracellular parasite of humans for which improper treatment develops in chronic infections and associated multiple diseases.
The genomic, proteomic and trascriptome analyses indicate that *C.t.* not only encodes but also produces at early stages of infection several protein complexes involved in Na+ circulation across *C.t.* plasma membrane. Some components of *C.t.* Na+ cycle could generate and maintain Na+ gradient across *C.t.* plasma membrane (i.e. Na+-transporting NADH:ubiquinone oxidoreductase, Na+-NQR and Na+/H+ antiporter NhaD). The multiple Na+/solute symporters and Na+-coupled V/A-ATPase could consume Na+ gradient across membrane for amino acids uptake and ATP synthesis at particular stages of developmental cycle. Because of the critical importants for *C.t.* cell physiology these Na+- translocating protein complexes are proposed to be the factors of *C.t.* pathogenicity. We aim to explore the role of putative Na+ transporting protein complexes for viability of *C.t.* cells during infection and other stages of *C.t.* cell cycle and to test the potency of these transporters to become novel drug target candidates for the treatment of *C.t.* infections.
We particularly focus on structural organization and regulation of *C.t.* V/A-ATPase, as an interesting drug target. We have produced and purified *C.t.* V/A-ATPase and its Vo/Ao and V1/A1 subdomains. Currently we perform characterization of the reversible assembly and catalytic properties of this V/A-ATPase. We have the first biochemical evidences about ion (Na+) specificity of this enzyme. Since no specific and medically relevant inhibitors of this Na+-type V/A-ATPase are currently available, we perform chemical ligand libraries screening in order to obtain highly potent inhibitors of *C.t.* V/A-ATPase.
Beside biochemical studies, in order to aid with rational drug design, we perform structural characterization of *C.t.* V/A-ATPase by means of X-ray crystallography.
After initial crystallization screening we have already obtained diffracting crystals for membrane Vo/Ao and soluble V1/A1 subdomains of the enzyme and tested these crystals at several INSTRUCT supported X-ray facilities.
This project was supported in 2106 by Instruct Internship and Instruct R&D pilot fellowships. During Instruct Biennial Structural Biology Meeting the current progress on the project will be presented.

Towards Structural Characterisation of Large DNA Virus OtV-5 Infecting the Marine Picoeukaryote *Ostreococcus tauri*

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Viruses are the most abundant biological entities in the oceans, with an estimated 10^8 particles per millilitre seawater. In aquatic environments, viruses of algae in general play a crucial role, controlling phytoplankton populations (algal blooms), promoting nutrient recycling and biogeochemical cycling, and driving evolutionary processes that shape biodiversity.

Tiny marine phytoplankton are ubiquitous and significant contributors to global primary production and biomass. *Ostreococcus tauri, a* unicellular, marine green alga, and the smallest known free-living eukaryote, is infected by Ostreococcus tauri virus (OtV-5), a large double-stranded DNA virus belonging to the family Phycodnaviridae[1].

In order to characterize and visualize OtV-5 and its interactions with the algal host cell during the different stages of infection and virus particle assembly, we employ an integrative structural biology approach by combining biochemical and biophysical techniques with state-of-the-art cryo-electron microscopy and –tomography.

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Acid- Sensing Ion Channel 1a (ASIC1a): a promising target in neurological Disorders

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Extracellular acidosis affects several neuroinflammatory/neurodegenerative disorders. Under physiological conditions, the extracellular pH levels in brain are maintained at 7.4 through various protons (H+) transporting mechanisms. However, during inflammation such levels are significantly reduced causing a long lasting acidosis in brain. Tissue acidosis is a common feature in pathological conditions such as multiple sclerosis (MS), stroke and epilepsy, which are characterized by massive cell loss, essentially leading to deterioration in quality and function of tissues. Several ion channels modulate this reduction of pH levels included the Acid-Sensing Ion Channels (ASICs). ASICs are voltage-independent, proton-gated cation channels expressed in neurons of the central (CNS)- and peripheral nervous system (PNS) of mammalian organisms. To date, six ASICs isoform (ASIC1a, ASIC1b, ASIC2a, ASIC2b, ASIC3 and ASIC4) encoded by 4 genes have been identified (Lingueglia et al., 2007; Li et al., 2014) that can assemble in functional homo- and heteromeric channels (Gru¨nder and Pusch, review 2015). Although ASICs isoforms are broadly expressed, CNS neurons preferentially express ASIC1a and ASIC2a/2b (Wemmie et al., 2002). The functional channel mediates Na+ and Ca2+ influx into the cells. Under pathological conditions, a robust increase of Na+ and Ca2+ intracellular concentration cause alteration of the cell behavior, including differentiation, autophagy and apoptosis (Li et al., 2013; Sun et al., 2011). Mounting evidences suggest that abnormal expression and activation of ASIC1a in brain leads to progression of neurodegenerative diseases (Xiong et al., 2004). Despite the considerable efforts and progresses in understanding the molecular mechanisms of such disorders, current therapeutic options are insufficient and no effective treatment drugs have emerged yet.

To date many studies have focused on the potential physiological roles of ASICs, however the precise mechanisms by which ASICs are activated and how the binding region(s) of different toxins (Chen et al., 2005; Diochot et al., 2004) and small molecules (Kuduk et al., 2009) on ASICs could block their activity in brain remain unclear. Besides, a new class of compounds, the diarylamidines, including diminazene (DA) has been described as potent inhibitors of ASICs (Chen et al., 2010). DA is potentially able to block the activity of ASIC1a/b and ASIC2 isoforms at low concentration. However, DA is poorly selective for different ASIC isoforms and shows a low BBB-permeability, thus the generation of new DA-inspired molecules being able to overcome the limitation for the development of innovative therapeutic compounds.

The main aim of my PhD project is to determine the ASIC1a isoform molecular features needed to design potent, isoform-selective, brain-penetrant DA analogs. Indeed, it is known that pharmacological inhibition of ASIC channels, using non-selective drugs, exerts beneficial effects, improves clinical outcomes and protects neurons from degeneration. The molecular mechanisms acting downstream of ASICs are far from being elucidated. Therefore, selectively targeting ASIC1 is crucial to clarify the role of ASIC isoforms in neuro-disorders. Structural analysis based on molecular modelling and protein

crystallography will allow the rational design of novel DA-inspired analogues with higher isoform-potency and selectivity.

Structure based development of a blood-stage malaria vaccine

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Many promising vaccine candidates from pathogenic viruses, bacteria, and parasites are unstable and cannot be produced cheaply for clinical use. For instance, *Plasmodium falciparum* reticulocyte-binding protein homolog 5 (PfRH5) is essential for erythrocyte invasion, is highly conserved among field isolates, and elicits antibodies that neutralize in vitro and protect in an animal model, making it a leading malaria vaccine candidate. However, functional RH5 is only expressible in eukaryotic systems and exhibits moderate temperature tolerance, limiting its usefulness in hot and low-income countries where malaria prevails. Current approaches to immunogen stabilization involve iterative application of rational or semirational design, random mutagenesis, and biochemical characterization. Typically, each round of optimization yields minor improvement in stability, and multiple rounds are required. In contrast, we developed a one-step design strategy using phylogenetic analysis and Rosetta atomistic calculations to design PfRH5 variants with improved packing and surface polarity. To demonstrate the robustness of this approach, we tested three PfRH5 designs, all of which showed improved stability relative to wild type. The best, bearing 18 mutations relative to PfRH5, expressed in a folded form in bacteria at >1 mg of protein per L of culture, and had 10–15°C higher thermal tolerance than wild type, while also retaining ligand binding and immunogenic properties indistinguishable from wild type, proving its value as an immunogen for a future generation of vaccines against the malaria blood stage. We envision that this efficient computational stability design methodology will also be used to enhance the biophysical properties of other recalcitrant vaccine candidates from emerging pathogens.

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Structural enzymological binding studies on the peptide substrate-binding domain (PSB) of human collagen prolyl 4-hydroxylase with proline-rich peptides

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Collagen prolyl 4-hydroxylases (C-P4Hs) catalyze the hydroxylation of the proline in -X-Pro-Gly- sequences. C-P4Hs play a central role in the synthesis of all collagens. Therefore C-P4Hs are considered potential targets for pharmacological inhibition to control excessive collagen accumulation in fibrotic diseases and severe scarring.[1] Vertebrate C-P4Hs are $\alpha_2\beta_2$ tetramers, the β-subunit being identical to protein-disulfide-isomerase (PDI). Up to now three isoforms of the catalytic α-subunit, which combine with the same β-subunit to form $[\alpha(I)]_2\beta_2$, $[\alpha(II)]_2\beta_2$ and $[\alpha(III)]_2\beta_2$ tetramers, have been characterized. The three isoforms have different tissue expression patterns. The physiological importance of these three isoforms is not well-known.[1]

A model of the human C-P4H-I $\alpha_2\beta_2$-assembly has been proposed.[2] The α-chain has three parts. At the N-terminus there is the dimerization domain (about 150 residues), followed by the peptide substrate-binding domain (the PSB-domain, in the middle, about 100 residues) and the catalytic domain (at the C-terminus, about 250 residues). The PSB domain has high affinity for proline-rich peptides, but the precise function with respect to the reaction mechanism is not known. To systematically study the PSB domain of the three C-P4H isoforms, recombinant human PSB-I, PSB-II and PSB-III were expressed and purified for circular dichroism (CD), isothermal titration calorimetry (iTC), surface plasmon resonance (SPR) measurements and crystallization studies, aimed at comparing their structures and the interaction with their proline-rich peptides.

Crystal structures of unliganded and proline-rich peptide complexed PSB-I have been determined earlier by us.[3] Here we report the crystal structures of unliganded and $(Pro)_9$-bound PSB-II. The overall structure of PSB-I and PSB-II, both consisting of five α helices, are very similar. However, there are marked differences in the mode of proline-rich peptide binding to the domain. In PSB-II the $(Pro)_9$ peptide has tight interactions with the peptide-binding groove residues only via its C-terminal residues, whereas in the $(Pro)_9$-PSB-I complex, also the N-terminal residues of the peptide are tightly interacting with the conserved tyrosines of the peptide-binding groove. This $(Pro)_9$-PSB-II structure explains the known fact that PSB-II has 10-fold lower affinity towards polyproline peptides compared to PSB-I.

Our biophysical and structural studies of the binding properties of the PSB domains of the three C-P4H isoforms to proline-rich peptides and collagenous substrate peptides will provide insight on the different biological and physiological roles of the three C-P4H isoforms. The structural information can provide also help to design specific inhibitors for the particular isoforms.

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Engineering two human proteins with β-trefoil fold for therapeutic applications

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The expression of glycoproteins containing immature truncated O-glycans such as the Thomsen-Friedenreich antigen (Ser/Thr-O-Galβ1–3GalNAc; T-antigen) and the Lewis antigen (sialyl-T-antigen) is a characteristic feature observed on almost all malignant epithelial cells. Those antigens can be recognized by lectins, a group of highly specific carbohydrate-binding proteins whose three-dimensional structure has been studied in our laboratory by X-ray crystallography. BEL β-trefoil is a lectin found in mushrooms that contains three binding sites for the T-antigen, its antiproliferative activity was demonstrated in various human tumor cell lines and it has also been employed for the targeting of antitumor drugs. Unlike other lectins with these properties, BEL β-trefoil presents a structural fold that is also found in human proteins, unlocking the opportunity to use protein engineering tools to design new anticancer therapeutics.
This work explores the possibility of modifying an existing human protein to recognize the carbohydrate antigens present on the surface of cancer cells, in order to reduce the potential immunogenicity risk that foreign lectins could have and allowing its future application in drug-delivery targeting. To reach this purpose, a truncated form of human N-acetylgalactosaminyltranferase-6 (GalNAc-T6) was produced to exploit its affinity to N-acetylgalactosamine for this new purpose.
Biophysical methods such as spectrofluorimetry and isothermal titration calorimetry were used to analyze the ability of the engineered protein to bind the T-antigen monosaccharides. The binding dissociation constant (Kd) of the protein-carbohydrate interaction was determined. The stability the protein was also studied through its thermodynamic parameters of unfolding using differential scanning calorimetry. Crystallization screenings were set up using a broad variety of precipitants in order to produce crystals to be used to study the three-dimensional structure of the engineered protein using X-ray diffraction. The crystals that were grown were taken to the European Synchrotron Radiation Facility (ESRF) in Grenoble (France) to carry out the diffraction experiments.
In conclusion, this work provides a new and interesting insight for the production of optimized protein therapeutics applied in drug-delivery methods for cancer treatment. The present biophysical data are the prerequisite for future studies regarding the biological properties of the engineered proteins and clinical parameters for their potential use in medicine.

Sirt1 can act as a wider range deacylase

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Sirtuins are NAD+ dependent deacetylases that govern genome regulation, metabolism and aging. While Sirt1-3 are robust deacetylases, sirt4-7 have weak to no deacetylase activity. Then it was found that Sirt3 and Sirt6 can remove long chain fatty acyl modifications from peptides or proteins. Sirt4 is reported to be a lipoamidase that can regulate pyruvate dehydrogenase complex activity and Sirt5 can remove succinylation modification on proteins. However, new function of Sirt1 except deacetylase activity is seldom reported. The mechanism of Sirt1 removing other modifications is unknown. Therefore, my study focus on the other deacylase activity of Sirt1 and the mechanism of Sirt1 deacylation. Our data suggest that Sirt1 can remove myristylation and lipoylation modifications on the H3K9 peptide with high efficiency. While delipoylation is both peptide sequence and Sirt1 C terminal region dependent, Sirt1 core domain only can achieve demyristoylation activity. Further study will focus on the crystal structure of Sirt1 with lipoylated and myristoylated peptides to elucidate the molecular mechanism. The physiology relevance of Sirt1 delipoylation and demyristoylation will also be studied. This study is supported by CRF HKU/C7037-14G and HKU/CRF/13G.

Recent Breakthroughs in the Structure/Function Studies of Acetylcholinesterase

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The synaptic enzyme acetylcholinesterase (AChE) terminates transmission at cholinergic synapses by rapidly hydrolysing acetylcholine. Examination of the 3D structure of AChE1 shows that the active site is located at the bottom of a deep and narrow gorge, lined largely by aromatic residues, with its peripheral anionic site located at the top, near the entrance to of the gorge. 3D structures of AChE have been determined for the *Torpedo*, *Electrophorus*, mouse, *Drosophila* and human enzymes. Overall, more than a hundred crystal structures of AChEs, and of covalent conjugates and reversible complexes with various inhibitors and substrate analogues have been determined. Although the 3D structure of the enzyme itself, and of its molecular dimer, are highly conserved, subtle structural differences are seen to occur upon the binding of certain inhibitors. These changes are well correlated with molecular dynamics data, and appear to be of functional significance.

Unfortunately, upon heterologous overexpression, many proteins misfold or aggregate, thus resulting in low functional yields. Human AChE is a typical case of a human protein that necessitates mammalian systems to obtain functional expression. Using a novel computational strategy, we designed an AChE variant bearing 51 mutations that improved core packing, surface polarity, and backbone rigidity (see Fig). This variant expressed at ~2,000-fold higher levels in *E. coli* compared to wild-type hAChE, and exhibited 20°C higher thermostability with no change in enzymatic properties or in the active-site configuration as determined by crystallography2,3.

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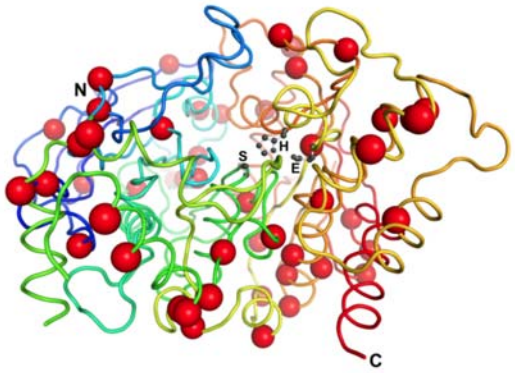

Fig. Crystal structure of a designed human AChE (PDB-ID 5HQ3). Fifty-one mutated residues distributed throughout the sequence are highlighted as red spheres.

Highlights of Instruct Image Processing Centre (I2PC) 2017

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The Image Processing Centre has provided service during 2016 to numerous Instruct projects, mostly in the area of Single Particle Analysis and Electron Tomography. Naturally, some of these projects have already resulted in publications in high impact journals. Particularly important is the contract being negotiated between FEI and I2PC to provide image processing support and training to the pharmaceutical industry.

We have organized and participated in several courses on Image Processing for Electron Microscopy. Particularly interesting have been the joint courses between CCP-EM and I2PC as well as between FEI and I2PC. We are also participating in the Necen-I2PC-FEI 2 months course on Electron Microscopy.

Regarding infrastructures, we have released our first version of Scipion (the software infrastructure that integrates different EM software packages for image processing), published in Journal of Structural Biology. The base of users is rapidly increasing worldwide. Currently, we are about to release the second version, 1.1. This development has been accompanied by Scipion Cloud, a platform to execute Scipion in the Cloud, together with Scipion Web Tools, a platform to easy access to specific Scipion protocols without any installation. During the last two years, Scipion has successfully integrated many protocols from different software developers around the world (Xmipp, Relion, Eman, Frealign, Spider, Simple, Motioncorr, Gctf, Gautomatch, …) showing its capacity to serve as an integrative platform for image processing. We have also increased the integration of the image processing pipeline with the acquisition of the images through Scipion Box, a development that allows streaming processing of the data, as soon as they are acquired at the microscope, providing feedback about the acquisition quality and avoiding unnecessary resource allocations in case that the sample does not qualify for the structural study it aims.

Asymmetric flow field flow fractionation methods for virus purification

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Detailed biochemical and biophysical characterization of viruses requires viral preparations of high quantity and purity. The optimization of virus production and purification is an essential, but laborious and time-consuming process. Asymmetric flow field flow fractionation (AF4) is a rapid and attractive alternative method for virus purification. Its size-based separation is suitable for complexes with a wide size range up to ~1 µm. Since there is no stationary phase in AF4, and the separation occurs in liquid phase of which composition can be modified according to the sample requirements, AF4 is a gentle separation method that preserves viral infectivity. Here we optimized the AF4 conditions to be used for purification of a model virus, dsDNA bacteriophage PRD1 (~66 MDa), from various types of starting materials. PRD1 virion consists of a membrane vesicle that encloses a double-stranded (ds) DNA genome within the icosahedral protein shell with diameter of ~65 nm. Our results show that AF4 is well suited for PRD1 purification as monitored by purity, specific infectivity and recovery of infectious viruses (up to 60% recovered). The obtained purity after AF4 separation was comparable to the purity obtained by traditional ultracentrifugation methods. Short analysis time and high sample loads enabled us to use AF4 for preparative scale purification of PRD1. Furthermore, we also show that AF4 is suitable for a rapid and efficient purification of an enveloped dsRNA virus, bacteriophage phi6 (diameter of ~85 nm, ~99 MDa) that shares structural and functional similarities with eukaryotic dsRNA viruses such as reovirus, blue tongue virus and rotavirus.

Discovery of novel inhibitors of the phosphatase activity of the soluble epoxide hydrolase

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The soluble epoxide Hydrolase (sEH) is an emerging pharmacological target. The enzyme is an antiparallel homodimer, where each of the two subunits is composed of a C-terminal hydrolase domain connected to an N-terminal phosphatase domain by a proline-rich linker. (Cronin et al. 2003) Although the physiological role of the hydrolase domain is well-investigated (Harris und Hammock 2013, S. 61), little is known about the phosphatase activity. One of the main reasons for this is the lack of high-affinity inhibitors, which can be used as chemical probes (Arrowsmith et al. 2015) to investigate its physiological role. In this paper, we present a new series of inhibitors for the human sEH- phosphatase domain. Starting from an HTS hit Oxaprozin we expanded the SAR using a fluorescence-based activity assay (Klingler et al. 2016). For two of the most potent inhbitors, we made ITC measurements to further characterize the interactions of the compound with the phosphatase. Additionally, we were able to co-crystalize the phosphatase domain with one of our most potent inhibitors under acetic conditions and without phosphate in the buffer.

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Virus-free protein production in insect cells

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The benefits of the Baculovirus system for the production of intracellular and secreted proteins are well known. The human HEK293 cell line represents a valuable alternative, especially for secreted proteins, and allows for virus-free transient transfection with plasmids.

Recently, virus-free, plasmid based transfection of insect cells has been optimized considerably. This system represents a useful alternative that combines advantages of the Baculovirus system and transient transfection of HEK293 cells.

Examples of proteins produced by transient transfection of HiFive insect cells will be presented, including intracellular and secreted production.

RF/TPCR: versatile tools for DNA manipulation

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DNA manipulations are essential research tool in biomolecular sciences. At the Israel Structural Proteomics Center (ISPC) we are using the Restriction Free (RF) and Transfer PCR (TPCR) methodologies for a variety of applications in manipulating the DNA sequence. These methods allow integration of a gene into any plasmid and at any position by whole plasmid amplification of the insert and the target vector. Based on the above strategies we have developed new applications, which include simultaneous cloning of several DNA fragments into distinct positions, simultaneous multi-component assembly and parallel cloning of the same PCR product into series of expression vectors. We have further expanded the applications for protein engineering including multiple alterations of the target gene, simultaneous multiple-site mutagenesis and simultaneous introduction of deletions and insertions at different positions. Using these strategies we have facilitated standard protocols for DNA manipulation and protein expression.
We found that there are differences in the performance of different polymerases. We have been using on a routine basis the Phusion (Thermo Fisher Scientific) polymerase. However, for certain sequences with high G/C content this enzyme may encountered difficulties in amplification even in the presence of DMSO. Recently, we started to use alternative polymerases such as the Q5 high-fidelity polymerase (NEB) and for the most challenging complex sequences the KAPA polymerase (VWR).

We are using on a routine basis the RF/TPCR methods for cloning of many thousands of genes or DNA fragments, from different sources and varying in size from few tens of bp up to 10 Kb and above. We rarely encountered with difficulties, which require using alternative approaches for cloning. The development of the TPCR, which is a modification of the RF methodology, enabled wide spectrum of mutagenesis applications. Starting from the basic single site mutagenesis evolving to generation of combinatorial and random DNA libraries.
We applied recently the RF/TPCR methodology to generate more than thousand gRNAs by high-throughput cloning. These plasmids containing transgenic gRNAs designed to manipulate Drosophila's genome by CRISPR. Those plasmids are later injected into Drosophila embryos, forming a homozygous loss-of-function mutant clones within an otherwise wild type animal.

Structure-guided identification of dual receptor binding PfEMP1 that are associated with cerebral malaria

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Cerebral malaria is a devastating disease in which red blood cells infected with the malaria parasite, *Plasmodium falciparum* adhere to and accumulate within tiny capillaries of the brain. This is mediated by the PfEMP1 proteins, a family of ~60 highly variant, multi-domain parasite proteins that are presented on the surface of infected cells and interact with various endothelial receptors. In this study, we reveal how the power of structural studies has allowed us to identify a novel binding phenotype associated with cerebral malaria.

A major goal in understanding the disease has been to determine whether PfEMP1 with specific receptor adhesion phenotypes are associated with cerebral malaria. This has been hindered by the high variability of PfEMP1 proteins and the inability to directly predict, using sequence information, the adhesion traits of PfEMP1 expressed in patients. To overcome this obstacle, we aimed to understand how PfEMP1 interact with ICAM-1, an important candidate receptor for endothelial adhesion of infected red blood cells in the brain.

For this, we determined the crystal structure of a PfEMP1 domain bound to human ICAM-1. This revealed a complex and elongated binding site consisting of three distinct subsites and through structure guided mutagenesis, SPR and sequence analysis we identified the key molecular determinants used by PfEMP1 to bind ICAM-1. Surprisingly, these features are highly conserved, allowing us to predict ICAM-1 binding PfEMP1 from a specific sequence motif alone for the first time. We used this motif to search a database of all known PfEMP1 sequences from multiple parasite genomes and identified over 100 PfEMP1 that were previously not known to bind ICAM-1. Intriguingly, all of these PfEMP1 also contained a domain that allows them to bind to a second receptor, EPCR. We show that these PfEMP1 are indeed able to synergistically bind both receptors and that this influences parasite adhesion.

To test whether the ability to simultaneously bind these two receptors has consequences for patients infected with parasites expressing such PfEMP1, we used the sequence motif to study the PfEMP1 proteins expressed in young African patients with varying malaria symptoms. We found a striking correlation between the expression of this novel class of dual receptor binding PfEMP1 and the likelihood of developing cerebral malaria. This reveals an important binding phenotype that could be targeted as part of a strategy to prevent cerebral malaria and highlights the remarkably conserved ICAM-1 binding site as potential immunogen for inclusion in such a vaccine.

Expression of RNA---dependent---RNA---polymerase of chronic bee paralysis virus using bacterial and baculovirus expression systems

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Colony collapse disorder (CCD), which results in rapid decline in the number of bee colonies, has become a global problem for bees and beekeepers. One of the viruses causing CCD is chronic bee paralysis virus (CBPV). Up to date, there is limited information about the structure and replication of this virus. Solving the structure of the RNA polymerase will allow structure-based development of anti-CBPV therapeutics targeting replication of the virus. Here we present approaches for recombinant expression of RNA-dependent-RNA-polymerase of CBPV. The RNA-dependent-RNApolymerase expressed in *E. coli* was insoluble. In contrast the RNA polymerase produced in insect *SF9* cells by the baculovirus expression system was partially soluble and is suitable for purification and following crystallization and structural analysis.

Characterization of Deformed wing virus 3C protease and its precursor 3CD

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Deformed wing virus (DWV) is one of the main threats for western honey bee (*Apis mellifera*). In association with the parasitic mite Varroa destructor, the virus causes honeybee winter mortality and colony collapse disorder. DWV genome encodes a single polyprotein that is co-translationally and post-translationally cleaved by virus 3C protease ($3C^{pro}$) or its precursor 3CD. $3C^{pro}$ autocatalytically releases itself from the polyprotein by proteolytic cleavage. We identified residues forming catalytic dyad – H63 and C200 of $3C^{pro}$ and prepared single and double mutants to observe changes in cleavage activity.

The structure of the *Legionella pneumophila* protein WipA reveals an unexpected tyrosine phosphatase effector

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Bacterial infections from the pathogen *Legionella pneumophila* range from mild illness (Pontiac fever) to a severe and potentially lethal pneumonia (Legionnaire's disease). The major virulence factor of *L. pneumophila* is a type IVb secretion system that is able to secrete more than 300 effector proteins into the host organism. These proteins interfere with a variety of cellular processes in the host cell in order to promote bacterial survival and replication inside the eukaryotic cell. The structural and functional elucidation therefore of *Legionella* effectors is crucial in understanding the virulence mechanisms of *Legionella* and the prevention of infections.

Here we describe the crystal structure and biochemical properties of WipA (or IcmW-interacting protein A; lpg2718), revealing a two-domain structure comprising a metallophosphoesterase attached to an 85 Å long α-helical hairpin forming a coiled-coil at its tip. The latter is possibly serving as a binding site for host-cell interaction partners. The phosphatase domain adopts the conserved fold of serine/threonine phosphatases displaying similarities to the PP1, PP2α αnd PP5 subfamilies. Kinetic studies on the *wild-type protein* and mutants of the protein suggest high catalytic rates and a clear role of specific amino-acids in the active site. When testing however the WipA against different phospho-peptides we observed a seven-fold specificity against phosphor-tyrosine peptides instead of the expected specificity against phospho-threonine/serines. Mutations in the proximity of the active site support the specificity for phosphotyrosine peptides. Furthermore, we confirmed that WipA is able to dephosphorylate the phosphor-tyrosine pY706 in the kinase domain of the FGFR3 clearly suggesting that WipA protein is a unique phosphatase with of a serine/threonine fold but with a specificity against phospho-tyrosines.

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Cervical cancer is the second most prevalent cancer in women worldwide. In more than 60% of the cases, it is caused by Human Papillomavirus type 16 infection (HPV-16). This virus is considered as high oncogenic risk, with mucosal tropism. However, there is a large variety of papillomaviruses leading to different phenotypes, such as warts, condylomas, or cancers (head and neck, skin, cervix). Through an innovative high-throughput assay developped in the team, we explored on a quantitative manner protein-motif interaction patterns of E6 oncoprotein from different PV types. In particular, E6 forms a hydrophobic pocket able to interact with proteins containing LxxLL acidic motifs. One of these LxxLL-motif-containing proteins is the ubiquitin ligase E6AP, which is first recruited by E6 from HPV and then leads the tumor suppressor p53 to proteasomal degradation. By understanding the key factors ruling the interaction preferences of each E6 protein, we can decipher how E6 interactome impacts oncogenic risk and tropism.

Cryo-electron microscopy structure of archaeal virus APBV1

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Archaeal viruses have evolved to infect hosts often thriving in extreme conditions. For example, viruses infecting hyperthermophilic archeons can resist extreme temperatures. Rod-shaped virus APBV1 (*Aeroperum pernix* bacilliform virus 1) infects a hyperthermophile *Aeroperum pernix*, which grows optimally at 100°C [1]. Due to shortage of information on archaeal virion structures, key determinants behind temperature resistance have remained elusive. Our cryo-electron microscopy analysis provided details of unique structural organisation of APBV1 which allowed better understanding of viral adaptation to hostile environment.

As major virion protein virus is a very small protein (10kDa) the APBV1 particles appeared as smooth rods on microphotographs recorded initially on CCD camera. Determination of helical parameters became possible only after the data was collected with direct electron detector (Strubi, Oxford). Helical parameters established from the Fourier-Bessel analysis of 2D class averages and corresponding power spectra allowed to obtain experimantal cryo-EM map at 3.7Å resolution and to build a model of the major virion protein (VP1) *de novo* [2]. The curvature of the alpha helices of VP1 is critical for incorporation of subunits in the helical lattice without gaps. The extreme thermostability of APBV1 is based on cooperative assembly of multiple VP1 subunits arranged in a helical array. Tight packing of the subunits held together by extended hydrophobic contacts makes the virion very rigid and remarkably stable.

PROTEIN DYNAMICS ARE ESSENTIAL FOR CATALYSIS AND ALLOSTERIC REGULATION OF *M. tuberculosis* ATP-PHOSPHORIBOSYLTRANSFERASE

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Allosteric regulation is a conserved and efficient way of controlling enzymatic activity. ATP-phosphoribosyltransferase (ATP-PRT) catalyses the first step of L-histidine biosynthesis. This enzyme is essential for *Mycobacterium tuberculosis* (Mtb), the causative agent of human Tuberculosis, but absent in humans. ATP-PRT is a hexamer in solution and, like other enzymes regulated via ferrodoxin-like (FL) domains, interconverts between an open active conformation and a closed inactive conformation, and binding of L-histidine, its feedback allosteric inhibitor, shifts the equilibrium towards the closed state. However, the exact mechanism by which inhibition-linked conformational changes regulate enzymatic activity is still poorly understood. With the aim of investigating the molecular basis of allosteric regulation of ATP-PRT by L-histidine, we performed a search for novel allosteric regulators by carrying out an innovative *in vitro* screening employing L-histidine analogues. We report the identification of 3-(2-Thienyl-L-alanine) (TIH) as a non-essential small-molecule allosteric activator of ATP-PRT that increases activity by increasing the catalytic rate up to 5-fold. TIH competes with L-histidine for the same allosteric site and fully restores the activity of the L-His inhibited enzyme. Surprisingly, both ion-mobility data and crystal structures of ATP-PRT complexes with either L-His or TIH reveal that both 'activated' and 'inhibited' hexamers are in a closed conformation suggesting an uncoupling between the ligand-induced conformational changes and the activation state of the enzyme. We will discuss the implications of these findings for the current model of ATP-PRT allosteric regulation, and will present preliminary data from solution studies aimed to propose a revised interpretation of this mechanism. These findings might also be relevant for other FL domain-containing enzymes.

Structural insights into the PHDv-C5HCH tandem domain of the NSD lysine methyltranferase family

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The NSD lysine methyltranferase proteins (NSD1, NSD2, NSD3) contain several chromatin-related modules (SET , PWWP and PHD domains) and they are implicated in developmental diseases and cancer, but their mechanisms of action is still unclear. We have started a systematic investigation of the structural/functional role of the PHD fingers of the NSD family. We are focusing particularly on the tandem PHD fingers (PHDv- C5HCH), since interactions mediated by these domains are crucial for gene regulation and tumorigenesis. The PHDVC5HCH domains of the NSD proteins share a high sequence identity (~60%), but they have a divergent role in the recognition of modified histones and of the co-repressor factor Nizp1 by its C2HR module. Our data reveal for PHDVC5HCHNSD1 the non-specific interaction with H3 histone tail and the high affinity for C2HRNizp1 (Kd~4μM), conversely PHDVC5HCHNSD2 and PHDVC5HCHNSD3 bind with a low affinity in two different binding pocket both the H3 histone tail (H3K4me0(1-21) and H3K9me3(1-21), respectively) and C2HRNizp1. On NSD PHDVC5HCH the C2HRNizp1 binding site localizes at the interface between the two PHD fingers in a distinct region from the canonical histone binding site. Additionally, NMR titrations indicate that H3 histone tail does not affect the interaction between PHDVC5HCHNSD1 and C2HRNizp1, whereas in NSD3 binding to H3K9me3(1-21) reduces the affinity of PHDVC5HCHNSD3 for C2HRNizp1, via an allosteric mechanism. In conclusion, our data propose a regulative scenario in which the same NSD protein domain can differently regulate the recruitment of co-factors necessary for gene transcription.

Evolution and functions of SMC complexes: new SMC5/6 insights

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The SMC (structure maintenance of chromosome) proteins are conserved from bacteria to humans. They associate with kleisin and kleisin-interacting proteins, forming different complexes (cohesin, condensin and SMC5/6 in eukaryotes). The SMC/kleisin complexes form elongated annular structures, which are critical for DNA replication, chromosome compaction and segregation, genome maintenance, and the regulation of gene expression. We describe marked structural similarities between bacterial and eukaryotic SMC/kleisin partner proteins (designated as "KITE" proteins for Kleisin Interacting Tandem-winged-helix Elements of SMC complexes). Kite proteins are integral parts of all prokaryotic SMC complexes and eukaryotic Smc5/6, but not cohesin and condensin complexes. Based on architectural rather than sequence similarity, we propose an adapted model for the evolution of the SMC protein complexes. In addition, we compare functional similarities between bacterial Smc/ScpAB and eukaryotic Smc5/6 in organization and dynamics of DNA, particularly in replication progression.
We discovered several important features of SMC5/6 kite subunits: their role in structural organization of the SMC5/6 complex, their DNA-binding ability, their evolution from bacteria to novel mammalian protein superfamily and new chromosome breakage syndrome associated with human Nse3-kite subunit mutations.

Ackonwledgement: Internal Masaryk University grant (MU/0822/2015)

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Characterization of lectin AFL from opportunistic pathogen *Aspergillus fumigatus* – combining a broad spectrum of biophysical methods

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Detailed protein characterization is a pivotal step for understanding its role in organism, deciphering its functions and its utilization in medicine or industry. The methodology evolves constantly with the progress in biology, physics, mathematics and informational technologies. Nowadays, a broad spectrum of biophysical techniques is available to determine protein purity, homogeneity, folding and stability, to help in structure solution or to examine protein activity and binding properties. Herewith, we demonstrate a case of the lectin AFL from the *Aspergillus fumigatus* fungus. This opportunistic pathogen is found worldwide and causes severe infections in immunocompromised humans, especially those after transplantations, patients with cancer or AIDS. Being a lectin, AFL can be responsible for host recognition in early stages of infection and as such is a potential virulent factor. The combination of various techniques including GPC, DLS, DSF, TSA, DSC, AUC, CD, ITC, SPR, MST and fluorescence microscopy allows for a complete description of the protein behavior. This may bring an essential information to decipher the processes causing *A. fumigatus* pathogenicity and be a key to the development of potential therapeutics.

This research was carried out under the project CEITEC 2020 (LQ1601) with financial support from the Ministry of Education, Youth and Sports of the Czech Republic under the National Sustainability Program II. The research received financial support from the Czech Infrastructure for Integrative Structural Biology project (LM2015043).

Insight into the structural features of pegylated asparaginase: a new route for the structural characterization of large pegylated protein therapeutics

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PEGylated proteins are widely used in biomedicine but, in spite of their importance, no atomic-level information is available since they are generally resistant to structural characterization approaches. Recently, we have shown that pelleted pegylated proteins provide high quality solid state multi-dimensional NMR spectra, which allows assessment of the structural integrity of proteins when PEGylated for therapeutic or diagnostic use.[1] However, a more detailed structural characterization requires an extensive resonance assignment and the collection of long-range structural restraints.
Here we describe a new route for the structural characterization of large pegylated proteins/assemblies based on an integrated use of solid-state and solution NMR.
The NMR experiments performed on the native enzyme and on its pegylated form allowed us to assign about the 74% of the residues. The structural model calculated by using the restrains obtained from solid state ^{13}C-^{13}C correlation spectra at different mixing times has been used to assess the structure preservation.

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Structural and functional studies of pore forming proteins

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Pore-forming proteins (PFPs) are found in all kingdoms of life and in most cases, they are used as toxins to attack and invade other organisms, or to defend against them. As such, they can be found in many pathogenic bacteria as well as in animal venoms. Those that do not act as toxins are involved in other physiological processes such as proper functionality of the immune system, neuronal development or digestion of food.

Generally, PFPs act in three major steps. First, they are synthesized by the originating organisms as soluble units (mostly monomers, sometimes dimers or oligomers). Second, upon binding of soluble units to lipid membranes, they form oligomers at the surface of the membrane. In most cases, these membrane-bound complexes form an ordered complex called a prepore, which is not yet functional. Finally, these membrane bound complexes undergo conformational changes and become inserted into the lipid membrane bilayer and form a functional pore. The binding to lipid membranes sometimes depends on presence of specific receptors, such as lipids (sphingomylein, cholesterol, etc), glycolipids, or (glyco-) proteins.

PFPs are traditionally classified into two major classes based on secondary structure elements that build the transmembrane part of the pore, with α-PFPs containing pores built of a cluster of α-helices, and β-PFPs with pores built of a β-barrel. Both classes are further divided in several families of proteins based on the sequence and structural similarity of the soluble monomeric forms. The number of protomers building these pores, ranging from 4 to 50, directs their size, which can span between 1-30 nm in diameter and this, together with the shape and charge of the pore, affects the nature of molecules that can translocate through the pore. Due to their interesting physico-chemical properties, PFPs may also serve as tools in medical research and nanobiotechnological applications.

In our group, we study PFPs that are members of MACPF/CDC superfamily (β-PFPs with large pores), actinoporin-like family (α-PFPs with small pores) and aerolysin-like family (β-PFPs with small pores). In order to understand their structure, function and in particular the mechanism of pore assembly, we use several methodological approaches, including bioinformatics, molecular biology, biochemistry and biophysics as well as structural and cell biology. Our recent results on listeriolysin O (CDC family) and lysenin (aerolysin family) will be presented.

Structural studies of J-DNA binding protein (JBP1)

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Base J (β-D-glucosyl-hydroxymethyluracil) replaces 1% of Thymidine (T) in the genome of all kinetoplastid flagellates such as *Trypanosoma, Leishmania* and *Crithidia*. In *Leishmania,* 99% of J is located in telomeric repeats, whereas 1% is in internal chromosomal positions (iJ) and is responsible for correct transcription termination. Biosynthesis of base J occurs in two steps. The first step includes hydroxylation of specific T bases forming hydroxymethyluracil (hmU). In the second step, a glucose molecule is added to hmU by a glucosyl transferase, resulting in base J.

In the hydroxylation step, two proteins are involved: JBP1 and JBP2. Both have an N-terminal thymidine hydroxylase (TH) domain, but only JBP1 has a J-DNA binding domain (JDBD). Crystallographic analysis of JBP1 revealed the structure J-DNA binding domain (J-DNA) that recognizes and binds onto J-DNA with ~10nM affinity and high specificity (~10,000 times) over normal J-DNA. The crystal structure of JDBD revealed a novel variant of the helix-turn-helix (HTH) domain, a helical bouquet, being responsible for J-DNA binding. A single residue (Asp525) is responsible for both affinity and specificity, towards J-DNA. However, to understand the molecular mechanism of J-DNA binding, structural information of the full length JBP1, is necessary.

Here, we show that removing JDBD from JBP1, results in a protein (Δ-JDBD) that contains the N-terminal TH domain tightly associated with the C-terminal region. Small Angle X-ray Scattering (SAXS) experiments on JBP1 and JDBD (in the presence and absence of J-DNA), and on Δ-JDBD, allowed us to generate a low-resolution three-dimensional model. Our results show that the C-terminus of JBP1, folds together with the N-terminal part that contains the TH function, creating a single compact folding unit. Based on the results above we hypothesized that JDBD acts as an autonomous folding unit that can explain the flexible nature of the full length JBP1, which has been proven a difficult target to crystallize. To remove or decrease this flexibility we decided to follow an approach that is widely used in the field of membrane proteins and particularly the G-protein coupled receptors (GPCRs): usage of fusion partners that would replace flexible regions of GPCRs was one of the solutions to promote the necessary crystal contacts that are required for crystallogenesis. Here we replaced the flexible JDBD domain with Rubredoxin, to decrease the flexibility of JBP1 and provide the necessary network of crystal contacts. This approach has been successful in growing crystals of the fusion Δ-JDBD-Rub protein.

West-life Virtual Folder - components and tools

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West-Life is a H2020 Virtual Research Environment project that will provide the application level services specific to uses cases in structural biology. The proposed presentation will introduce it's one part Virtual Folder and it's current capabilities to perform tasks and workflows of structural determination, model building, model refinement on data stored within different storage providers, currently supporting academic EUDAT (it's B2DROP service), commercial DROPBOX and others. The Virtual Folder leverages on software suites of CCP4, SCIPION and other.

Characterization of Mps1 kinase by NMR spectroscopy

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During cell division, all chromosomes have to line up and get attached through their kinetochores to spindle microtubules. Errors in this process lead to developmental defects and cancer. The spindle assembly checkpoint (SAC) is a signalling mechanism that inhibits the progress of cell division until all chromosomes are bipolarly attached to spindle microtubules. Accumulating evidence shows that the predominant SAC kinase, Mps1 role directly "senses" by competing with spindle microtubules, whether chromosomes are attached or not.[1,2] My research aims to understand how Mps1 regulates mitosis and the SAC machinery at the atomic level. Therefore, we have assigned the backbone of the N-terminal localization module of Mps1. The comparison of the HSQC spectra of the NTE-TPR (#1–239) and TPR (#62–239) showed significant chemical shift changes in the TPR domain, indicating the interaction between NTE and TPR.

Acknowledgments: The work is supported by the NWO-VENI grant 722.015.008

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