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Instruct Biennial Structural Biology Conference
Cascais, Lisbon, Portugal 23 – 24th May 2024

Abstract Booklet

Instruct Biennial Structural Biology Conference

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8	Instruct Centre NL is the Dutch national Instruct Centre. Instruct-NL includes three access sites offering a wide range of structural biology methods, as well as a vast national stakeholder community.	Instruct Centre NL	
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Speakers

RNA Structural Biology

Harald Schwalbe

Goethe-University Frankfurt, Instruct-ERIC, Oxford.

The importance of RNA structural biology is increasingly recognized. Yet, it lacks behind protein structure biology for various regions. In this contribution, integrated methods combining NMR and MD as well as NMR, SAXS and cryo-EM will be presented. Focus will be given on the RNA structure space of SARS-CoV-2 RNAs. Neither RNA 2nd nor 3rd structure outside regular A-form helical segments can be predicted reliably. Further, ensemble descriptions of RNA are needed to capture all functional states of RNAs. RNAs undergo modifications and they react to experimental clues. Examples will be given on how RNA modification change RNA interactions and how RNA 2nd structure is both, shifting pKa for protonation substantially and allowing novel base pairing interactions.

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doi: 10.1261/rna.079902.123.

NMR ^{1}H,^{19}F-based screening of the four stem-looped structure 5_SL1-SL4 located in the 5'-untranslated region of SARS-CoV 2 RNA. Hymon D, Martins J, Richter C, Sreeramulu S, Wacker A, Ferner J, Patwardhan NN, Hargrove AE, **Schwalbe H.** RSC Med Chem. 2023 Nov 28;15(1):165-177.
doi: 10.1039/d3md00322a.

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doi: 10.1093/nar/gkad762.

Integrated NMR/Molecular Dynamics Determination of the Ensemble Conformation of a Thermodynamically Stable CUUG RNA Tetraloop.
Oxenfarth A, Kümmerer F, Bottaro S, Schnieders R, Pinter G, Jonker HRA, Fürtig B, Richter C, Blackledge M, Lindorff-Larsen K, **Schwalbe H.** J Am Chem Soc. 2023 Aug 2;145(30):16557-16572.
doi: 10.1021/jacs.3c03578.

After the AlphaFold revolution: AI-enabled adventures in integrative structural biology

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The Netherlands Cancer Institute (NKI)

How can we harness the full potential of AlphaFold, beyond straightforward structure prediction? The simple observation of a missing iron atom and the oxoglutarate ligand on the predicted structure of the J-DNA binding protein, led us to develop AlphaFill and the LegoFill-AI for predicting binding of physiological ligands. The same predicted structure, also led us to new insights on how it binds to DNA, by observing an unexpected intra-molecular interaction of the N-terminus with the central DNA-binding domain. Motivated by that approach, we ventured on using AlphaFold in predicting inter-molecular interactions and discovered how multiple repeats of Talin interact with the Caskin C-terminus. Next, these insights led us to computationally test various hypotheses “fishing” for cohesin domain interactions, revealing new regulators of its function during mitotic entry. Making such predictions computationally efficient, available to all, and easy to interpret for biologists is our new venture, in an effort to navigate the complex landscape that co-develops between experimental structures, structural predictions and functional validation.

T3

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The potential of AI for biomolecular structure determination, prediction and validation

Andrea Thorn

University of Hamburg, Germany

Structural biology is key to understanding basic processes of life and a major driver for the development of new therapies. However, these structures do not directly result from the experiment, but are merely models which explain the observed data according to a priori knowledge. Consequently, our structures are only as good as our limited understanding of the underlying principles.

However, in crystallography, the gap between model and reality remains clearly evident in hard to interpret maps and large R values. In cryo-EM, model properties do not align well with the actual specimen. Machine learning methods, in particular convolutional neural networks, have been applied to a variety of problems in the electron cryo microscopic and crystallographic structure solution of biological macromolecules. However, until recently, their acceptance by the community was limited to tasks where they replaced repetitive work and visual checks were easy, such as particle picking, crystal centering or crystal recognition. With Artificial Intelligence (AI) based protein fold prediction now revolutionizing the field, it is clear that their scope could be much wider, including structure determination, prediction and validation. Biomolecular structure determination could potentially profit immensely from AI, which may even pave the way to joint analyses of data from different labs and methods, which would significantly advance our understanding of the molecules that govern key biological processes.

Whether we will be able to exploit this potential fully will depend on the manner in which we use machine learning: training data must be well-formulated, methods need to utilize appropriate architectures, and outputs must be critically assessed, which may even require explaining AI decisions.

Exploiting the micro-crystal toolbox to generate a mechanistic understanding of GmhA heptose biosynthesis in *Burkholderia pseudomallei.*

Courtney J. Tremlett[1], William Stuart[1], Nicholas J. Harmer[1]

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Antimicrobial resistance is one of the greatest threats to global health. Gram-negative bacteria are leading the way with many species possessing multi-drug resistance resulting in plummeting patient prognosis. Gram-negative bacteria possess a unique cell wall which is integral to their virulence and viability, making it an attractive therapeutic target. Sugar synthesis across gram-negatives is highly conserved, making this a target for a broad-spectrum antibiotic that can be used in mixed species infections, such as cystic fibrosis. Our target organism, *Burkholderia pseudomallei*, uses sedoheptulose-7-phosphate isomerase (GmhA) to form sugars in the LPS of the bacterium and by inhibiting this, the membrane will be more susceptible to current therapeutics. We know the protein converts these sugars in a 2-step fashion but the exact mechanism is unknown.

We are applying a multi-faceted approach by utilising micro/nanocrystal techniques available at both Diamond and ESRF to build a molecular movie of GmhA activity. Through this journey we have learnt how to manipulate our protein to form crystals ranging from 200 nm to 0.3 mm in size. Additionally, we have optimised sample preparation pathways to obtain 1 to>1000 crystals per 0.8 µL microbatch drop. Thus far we have built a library of samples for VMXm, serial crystallography, MicroED and single crystal methods. Each of these methods have come with their own advantages and challenges to overcome. Combining these techniques has led to the deepened understanding of GmhA negative and positive co-operativity and how we can possibly exploit mechanistic steps in inhibitor design.

Structural basis for inhibition of the SARS-CoV-2 nsp16 by substrate-based dual site inhibitors

Gints Kalnins[1], Laura Drunka[2], Anna L. Bula[2], Diana Zelencova-Gopejenko[2], Olga Bobileva[2], Mihails Sisovs[1], Kaspars Tars[1,3], Aigars Jirgensons[2], Kristaps Jaudzems[2,3], Raitis Bobrovs[2*]

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3. University of Latvia, Jelgavas 1, Riga LV-1004, Latvia

Coronaviruses, including SARS-CoV-2, possess an mRNA 5' capping apparatus capable of mimicking the natural eukaryotic capping signature. Two SAM-dependent methylating enzymes play important roles in this process: nsp14 methylates the N7 of the guanosine cap, and nsp16-nsp10 methylates the 2'O of the two subsequent nucleotides of viral mRNA. The 2'O-methylation performed by nsp16-nsp10 is crucial for the escape of the viral RNA from innate immunity. Inhibition of this enzymatic activity has been proposed as a way to combat coronaviruses.

In our study, we employed X-ray crystallography to analyze the binding of the SAM analogues to the active site of nsp16-nsp10. We obtained thirteen novel 3D crystal structures of the nsp16-nsp10 complexes with SAM-derived inhibitors, demonstrated different conformations of the methionine substituting part of the molecules, and confirmed that simultaneous dual-site targeting of both SAM and RNA sites correlates with higher inhibitory potential.

Acknowledgement: This work was supported by projects VPP-COVID-2020/1-0014, VPP-EM-BIOMEDICĪNA-2022/1-0001 and ANM_K_PG_28 grants.

Biophysical experiments and biomolecular simulations: A perfect match?

Kresten Lindorff-Larsen[1]

1. Structural Biology and NMR Laboratory, Linderstrøm-Lang Centre for Protein Science, Univ. of Copenhagen

Biological macromolecules are dynamic entities whose functions depends on both their structures and motions. In my talk I will discuss methods for how simulations and experiments can be used synergistically to study biomolecular dynamics. Despite progress in the accuracy of force fields and sampling methods, one may still find that a simulation does not quantitatively match experiments. Then, experiments and simulations may be combined in a direct fashion to provide a description of the molecular motions that combines the details of atomic simulations with the accuracy afforded by experiments. The resulting conformational ensembles may provide novel insight into biomolecular systems that are not obtainable by simulations of experiments alone. I will discuss how this may be achieved for “static” ensemble-averaged experimental data, and extensions of these methods to systems that contain both conformational and compositional heterogeneity. I will discuss progress on studies of experimental data such as NMR relaxation and fluorescence energy transfer that also depends on the timescales of motions. Finally, I will discuss initial applications for using simulations to interpret time-resolved experiments.

Structural biology at the level of proteoforms and their complexes using single molecule mass spectrometry

Prof. Neil Kelleher

Northwestern University, IL, USA

The adaptation of the Orbitrap mass spectrometer to assign charge on individual ions represents a transformative advance, accelerating efforts to decipher complex biological systems. The “top-down” approach, which maximizes information retention prior to analysis, is exemplified by native individual ion mass spectrometry (I2MS). I2MS has emerged as an indispensable structural biology tool, enabling meticulous characterization of intact molecular assemblies. The Nuc-MS platform allows comprehensive profiling of undigested, non-denatured nucleosomes, elucidating compositional variations in histone variants and post-translational modifications. This seminar will describe the use of peptide MS, intact histone MS and Nuc-MS to quantify dramatic changes in mononucleosomes versus those immuno-enriched for hetero- and eu-chromatic marks (like H3K27me3 and H3K4me3, respectively). Additionally, I2MS facilitates structural analysis of intact adeno-associated viruses, providing insights into their molecular architectures and cargoes. These examples highlight the enhanced capabilities of I2MS for characterization of protein complexes and align with the aims of the Human Proteoform Project, which seeks to unravel proteoform complexities and molecular mechanisms using the latest technologies for scalable and systematic mapping of proteoforms to promote human health.

From Structures to *In Situ*- latest developments in cryoEM

Rebecca Thompson

Director Sales Development- Life Sciences Electron Microscopy- EMEA- Thermo Fisher Scientific

Over the last decade, cryo-electron microscopy (cryo-EM) has become the method of choice for structural characterization of proteins and protein complexes, revolutionising our understanding of molecular architecture. Single particle cryoEM is continuing to improve the quantity, range and quality of structures that can be determined. Increasingly, there is a greater emphasis on examining samples within their native cellular context, which has become possible by the adoption of cryo-electron tomography (cryo-ET) coupled with focused ion beam (FIB) milling. Most biological samples are too thick to be directly analysed by transmission electron microscopy. Utilising dual-beam systems which are equipped with both a scanning electron column and a focused ion beam column allows the production of thin slices of cells or tissues known as lamellae. These can be imaged in high end 200kV and 300kV TEM systems which, when combined with direct electron detectors and energy filters, are capable of producing data that not only provides contextual information about the sample, but also allows sub-tomogram averaging to obtain structural information. This presentation will provide an overview of the latest technological developments in electron microscopy.

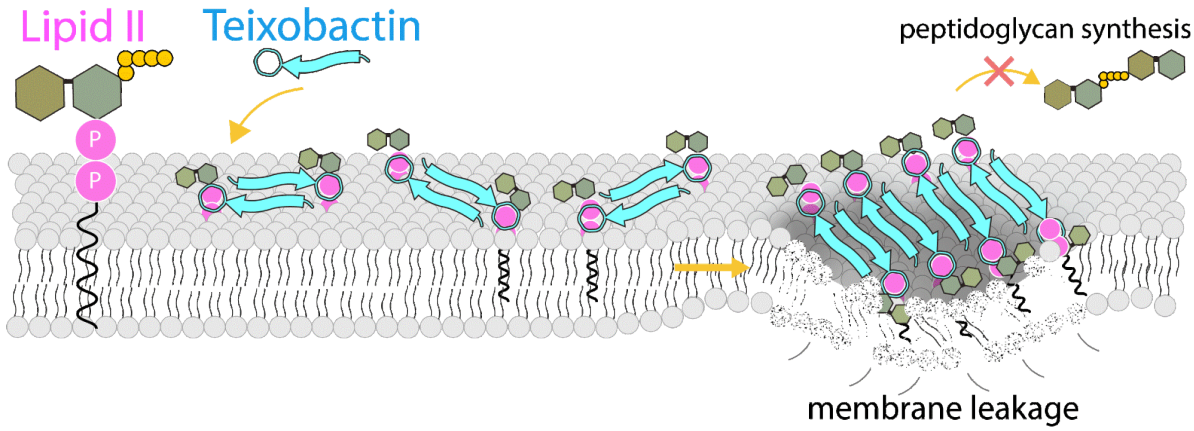
Cracking Nature's Recipes to Design Lipid-Targeting Antibiotics

Markus Weingarth

[1]NMR Spectroscopy Group, Utrecht University, Utrecht, the Netherlands

Antimicrobial resistance is a global health threat, calling for new antibiotics. Good candidates could be compounds that target special lipids that only exist in bacterial, but not in human cell membranes. These drugs kill pathogens without detectable resistance, which has generated considerable interest.

Using solid-state NMR and microscopy, our group has introduced approaches to study lipid-targeting antibiotics across different length-scales in membranes[1]. Recently, we determined the killing mechanism of teixobactin[2,3]. We showed that teixobactin kills bacteria by forming supramolecular fibrils that compromise the bacterial membrane. In addition, we show the supramolecular mechanism of Clovibactin, a novel antibiotic from 'unculturable' bacteria[4]. Finally, on the example of plectasin, an antibiotic from fungi, we present a general conceptional framework on the supra-mechanisms of lipid-targeting antibiotics[5].



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Disruption of the mitochondrial network in a mouse model of Huntington's Disease visualized by in-tissue multiscale 3D electron microscopy

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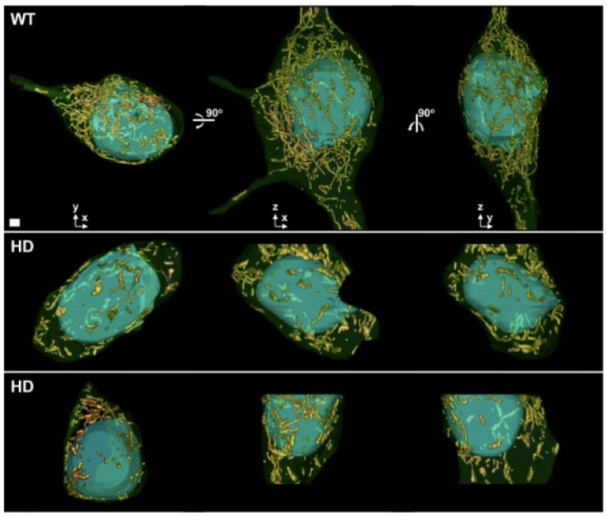
Huntington's disease (HD) is an inherited neurodegenerative disorder caused by an expanded CAG repeat in the coding sequence of huntingtin protein. Initially, it predominantly affects medium-sized spiny neurons (MSSNs) of the corpus striatum. No effective treatment is still available, thus urging the identification of potential therapeutic targets. While evidence of mitochondrial structural alterations in HD exists, previous studies mainly employed 2D approaches and were performed outside the strictly native brain context. In this study, we adopted a novel multiscale approach to conduct a comprehensive 3D in situ structural analysis of mitochondrial disturbances in a mouse model of HD. We investigated MSSNs within brain tissue under optimal structural conditions utilizing state-of-the-art 3D imaging technologies, specifically FIB/SEM for the complete imaging of neuronal somas and Electron Tomography for detailed morphological examination, and image processing-based quantitative analysis. Our findings suggest a disruption of the mitochondrial network towards fragmentation in HD. The network of interlaced, slim and long mitochondria observed in healthy conditions transforms into isolated, swollen and short entities, with internal cristae disorganization, cavities and abnormally large matrix granules.



3D visualization of the FIB/SEM volumes. Three different views of MSSNs are presented in the top (WT), middle (HD) and bottom (HD) rows, respectively. The leftmost views show the volumes with their Z axis running through the depth, a 90° rotation around the horizontal axis results in the views at the central panels, and a subsequent 90° rotation around the vertical axis produces the rightmost views. Segmented mitochondria are depicted with isosurface representation in gold color. Plasma and nuclear membranes are displayed in 85% transparent green and 50% transparent cyan, respectively, allowing visualization of the mitochondria behind the nucleus. The missing wedge in the volume shown in the middle row (central panel) is caused by a technical drift while FIB/SEM acquisition. Bar: 1 µm.

NMR-based investigation of intrinsically disordered regions of modular proteins for tailored drug-design

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Numerous RNA-binding proteins (RBPs) exhibit modular structures containing folded domains and intrinsically disordered regions (IDRs). Investigating the role of these domains and their potential interaction is essential for understanding protein function and developing intervention strategies. The Nucleocapsid protein (N) of SARS-CoV-2 is a pivotal example of RBP. Its complex structure encompasses two folded domains and three IDRs. In particular, the globular N-terminal domain (NTD) is responsible for the viral RNA interaction and the two flanking IDRs play an important synergic role (1). The aim of my PhD project is to design and synthesize molecules able to interfere with the protein function, monitoring the interaction through solution NMR titrations. In particular, taking into account the structural characteristics of the protein, a first peptide was designed to simulate the main interactions driving the viral RNA binding. Then, a series of different peptides with slight modifications were synthesized to discern residues or motifs essential to interact in the protein target site. This collection of peptides has been tested by NMR titrations to identify the sequence displaying the highest affinity with the protein NTD and also with the NTR construct, comprising also the two flanking IDRs. Different NMR experiments were performed to enable the simultaneous observation of globular and disordered regions both with atomic resolution; in particular the interaction was followed through ^{1}H-^{15}N HSQC experiments but also by exploiting the ^{13}C detection, essential to investigate flexible regions. This study is now being improved with the design and synthesis of a peptide-PNA chimera, replacing certain amino acid residues of the parental peptide sequence with four PNA building blocks, selecting four G as nucleobases (2), aiming to better mimic the RNA nature. This promising molecule was tested with both protein constructs in the same experimental conditions. For now, this study yielded two main and clear results: the comparison among the titrations carried out first with the peptides and then with the peptide-PNA chimera has revealed a significantly greater affinity between the protein and the chimera with the respect to the peptide molecules. Additionally, in both cases, the presence of IDRs in the protein NTR construct has shown visibly more pronounced effects in the HSQC spectra compared to the NTD alone, suggesting an important contribution of these flexible regions in the interaction.

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Unveiling O2 damage on Mo/W Formate dehydrogenases and their innate protection mechanism

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The reversible interconversion of CO2 into formate by Mo/W-Formate dehydrogenases (Fdhs) placed these enzymes on the spotlight, probing a promising route not only for green-house gas sequestration but also a sustainable way to produce fuel. Formic acid is a safe option for hydrogen storage/delivery (53g H2/L) in cell power applications [1]. W-FdhAB is a periplasmic heterodimer and the main responsible for CO2 reduction in *D. vulgaris (Dv)* [2]. It comprises two pyranopterin cofactors in the W active site (bisMGD, selenocysteine and a sulfido ligand) and four [4Fe-4S] clusters responsible for electron transfer.

Due to its robustness and high catalytic activity, *Dv*FdhAB is a suitable model for biocatalytic applications for CO2 reduction [3, 4]. However, it is known that metal-dependent Fdhs are oxygen sensitive and easily inactivated when exposed to O2, thus hampering their productive use as a biocatalyst for industrial CO2 reduction to formate. Nonetheless, the exact chemical and structural consequences of O2 damage are unknown but crucial to help devise a protection mechanism and optimize the biocatalyst. Our recent study [5], combining biochemical, spectroscopic, and structural studies of *Dv*FdhAB, when exposed to oxygen, reveals that O2 inactivation is promoted by the presence of either substrate and involves forming a new active site species, reproducibly captured in the crystal structures, where the SeCys ligand is displaced from tungsten coordination and replaced by a dioxygen or peroxide molecule. Furthermore, these results prove that oxidative inactivation does not require reduction of the metal, as widely assumed, as it can also occur in the oxidized state in the presence of CO2 [5].

*Dv*FdhAB is considerably more oxygen-tolerant than other Fdhs and can be purified aerobically in the absence of substrates [3]. In fact, the formation of a conserved disulfide bond, uncovered by our team [6], reduces enzyme activity and protects it from oxidative inactivation. *Dv*FdhAB can protect itself from transient O2 damage when exposed to physiological concentrations of formate (low µM). Our structural studies disclosed the allosteric mechanism responsible for transducing the signal from the surface exposed disulfide bond to the deeply buried active site [6].

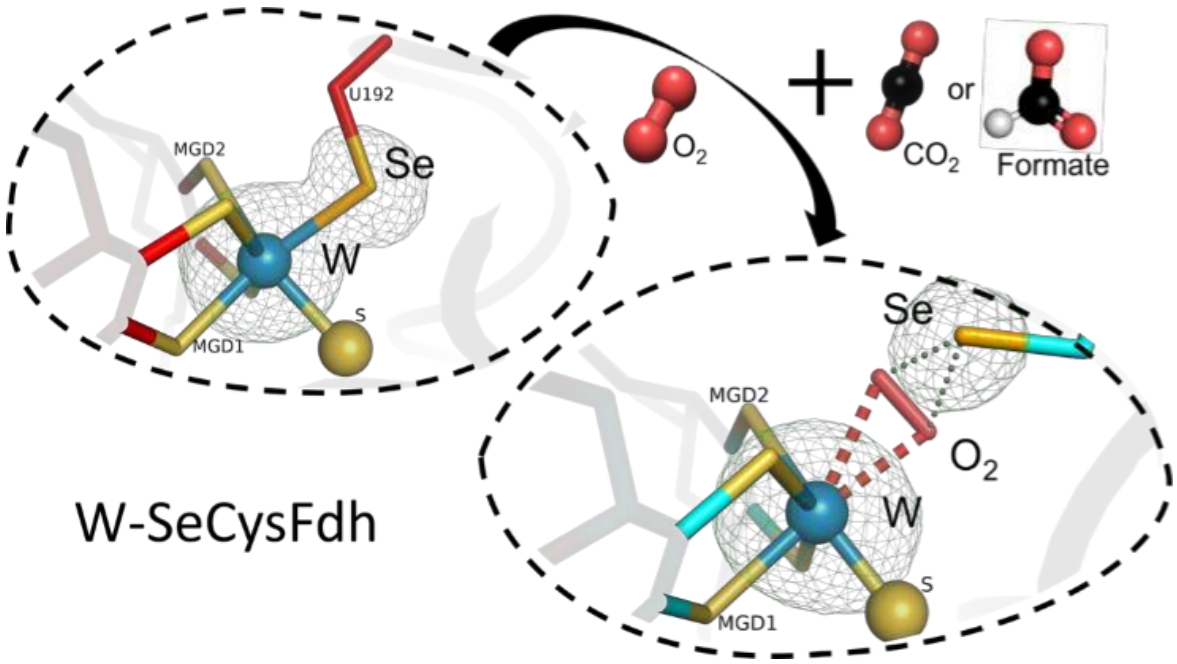

Fig 1. Displacement of the catalytic SeCys in W-FdhAB active site, induced by co-exposure to O_2 and either substrate (formate or CO_2). Anomalous difference map contoured at 5σ (green mesh) [5].

Acknowledgments: This work is financed by national funds from FCT - Fundação para a Ciência e a Tecnologia, I.P., through fellowships. 2023.00286.BD (G.V.-A.), in the scope of the project PTDC/BII-BBF/2050/2020, and UIDP/04378/2020 and UIDB/04378/2020 of the Research Unit on Applied Molecular Biosciences - UCIBIO and LA/P/0140/2020 of the Associate Laboratory Institute for Health and Bioeconomy - i4HB.

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Using NMR spectroscopy to study the pathological linkage in alpha-1-antitrypsin polymers

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The glycoprotein alpha1-antitrypsin (AAT) is a 52 kDa serine protease inhibitor found at high concentrations in human plasma. The Z mutation (E342K) occurs in 1 in 1700 Northern Europeans and promotes ordered aggregation ('polymerisation') leading to liver cirrhosis and early-onset emphysema. Solution NMR investigations of the monomeric states of the wild-type and Z variants, using 2D ^{1}H,^{13}C experiments at natural abundance on AAT samples purified from patient donors have allowed us to probe structural and dynamic features at the earliest stages of misfolding and polymerisation (Jagger, Nat Commun 2020). However, the structure of the polymer itself is currently unknown, yet critical to a full understanding of the polymerisation mechanism and application to ongoing drug development efforts.

Polymerisation of isotopically enriched, methyl-labelled AAT was induced artificially at elevated temperature and followed in real-time by 1D ^{1}H NMR, translational diffusion and ^{1}H, ^{13}C HMQC experiments. This analysis yielded the first high-resolution ^{1}H, ^{13}C spectrum of AAT polymers. These spectra overlaid closely with a proteolytically cleaved, monomeric form of the protein, from which we could obtain and transfer methyl resonance assignments. As it is not certain that recombinant samples accurately recapitulate what is found within patients, we purified pathological polymers from liver explant for comparison. These were fractionated into varying polymer chain lengths for characterisation by ^{1}H NMR. Using a methyl-selective Ernst angle excitation to improve sensitivity, we compared the high-field methyl resonances of ex vivo polymers with the artificial polymer standards. These showed resonance equivalence that could only be explained by a mechanism in which there is full insertion of the RCL and intermolecular domain swap of the C-terminus of the molecule. These data explain the mechanism of aberrant polymer formation in Z AAT deficiency.

Here, we have used NMR techniques to perform the first direct elucidation of the internal structure of the AAT polymer subunit. Further work is being completed on 2D CH solid state spectrum of the liver-derived AAT polymers at natural isotopic abundance.

Structural Biology: Go Bigger, Go Native

Tzviya Zeev-Ben-Mordehai

Bijvoet Center for Biomolecular Research

Cryo-electron microscopy (cryoEM) is emerging as a discovery tool for de-novo identification of proteins. We have used cryoEM to resolve the doublet microtubules (DMTs) structure of sperm axoneme, a large multi-protein molecular machine that powers sperm. To this end, we developed a unique single particle analysis approach that does not involve purification of the complex. Using this approach, we identify >90 new proteins decorating sperm DMTs; at least 15 are sperm specific and many are linked to infertility. The methods we use for sample preparation, data collection, and protein identification using the latest deep learning-based algorithms exemplify the post-AlphaFold era of experimental structural biology, where focus shifts to large multi-protein complexes that cannot be isolated from the cellular environment.

Phase separation and emergent properties of condensates

Tanja Mittag[1]

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Phase separation compartmentalizes cells via the formation of membraneless organelles, also called biomolecular condensates. Phase separation influences many fundamental biological processes, from transcription to sorting of molecules and the stress response. Phase separation is mediated by a combination of associative and segregative phase transitions, or networking and a density transition, which together result in a percolated dense phase. Condensates therefore have emergent properties, i.e., properties that small complexes do not possess. These include material properties, which can be characterized in terms of dynamical moduli and can change as a function of the age of the condensate. While the lifetimes of small complexes are determined by the affinities of their constituent molecules, condensates cross the percolation line and therefore have long lifetimes. The mobility of individual biomolecules in the dense phase and between dilute and dense phases, are also determined by their interactions and can be high. Condensates have internal network structures and interfaces with the coexisting dilute phase, and the latter are characterized by distinct conformations of the biomolecules and can mediate specific biochemical functions. Not only biomolecules but also ions can partition asymmetrically between coexisting phases, and this depends on the ion concentration, salt type and the charge of the biomolecules that form the condensates. Asymmetric partitioning results in an electric potential between phases and across the interface. These membrane-like, interphase electric potentials predict that condensates can be responsive to changes in osmotic pressure and to changes in electrochemical potentials. Phase separation thus enables the formation of large assemblies with long lifetimes, high internal mobility, and biochemically active interfaces with electric potentials. I will discuss some of these recent insights and how they relate to condensate function and relationship to diseases.

Atomic resolution molecular imaging based on soft-landing electrospray ion beam deposition (ESIBD).

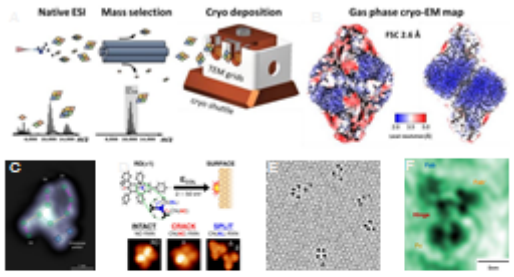
Stephan Rauschenbach

University of Oxford, Department of Chemistry, Kavli Institute of Nanoscience Discovery, Oxford, United Kingdom

Electrospray ion beam deposition (ESIBD), the deposition of intact molecular ions created by electrospray ionisation onto solid surfaces in vacuum, has been introduced in our lab as a tool for the handling of large and complex, usually non-volatile molecules.[1] Initially, the high-resolution single-molecule imaging by scanning probe microscopy (SPM) has been the major application. Here ESIBD proved successful in the investigation of structure, conformation, and properties of proteins, peptides, saccharides, and synthetic molecules. [2,3]

ESIBD's high level of control over molecular ion beam and environment in opens new avenues in molecular imaging. Native ESI enables the chemically selective enrichment of folded proteins and proteins complexes for structural investigation by electron microscopy imaging (cryoEM)[4,5], and low energy electron holography (LEEH).

Optimized conditions for native deposition promote imaging of individual proteins at a resolution sufficient for the construction of atomic models from cryoEM data.[5] The structure obtained from cryoEM after embedding the landed proteins in ice grown from the gas phase shows a fold and subunit arrangement which is remarkably similar to the solution structure. Small conformational changes cause differences mostly at the protein surface and interfaces. We find the closing of cavities and crevices' due to self-interaction in absence of water, a change reversed in MD simulations to find the native structure again.



Molecular imaging based on ESIBD. ***(A)*** *Scheme of native ESIBD deposition onto cryoEM grids.[1]* ***(B)*** *3D density of b-gal prepared by native ESIBD.[5]* ***(C)*** *STM image of a branched 11-mer glycan.[2]* ***(D)*** *Hyperthermal chemistry: Intact Reichardt's Dye molecule and products of the hyperthermal collision in STM images.* ***(E)*** *Transmission electron microscopy of atomic clusters on freestanding graphene.* ***(F)*** *Low energy electron holography image of an antibody.*

References: [1] Rauschenbach, **Annu Rev Anal Chem** 9 (2016) 16.1-16.26. [2] Wu, **Nature** 582 (2020) 375–378. [3] Anggara, **Science** 382 (2023) 219-223. [4] Esser, **PNAS Nexus** 1 (2022) pgac153. [5] Esser, **Science Advances** 10 (2024) eadl4628.

Cryo-EM Reveals the Structure and Infection Mechanism of Phage LUZ19

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Central European Institute of Technology, Masaryk University, Kamenice 753/5, 625 00 Brno, Czech Republic

Infections caused by antibiotic-resistant bacteria are a growing health concern. As antibiotic treatments become less effective, alternative approaches are explored. One of the promising methods is phage therapy. However, our understanding of phage–bacterium interactions is limited, and phage therapy is currently considered an experimental treatment. We use cryo-electron microscopy to study the structure of bacteriophage LUZ19 and the process of LUZ19 infection of PAO1, a clinically relevant strain of P. aeruginosa. The virion particle is composed of an icosahedral capsid and a tail decorated by six long tail fibres. The capsid is built from major capsid, head cement, and flexible head decoration proteins, and encloses a 43.5 kbp-long dsDNA genome and an inner core complex. The symmetry mismatch between the capsid and the tail is mediated by a dodecameric portal complex which occupies one vertex of the capsid. The portal complex interacts with the adaptor dodecamer of the tail through its “stem” helices which interlock with the adaptor C- termini. LUZ19 tail extends to a hexameric tail nozzle which is decorated with six flexible tail fibres. The nozzle and the tail fibres mediate the attachment of phage LUZ19 to the type IV pili of PAO1. The contraction of the pili carries the phages to the cell surface, where they irreversibly attach and infect the host cell. Studying phage LUZ19's structure and its infection mechanism aims to deepen our understanding of phage-bacterium interaction.

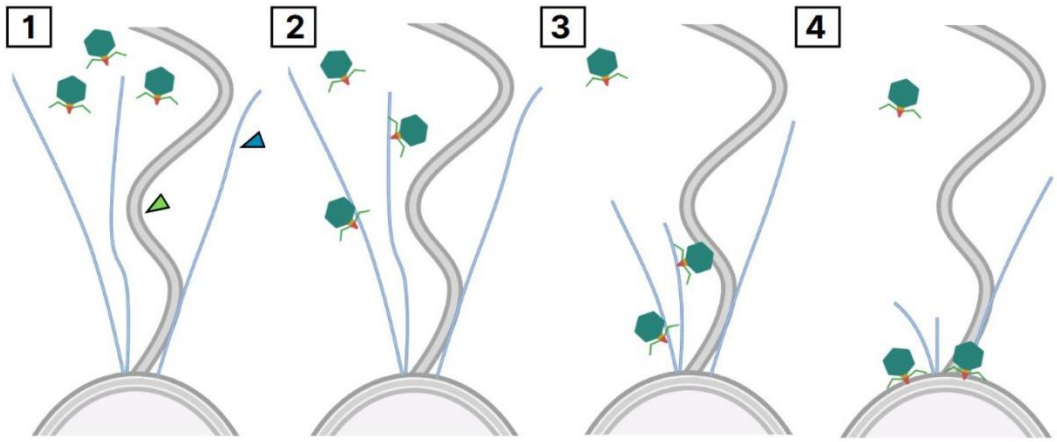

Figure 1. Graphic scheme of the phage attachment process. 1. Free-floating LUZ19 phages. 2. Attachment of LUZ19 to type IV pili of a PAO1 cell. 3. Contraction of pili towards the cell. 4. Irreversible attachment of phages to the cell surface.

Cryo-electron Microscopy and Tomography core facility (CEMCOF) of CEITEC Masaryk University is gratefully acknowledged for obtaining the scientific data presented in this poster. This work was supported by the project “National Institute of Virology and Bacteriology” (Programme EXCELES, ID Project No. LX22NPO5103) - Funded by the European Union - Next Generation EU.

Time-resolved structural transitions of the multidrug transporter BmrA using rapid kinetics and time-resolved cryoEM.

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According to the World Health Organization (WHO), antibiotic resistance is a predominant and worrying health problem for humanity due to the emergence of multi-resistant bacteria. The first line of defence of these bacteria is the overexpression of ABC (ATP-Binding Cassette) efflux pumps that expel antibiotics out of the bacteria below their cytotoxicity threshold. The drug is thus inactive, and the bacteria can develop, and with this low exposition to the drug can start to acquire resistance by mutating the target. The Bacillus subtilis ABC transporter BmrA confers resistance to cervimycin C, an antibiotic secreted by Streptomyces tendæ, a natural competitor for the same biotope. BmrA has a broad specificity for a variety of structurally and chemically unrelated molecules conferring to the pump a multidrug efflux capacity.

To better understand the details of the mechanism carried out by BmrA, the team solved its X-ray and cryo-EM structures, the latter in presence of a substrate in pre-release state, never isolated before. These data shed light on how BmrA handles its ligands and expels them. The team recently showed that BmrA structurally deforms in specific regions to accommodate its ligand rhodamine 6G and proposed a new mechanism of structural adaptation to the substrate using intrinsic structural plasticity [1]. In the present work, we aim at defining and characterizing the biomechanical parameters of the drug efflux mechanism in 4D. I first identified the conditions allowing me to identify the conformational changes biochemically and enzymatically. Then I moved on and characterized structurally the conformational changes using time-resolved cryoEM. This multidisciplinary and integrative approach will give us access to the structural dynamics of the pump in the millisecond to second time-scale, and will further our understanding of drug handling by these transporters in 4D.

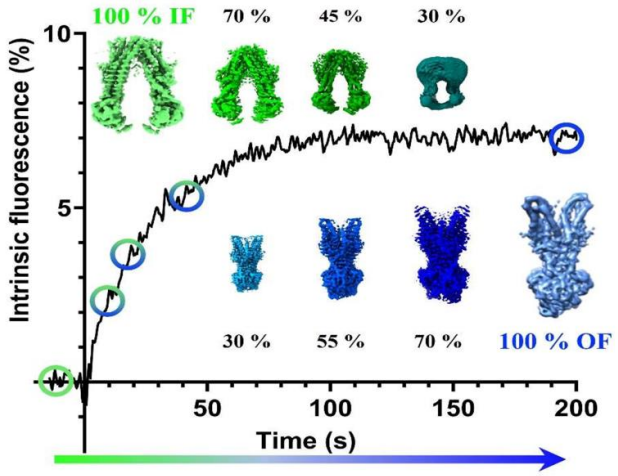

Fig. 1. Observation of the transition inward-facing to outward-facing conformation of BmrA thanks to biochemical and structural approaches in time-resolved.

[1] Chaptal V, and al. 2022. Substrate-bound and substrate-free outward-facing structures of a multidrug ABC exporter. Sci Adv. 8(4):eabg9215.

Tracking transcription-translation coupling in real-time

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A central question in biology is how macromolecular machines function cooperatively. In bacteria, transcription and translation occur in the same cellular compartment, and can be physically and functionally coupled. While several recently published high-resolution structures of the ribosome-RNA polymerase (RNAP) complex provided first mechanistic insights into the coupling process, we lack knowledge of how these structural snapshots are placed along a dynamic reaction trajectory. Here, we reconstitute a complete and active transcription-translation system and develop multi-color single-molecule fluorescence microscopy experiments to directly and simultaneously track transcription elongation, translation elongation and the physical and functional coupling between the ribosome and the RNAP in real-time (Qureshi & Duss, bioRxiv, 2023, 10.1101/2023.12.07.570708).

Our data show that physical coupling between ribosome and RNAP can occur over hundreds of nucleotides of intervening mRNA, by mRNA looping, a process facilitated by transcription factor NusG. We detect active transcription elongation during mRNA looping and show that transcription factor NusA-paused RNAPs can be activated by the ribosome by long-range physical coupling. On the other hand, the ribosome slows down while colliding with the RNAP, a state with no intervening mRNA between both machines and physical coupling between both machineries becomes more transient once the RNAP escapes from a collision. We hereby provide an alternative explanation on how the ribosome can efficiently rescue RNAP from frequent pausing without requiring collisions by a closely trailing ribosome. Overall, our dynamic data mechanistically highlight an example of how two central macromolecular machines, the ribosome and RNAP, can physically and functionally cooperate to optimize gene expression.

Seeing and Touching Adenovirus: Complementary Approaches for Understanding Assembly and Disassembly of a Complex Virus

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We investigate the structural and physical principles governing adenovirus assembly. The adenovirus infectious particle (95 nm diameter, over 10 different protein components) is among the most complex of the icosahedral, non-enveloped viruses. Within the *pseudoT* = 25 icosahedral shell, the 35 kbp dsDNA genome is packed together with ~25 MDa of positively charged core proteins V, VII and µ. Understanding adenovirus assembly and disassembly poses many challenges due to the virion complexity. Addressing these challenges requires the use of complementary techniques.

Cryo-electron microscopy (cryo-EM) structures are broadening our understanding of adenovirus capsid variability along evolution, but little is known about the organization of the non-icosahedral nucleoproteic core and its influence in the infectious cycle. Atomic force microscopy (AFM) probes the biomechanics of virus particles, while simultaneously inducing and monitoring their disassembly in real time. I will present an overview of our work, where we combine electron microscopy in its various flavors (from observation of resin-embedded infected cell sections to cryo-EM three-dimensional reconstruction) with AFM to analyze adenovirus capsid architecture, assembly and *in vitro* disassembly. This combination has provided the first glimpses on how the adenovirus core is organized and tethered to the capsid, how this organization and tethering change during assembly, and how these changes

AI driven workflows for biomolecular NMR

Daniel Mathieu

Bruker BioSpin

We have teamed up with the group of Roland Riek at the ETH in Zurich in order to provide users with easy to use and secure access to ARTINA, a cloud-based AI supported platform, which provides a fully integrated workflow for spectra analysis, resonance assignment as well as 3D structure determination. Being able to provide structures as well as assignments, which are the basis for all downstream data analysis, other results such as protein dynamics, minor populated states or chemical and conformational exchange can be obtained in a matter of days, as compared to weeks or months before.

HDX-MS as a tool for small molecule binding characterization: monitoring modulation of human Cyclophilin D

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3. Merck Healthcare KGaA, Frankfurter Strasse 250, 64293 Darmstadt, Germany.

Hydrogen deuterium exchange mass spectrometry (HDX-MS) is emerging as a powerful biophysical technique for probing protein interactions, structure, and conformational dynamics. While HDX-MS is well-established for the characterization of potent compounds binding to their target proteins there are only few examples in the literature regarding the application of HDX-MS to the study of low-affinity fragments and none describing the usage of HDX-MS for molecules with affinities in the mM range.

In our work, we use human Cyclophilin D (CypD) as a system to explore the possibility of using HDX-MS to characterize the binding of fragments with mM binding affinities. CypD is the mitochondrial isoform of Cyclophilins which plays an important role in the execution of cell death by regulating the mitochondrial permeability transition pore. Mitochondrial dysfunction has been implicated in a cascade of cellular processes related to several diseases such as multiple sclerosis and cardiovascular disease, making CypD an interesting target for therapeutic intervention.

HDX-MS was used to monitor the binding of fragments targeting three different pockets of CypD. Despite the weak binding affinities of these molecules (mM range) we could observe reduction in deuteration levels in CypD regions that match the different binding sites of the fragments. In a second step, we tested fragments with an unknow binding site. Careful analysis of the HDX-MS profiles obtained enabled the mapping of the different binding sites. Altogether our results show that HDX-MS could be a valuable tool in fragment-based drug design projects for the design and improvement of low-affinity CypD inhibitors.

The Regulatory Cortactin-WIP complex: Structure and pharmaceutical implications

Chana G. Sokolik[1], Shams Twafra[2], Hava Gil-Henn[2], **Jordan H. Chill**[1]

Bar Ilan University, Israel

Regulatory complexes are by nature of intermediate affinity and tend to challenge structure determination by crystallography or cryo-EM methods, whereas solution NMR is well-suited to handle such structures. A case in point is the complex formed between the C-terminal SH3 domain of cortactin (CortSH3), responsible for actin polymerization and a biomarker for invasive cancers [1], and WASp-interacting protein (WIP), a cellular multitasker regulating actin cytoskeleton remodelling and a 'hub' of protein–protein interactions impacting health and disease [2,3]. While this binding event was previously mapped to the proline rich region of WIP [4], to date no structural evidence has supported this hypothesis. Employing NMR, we have identified the WIP cortactin binding motif and elucidated the structure of the WIP/cortactin complex. Strikingly, WIP binds the cortactin N-terminal SH3 domain in a combined canonical/non-canonical mode that gives rise to a 'fuzzy' complex of intermediate affinity (Figure 1). By scanning mutagenesis, we demonstrate the contribution of the various molecular determinants to the binding energy. This molecular view of the CortSH3/WIP interaction has recently acquired pharmaceutical importance. We have found that the non-receptor tyrosine kinase Pyk2 mediates cortactin involvement in invadopodia formation and the metastatic phase of breast cancer, and a Pyk2-derived peptide inhibits the CortSH3/Pyk2 interaction and greatly reduces formation of metastases [5]. The combined information from both CortSH3/Pyk2 and CortSH3/WIP structures becomes a powerful driving force for developing peptidic inhibitors with pharmaceutical potential for halting the often lethal metastatic progression of breast cancer. Current efforts in developing this research avenue suggest NMR-based methods play an important role in both structural studies and in drug-design efforts.

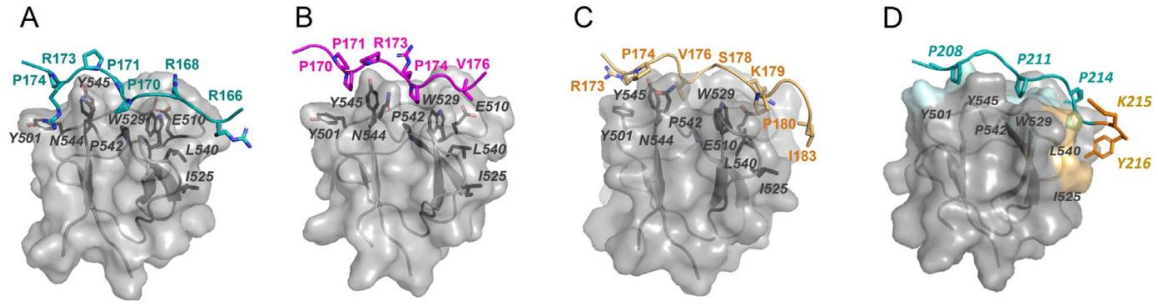

Figure 1. Structures of the cortactin C-terminal SH3 domain (grey, surface representation) with **(A-C)** WIP$^{165\text{-}183}$ in teal/magenta/light orange, **(D)** Pyk2$^{202\text{-}220}$ (in cyan/orange).

1. Daly, R. J. Cortactin Signalling and Dynamic Actin Networks; 2004, Vol. 382, **2.** Antón, I. M.; Jones, G. E. Eur. J. Cell Biol. 2006, 85 (3–4), 295–304, **3.** Sokolik, C. G.; Qassem, N.; Chill, J. H. Biomolecules 2020, 10 (7), 1–19, **4.** Kinley, A. W. et al, Curr. Biol. 2003, 13 (5), 384–393, **5.** Twafra, S.; Sokolik, C. G. et al, Chill, J.H.; Gil-Henn, H. Oncogene, 2023, 42 (4), 337.

Dissecting the Wood-Ljungdahl Pathway - Molecular Basis of Acetogenesis

Jan Schuller

SYNMIKRO Research Centre

Research in our laboratory is focused on unravelling the diverse and ingenious mechanisms that nature has evolved to capture and utilise the greenhouse gas CO_2 for biochemical processes. Among the often overlooked heroes of CO_2 fixation, with significant ecological and biotechnological importance, are anaerobic bacteria and archaea. In particular, acetogens are a specialised group of strictly anaerobic bacteria that excel at converting CO_2 to acetic acid using electrons derived from molecular hydrogen (H_2) or carbon monoxide (CO), major components of industrial waste gases, making them promising candidates for carbon recycling processes.

In recent years, our research has focused on exploring the key molecular machinery involved in acetogenesis. Using redox-controlled cryo-EM, we have uncovered common principles in acetogenic lifestyles, highlighting the central role of enzymatically decorated nanowires and redox-induced conformational changes. This knowledge enhances our understanding of how acetogens thrive in conditions at the thermodynamic limit of life, similar to the environment on early Earth where life evolved in the absence of oxygen.

Capsid inhibitors of coxsackievirus A9

Chiara Tammaro [1], Zlatka Plavec [2], Laura Myllymäki [3], Cristopher Mitchell [2], Sara Consalvi [1], Mariangela Biava[1], Alessia Ciogli [1], Pietro Sciò [1], Antonio Coluccia [1], Cienna Buckner [2], Ausra Domanska, Valtteri Leppilampi, Mira Laajala [3], Varpu Marjomäki [3], **Sarah J. Butcher [2]** and Giovanna Poce [1]

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2. Faculty of Biological and Environmental Sciences, Molecular and Integrative Bioscience Research Programme, & Helsinki Institute of Life Sciences-Institute of Biotechnology, University of Helsinki, Helsinki, Finland
3. Department of Biological and Environmental Sciences, Nanoscience Center, University of Jyväskylä, Jyväskylä, Finland

Enterovirus infections are common in humans, yet there are no approved antiviral treatments. In this study we concentrated on inhibition of one of the Enterovirus B, namely coxsackievirus A9, using a combination of medicinal chemistry, virus inhibition assays, structure determination from cryogenic electron microscopy and molecular modelling. to determine the structure activity relationships for a hit expansion from promising N-phenyl benzamides. Of the 29 compounds synthesized, 11 had EC50 values between 0.68-14.06 μM, and of these, 7 had CC50 values higher than 200 μM. The reconstructions confirmed that the hydrophobic pocket in VP1 is the target for the compounds, with clearly resolved density for 5 of the compounds with only a minor rearrangement of Tyr210 occurring on binding. Antiviral activity thus occurs through capsid stabilization, preventing capsid expansion and subsequent release of the genome. The N-phenyl benzamide backbone is amenable to modification for the development of better antiviral molecules.

Subcellular elemental mapping using correlative cryoSEM-CryoNanoSIMS to gain insights into salinity stress in plants

Priya Ramakrishna[1], Etienne Bellani[3], Damien De Bellis[2,3], Cristina Martin Olmos[1,4], Stéphane Escrig[1], Christel Genoud[2], Niko Geldner[3], Anders Meibom[1,4]

1. Laboratory for Biological Geochemistry, Ecole Polytechnique Fédérale de Lausanne (EPFL), Lausanne, Switzerland
2. Electron Microscopy Facility, University of Lausanne, Switzerland
3. Department of Plant Molecular Biology, University of Lausanne, Switzerland
4. Center for Advanced Surface Analysis, Institute of Earth Sciences, University of Lausanne, Switzerland

Sodium is an unusual nutrient for life. It is an essential element for animals, while most plants avoid it at all costs. Salinity stress (mainly sodium) causes significant loss to agricultural productivity globally, and while we understand sodium is toxic to plants, the precise mechanisms of this toxicity remain poorly understood. One reason being the lack of analytical capacity to quantify these elements with sufficient specificity and resolution in the native state. Nanoscale Secondary Ion Mass Spectrometry (NanoSIMS) is an imaging technique that produces quantified isotopic/elemental maps and often used to understand complex transport and metabolic processes in living organisms. However, a challenge has been the need for sample fixation and embedding of biological samples for their observation at room temperature, which inevitably leads to severe changes in the distribution of many ions. The recent development of the CryoNanoSIMS at EPFL now enables examination of vitrified biological samples without loss or significant displacement of cell constituents, including soluble compounds. I will briefly describe the cryogenic-workflow and its application to visualize multiple physiologically important elements in plant cells. Using this approach, we show the role of a key sodium transporter in plants, required for regulating sodium distribution within the cell and gain insights into the fine-tuned mechanisms plants employ to respond to salinity stress.

Posters

Instruct-ERIC: Get access to cutting edge structural biology services in Europe.

Corinna Brockhaus [1], **Pauline Audergon** [1], John Dolan [1], Natalie Haley [1], Claudia Alen Amaro [1], Harald Schwalbe [1].

[1] Instruct-ERIC, Oxford House, Parkway Court, John Smith Drive, Oxford, OX4 2JY, UK.

Instruct-ERIC is a pan-European distributed research infrastructure making high-end technologies and methods in structural biology available to users. Instruct-ERIC is comprised of 17 Member Countries and organisations: Belgium, Czech Republic, EMBL, Finland, France, Germany, Greece, Israel, Italy, Latvia, Lithuania, Netherlands, Portugal, Slovakia, Slovenia, Spain and United Kingdom. Through its 11 specialist research centres and 23 facilities in Europe, Instruct-ERIC offers funded research visits, training, internships and R&D awards. Instruct also offers funded access to its infrastructure through European projects such as canSERV that provide access to a broad range of services for cancer-related research projects. By promoting integrative methods, Instruct-ERIC enables excellent science and technological development for the benefit of all life scientists.

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Introducing FlexibilityHub, a new service for the analysis of molecular motions in cryoEM data.

Gragera M., Herreros D., Melero R., Sorzano COS., Carazo JM.

CryoEM has become a well-established technique for solving macromolecular complexes at high resolution. By aligning and averaging thousands of individual projections of a biological specimen, single-particle analysis (SPA) allows for the generation of one or a few high-resolution structures from a single data collection. However, these static structures still represent discrete states within the complex motions that proteins typically undergo, despite the fact that the information on the dynamics of the sample is indeed contained in the data. Recently, the cryoEM community has begun to exploit this information, developing new image processing algorithms to gain insights into the conformational landscape of protein complexes and/or to correct for local motions, thereby increasing the resolution of moving subregions of a protein. Therefore, a growing demand is anticipated in the coming years from users seeking to unravel the molecular motions present in their samples of interest. We introduce FlexibilityHub, a new service/technology offered by the Instruct- ES centre, now available for users to utilize alongside our current SPA service. In this service, users are encouraged to provide a particle set that has undergone prior 2D and 3D classification but still exhibits a certain degree of flexibility, whether compositional or conformational. Subsequently, we will conduct a comprehensive study of the dataset's flexibility using state-of-the-art software packages such as Zernikes3D, cryoDRGN, opusDSD, cryoSPARC 3DVA, and more, in combination with methods for flexible reconstruction to correct for motions such as Relion5 - DynaMight, cryoSPARC - 3D Flex, and Xmipp - ZART. This multi-algorithmic approach is facilitated by Scipion, a cryoEM image processing framework developed by our team, which integrates various tools into a single platform. This new service is available in the Instruct catalogue under the name Electron Microscopy FlexibilityHub and this talk will show some of its current capabilities.

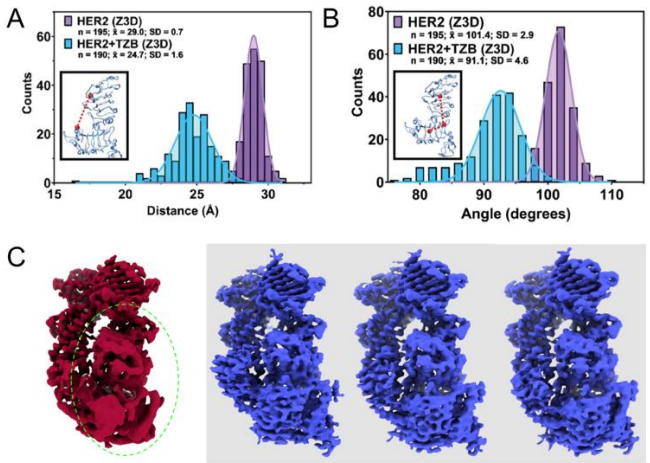

Fig. 1. Examples of applications of the methods applied in the FlexibilityHub service. A, B) Characterizing HER2 ECD response under different treatments by measuring interdomain distance. Flexibility analysis with Zernikes3D allowed for obtaining hundreds of maps from individual particles to increase the sample size and to have a more robust statistics. C) Example of flexible refinement. In red, consensus map with a subregion (dashed circle) showing poorer local resolution due to flexibility. In blue, three different reconstructions of with the same particles after correction of local motions with ZART, which resulted in better resolution in the region of interest.

The CryoEM CNB-CSIC Facility

Maria Teresa Bueno

Located within the esteemed National Centre for Biotechnology in Madrid, the CryoEM CNBCSIC facility plays a pivotal role as part of the INSTRUCT Node Spain. Collaborating closely with the Instruct Image Processing Centre (I2PC), our facility is proud to be included in the renowned Instruct-ERIC and iNEXT-discovery programs for infrastructures. We provide European researchers with exceptional access to advanced cryoelectron microscopy (cryo-EM) infrastructure. At our facility, we warmly welcome both seasoned experts and aspiring scientists, offering a unique opportunity to submit samples for meticulous cryoEM sample characterization and engage in high resolution single-particle analysis (SPA) data acquisition. In line with our dedication to innovation, we have expanded our services to include cryo-electron tomography (cryo-ET), cryo-correlative microscopy (cryo-CLEM), and microcrystal electron diffraction (microED) for molecules and small crystal proteins. Join us at the CryoEM CNB-CSIC facility as we embark on an exciting journey, unravelling the secrets of structural biology and uncovering groundbreaking discoveries.

The Oxford Particle Imaging Centre (OPIC), UK

Helen M.E. Duyvesteyn[1]

[1]University of Oxford

The OPIC facility is based close to Oxford City centre offers a wealth of resources for internal and external applicants. Working in synergy with eBIC (Harwell, UK), we have the unique capability of preparing and collecting TEM data on Biosafety-level 3 virus samples using high-end equipment. Over the past couple of years, we have undergone significant upgrades within our containment suite, including the installation and commissioning of the first commercial UK Arctis pFIB/SEM, Delmic clean station for handling samples under a clean nitrogen atmosphere and upgrade of our Krios G3i microscope with a state-of-the-art Falcon-4i/SelectrisX detector for rapid high quality data collection.

Access to our facility is available at request from academia and industry alike for competitive prices, and funding is available via Instruct-ERIC.

Structure and function of CDNF-BiP complex in ER protein folding regulation

Satoshi Fudo, Olesya Shpironok, Mart Saarma and **Tommi Kajander**

Institute of Biotechnology, Helsinki Institute of Life Sciences, University of Helsinki

Cerebral dopamine neurotrophic factor (CDNF) is regulator of endoplasmic reticulum (ER) stress and protein folding,and promotes neuronal survival. It has been found to interact with ER Hsp70 protein BiP, similar to the homologous mesencephalic astrocyte-derived neurotrophic factor (MANF). Here we present the crystal structures at 1.65 Å and 1.5 Å resolution of CDNF C-terminal domain (C-CDNF) in complex with BiP nucleotide binding domain (NBD) with and without bound ADP. These are also the first high resolution crystal structures of the C- CDNF region. It has been reported that MANF would act as an antagonist of nucleotide exchange in ER. We show the binding of CDNF to BiP with fluorescence complementation, microscale thermophoresis and analytical gel filtration and pull-down assays and demonstrate it has a similar binding mode to MANF. We also show that CDNF antagonizes the nucleotide exchange by BiP in a fluorescence assay. We suggest CDNF and MANF have overlapping functions in BiP chaperone cycle with some differences, e.g. MANF have been shown to bind sulfatide lipids through its N-terminal domain, while CDNF does not bind to sulfatide, but different lipids, this may result in differences in function. Overall, the binding site on BiP is very similar for both proteins, and based on the results and structural analysis we conclude the binding of CDNF interferes with the conformational change in the BiP chaperone cycle, and conversion of BiP to the ATP bound state, supporting a role in regulation of BiP chaperone function.

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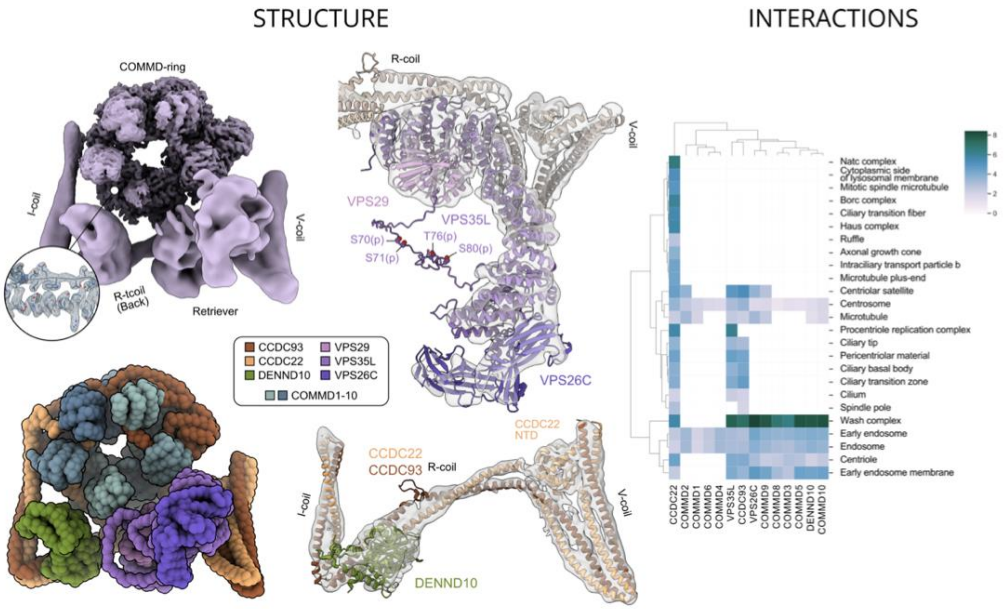
Structure and Interactions of the Commander Supercomplex

Esa-Pekka Kumpula[1#], Saara Laulumaa[1#], Juha T Huiskonen[1], Markku Varjosalo[1]

[1]*Institute of Biotechnology, Helsinki Institute of Life Science HiLIFE; 00014 University of Helsinki, Helsinki, Finland*
[#]*Equal contribution*

Regulation of transmembrane receptor fate through endosomal sorting underpins numerous cellular functions and pathologies, necessitating a precise understanding of the underlying molecular machinery. Endosomal sorting depends on several interacting protein complexes on endosomal membranes. One such vital complex is the 16-subunit heteromeric protein supercomplex, called Commander. It is an assembly of two subcomplexes, the COMMD/CCDC22/CCDC93-complex (CCC) and the Retriever complex, and the guanine nucleotide exchange factor protein DENND10. Retriever (VPS29, VPS35L and VPS26C) is a heterotrimeric cargo sorting complex analogous to Retromer, which functions in coalescence of cargos via interactions with cargo-recognizing sorting nexins on the surface of endosomes. The CCC-complex is a hetero-12-mer associated with sorting of certain receptors, but with an unknown function, while DENND10 is virtually unstudied. We determined the structure of the endogenous human Commander complex using cryo-electron microscopy and determined its interaction network in HEK293 cells using advanced mass spectrometry-based proteomics. We uncover functions of the complex in transcriptional regulation, ciliary biogenesis, centrosomal functions, centriole replication and regulation of intracellular transport. We also propose interaction interfaces with key interacting complexes. Our findings unveil a multifaceted role of the Commander complex in cellular function and offer a promising foundation for targeted therapeutic intervention strategies in associated pathologies.



Novel regulation of a membrane embedded phosphatase linked to outer membrane vesicle production in *Porphyromonas gingivalis*

YICHAO LIU[1*], Hoyan Lam[1], Sunjun Wang[1], Joseph Atherton[2], Joseph Aduse-Opoku[1], Mike Curtis[1], James Garnett[1]

[1]Centre for Host-Microbiome Interactions, Faculty of Dentistry, Oral and Craniofacial Sciences, King's College London, London, United Kingdom
[2]Randall Centre for Cell and Molecular Biophysics, King's College London, London, United Kingdom

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The Gram-negative oral pathogen *Porphyromonas gingivalis* (Pg) uses the type-IX secretion system (T9SS) and outer-membrane vesicles (OMVs) to export virulence factors (e.g. gingipains R and K). This is dependent on a conserved C-terminal domain within the cargo proteins, which are covalently attached to the cell surface via A-LPS (anionic-lipopolysaccharides, mediated by lipid A) or become subsequently sorted into OMVs via blebbing from the bacterial surface. We have shown the production of OMVs is associated with modifications to lipid A structure and this appears to be linked to the T9SS. LpxE is a lipid A 1-phosphatase broadly distributed in Gram-negative bacteria which modulates the phosphorylation status of lipid A. In *P. gingivalis*, $LpxE_{PG}$ is twice the size of other LpxEs with a long C-terminal extension of unknown function(s). $LpxE_{PG}$ was expressed as fusion protein His10-MBP-$LpxE_{PG}$ (100 kDa) in E. coli. The purified protein was confirmed by LC/MS-MS (liquid chromatography/tandem mass spectrometry). LpxEs are typically monomeric, but negative-stain TEM (transmission electron microscopy) and cryo-TEM studies indicate that MBP-$LpxE_{PG}$ forms a unique trimer-like compact complex of 120Å in length. This is consistent with the suggested molecular weight of 300-500 kDa from size exclusion chromatography with multi-angle light scattering (SEC-MALS). The C-terminal region of $LpxE_{PG}$ forms a small domain that sit on top of the inner membrane. This mediates trimerization, while also inserting within the inner-membrane and modifying the arrangement of the embedded helices within the N-terminal phosphatase. Different conformations of cryo-TEM maps indicate the C-terminus may regulate the structure and activity of the phosphatase. We are currently investigating point mutations and truncations in $LpxE_{PG}$ to elucidate the role of the C-terminal region. Through a pull-down assay using Pg lysates, we have also identified a potential interaction between LpxE and the type I fimbriae regulator protein, FimB, although the precise role of this interaction or its connection to LpxE linking with the T9SS remains unclear. This work presents new insights into bacterial secretion and outer membrane vesicle production and may have wider implications in other organisms with T9SS.

Modulating the *in vitro* oligomerization of SMAD4 with constructs derived from single-chain antibodies

Miriam Condeminas[1,2], Lidia Ruiz[1], Radoslaw Pluta[1], Carles Torner[1,3], Pau Martin-Malpartida[1] and Maria J. Macias[1,4*]

[1]Institute for Research in Biomedicine (IRB Barcelona), The Barcelona Institute of Science and Technology, Carrer de Baldiri Reixac 10, Barcelona 08028, Spain.
[2]Department of Medicine and Life Sciences, Universitat Pompeu Fabra (MELIS-UPF), Carrer del Doctor Aiguader 88, Barcelona 08003, Spain.
[3]Faculty of Biology of the University of Barcelona, Diagonal 643, Barcelona 08028, Spain.
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SMAD proteins are a family of transcription factors that mediate Transforming Growth Factor Beta (TGFβ) signaling. This pathway is essential, among other processes, for embryonic development, tissue homeostasis and regeneration, immune system maintenance and neuroprotection. Defects in the TGFβ cascade result in many pathologies, and SMAD missense mutations are known to inactivate TGFβ's tumor suppressor function in cancers and to cause rare diseases.

When the TGFβ hormones reach the pathway's receptors, a cascade is triggered that results in the phosphorylation of two C-terminal serines of receptor-activated SMADs (R-SMADs) such as SMAD3. These post-translational modifications change R-SMADs oligomerization properties so that they form heteromeric complexes with SMAD4, also known as the common-partner SMAD (Co-SMAD). These SMAD4/R-SMAD heteromers constitute transcriptionally active units that translocate into the nucleus and modulate the expression of TGFβ's downstream genes.

SMAD4 and the R-SMADs are proteins formed by around 500 amino acids and consist of two globular domains connected by a flexible and partially unstructured linker region. At the N-terminus, they present the DNA-binding Mad Homology 1 (MH1) domain and, at the C-terminus, the MH2 domain that together with the linker, establishes protein-protein interactions with other SMADs and with cofactors.

Thanks to an Instruct-ERIC project, we have discovered two Nanobodies that specifically recognize the MH2 domain of SMAD4. Nanobodies are recombinantly expressed VHHs from camelid single chain antibodies. This work was started by a short visit to the Nanobodies4Instruct facility of the VIB-VUB Center for Structural Biology in Brussels (Belgium) and continued in our laboratory. One of the discovered Nanobodies binds SMAD4 with a millimolar dissociation constant and blocks the interaction between SMAD4 and SMAD3, whereas the other is a nanomolar binder that enables the formation of heteromeric SMAD4-SMAD3 species. We have grafted these two Nanobodies into larger rigid scaffolds to generate Megabodies, which retain the binding properties of the Nanobody they originate from. We are currently investigating how these Megabodies will be useful to label SMAD4 in big complexes so that we can apply Cryogenic Electron Microscopy (cryoEM) to understand their structures. We are particularly interested in understanding the molecular basis of pathogenic mutations in reconstituted heteromers whose composition and stability are altered in disease.

Breaking Bad Bugs: Cooking Up a Cure for *Coxiella burnetii*

William Stuart[1], Christopher Jenkins[2], Phil Ireland[2], Izzy Norville[2], Nicholas Harmer[1]

[1]Living Systems Institute, University of Exeter, Stocker Road, Exeter
[2]Defence Science and Technology Laboratories, CBR Division, Porton Down, Salisbury

Coxiella burnetii is a highly pathogenic zoonotic bacterium, causing Q-fever in humans. Ruminants are the main animal reservoirs and previous outbreaks resulted in significant economic damage. Farm and veterinary workers are at highest risk, with infections in the wider community likely under-reported. There is no human vaccine available outside Australia. Current therapy for chronic infections relies on a long antibiotic course (at least 18 months). This work will produce a prioritised list of targets for drug discovery and demonstrate feasibility by generating initial hits to aid the development of a targeted therapeutic against *Coxiella burnetii*. We developed a pipeline to assess essential *C. burnetii* genes and have progressed a candidate to the initial steps of fragment-based lead discovery with X-ray crystallography.

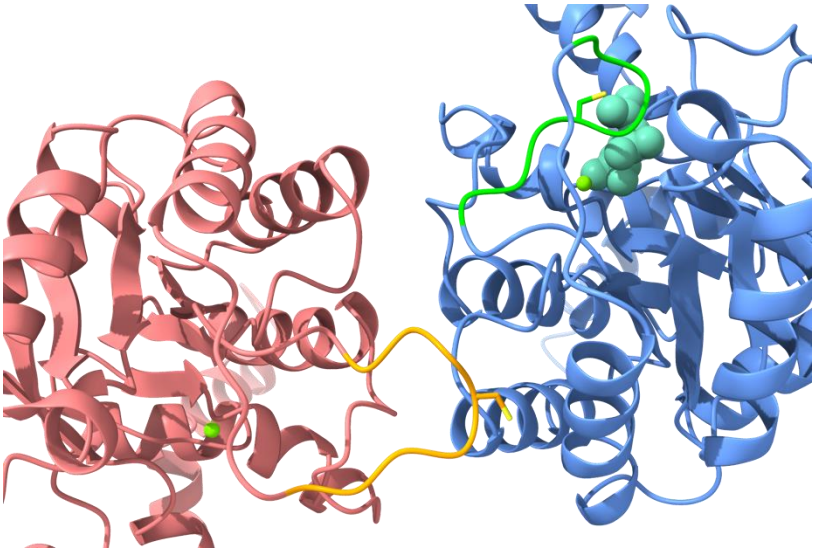

Figure 1: First substrate bound structure of an identified drug target against Coxiella burnetii.

Effect of cardiolipin on the morphology of liposomes hydrated in PBS

Alice Piccinini[1,2,3], Joachim Kohlbrecher[4], Dihia Moussaoui[5], **Anja Winter[2],** Sylvain Prévost[1]

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[2] School of Life Sciences, Keele University, ST55BG Staffordshire, United Kingdom
[3] Dept. of Medical Biotechnology and Translational Medicine, Università degli Studi di Milano, 20054 Milan, Italy
[4] Paul Scherrer Institut, 5232 Villigen, PSI, Switzerland
[5] ESRF -- The European Synchrotron, 38043 Grenoble, France

Lamellarity and shape are important factors in the formation of vesicles and determine their role in biological systems and application as drug delivery systems. Cardiolipin (CL) is a major lipid in many biological membranes and exerts a great influence on their structural organization due to its particular structure and physico-chemical properties.

Here, we used small-angle X-ray and neutron scattering to study the effects of CL with different acyl chain lengths and saturations ($CL_{14:0}$, $CL_{18:1}$, $CL_{18:2}$) on vesicle morphology and lamellarity in membrane models containing mixtures of phosphatidylcholine and phosphatidylethanolamine with different acyl chain lengths and saturations ($C_{14:0}$ and $C_{18:1}$). Measurements were performed in the presence of Phosphate Buffer Saline (PBS), at 37°C, to better reflect physiological conditions, which resulted in strong effects on vesicle morphology depending on the type and amount of CL used.

In non-extruded vesicles, the presence of 2.5-20 CL reduced inter-membrane correlations and increased perturbation of the membrane, an effect which is enhanced in the presence of matched shorter saturated acyl chains, and mainly unilamellar vesicles (ULV) are formed. In extruded vesicles, employed for SANS experiments, flattened vesicles are observed, partly due to the hypertonic effect of PBS, but also influenced by the type of CL added. Additionally, similar to our SAXS data, a reduction in lamellar stacking can be observed leading to formation of ULV ad BLV.

Our experimental data confirm a strong dependence on CL content and type in shaping the membrane microstructure, with an apparent optimum in the PC:CL mixture in terms of promoting reduced correlations, preferred curvature and elongation. However, the use of PBS caused distinct differences from previously published studies in water in terms of vesicle shape, and highlights the need to investigate vesicle formation under physiological conditions in order to be able to draw conclusions about membrane formation in biological systems.

Cryo-EM structure of Tailless Complex Polypeptide 1 Ring Complex (TRiC)

Mohammad Ghanem

Tailless complex polypeptide 1 ring complex (TRiC), also known as tailless complex polypeptide 1 (CCT), is a pseudo-symmetric hetero oligomer consisting of 2 rings of 8 protein subunits. TRiC is responsible for the folding of newly synthesized obligate substrates, such as actin and tubulin, and other opportunistic substrates such as Huntingtin protein (Htt), the mutant form of which is known for being the leading cause of Huntington's Disease (HD). Under certain physiological and pathological conditions, the capacity of TRiC to suppress the aggregation of mutant Htt diminishes. Understanding the crosstalk between TRiC and Htt could reveal the mechanism that underlies the development of HD. It was shown that TRiC directly interacts with the N terminal of Htt. However, it's unknown whether the binding occurs when TRiC is in a closed or open conformation. Thus, determining the structure of TRiC in both closed and open states will prove beneficial towards resolving the structure of its complex with Htt. The structure of closed TRiC has been revealed, whereas the structure of the open form is still unresolved, which is the main goal of my current research work on a cryo-EM data collected for TRiC. Additionally, I'm aiming at deciphering the intermediate states that actin shows over the folding process inside TRiC's folding chamber. This would uncover key mechanisms of subunit-substrate interaction of TRiC. I aspire to use the same approach to unlock the mechanism of interaction between TRiC and Htt.

Capsid inhibitors of coxsackievirus A9

Chiara Tammaro[a], Zlatka Plavec[b], Laura Myllymäki[c], Cristopher Mitchell[b], Sara Consalvi[a], Mariangela Biava[a], Alessia Ciogli[a], Pietro Sciò [a], Antonio Coluccia [a], Cienna Buckner[b], Ausra Domanska, Valtteri Leppilampi, Mira Laajala[c], Varpu Marjomäki[c], **Sarah J. Butcher[b]** and Giovanna Poce[a]

[a]Department of Drug Chemistry and Technologies, Sapienza University of Rome, Piazzale Aldo Moro 5, Roma 00185, Italy.
[b]Faculty of Biological and Environmental Sciences, Molecular and Integrative Bioscience Research Programme, & Helsinki Institute of Life Sciences-Institute of Biotechnology, University of Helsinki, Helsinki, Finland
[c]Department of Biological and Environmental Sciences, Nanoscience Center, University of Jyväskylä, Jyväskylä, Finland

Enterovirus infections are common in humans, yet there are no approved antiviral treatments. Inthis study we concentrated on inhibition of one of the Enterovirus B, namely coxsackievirus A9, using a combination of medicinal chemistry, virus inhibition assays, structure determination from cryogenic electron microscopy and molecular modelling. to determine the structure activity relationships for a hit expansion from promising N-phenyl benzamides. Of the 29 compounds synthesized, 11 had EC50 values between 0.68-14.06 μM, and of these, 7 had CC50 values higher than 200 μM. The reconstructions confirmed that the hydrophobic pocket in VP1 is the target for the compounds, with clearly resolved density for 5 of the compounds with only a minor rearrangement of Tyr210 occurring on binding. Antiviral activity thus occurs through capsid stabilization, preventing capsid expansion and subsequent release of the genome. The N-phenyl benzamide backbone is amenable to modification for the development of better antiviral molecules.

Instruct-ERIC network: Biophysical characterization of antigen-nanobody complexes

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Forest environments are exposed to multiple stressful factors of both abiotic and biotic nature which may lead to their massive decline [1]. Understanding the molecular mechanisms of specific stress conditions and monitoring the fluctuations of reliable forest plant biomarkers with affordable methods would be instrumental for assessing stress levels over the time. Ascorbate peroxidase (APX) represents a suitable plant biomarker. APX is a hydrogen peroxide-scavenging enzyme the critical role of which has been described in several plants, both herbaceous and woody. Its activity generally increases under oxidative stress during which its peroxide detoxifying function is part of the wider ascorbate-glutathione cycle [2]. The development of reagents to detect such fluctuations would help the evaluation of plant physiological conditions. In this study, nanobodies (Nbs) targeting APX have been identified. Nbs correspond to the variable domain of heavy chain-only antibodies derived from camelids. They are small (15 kDa), stable, and can be easily produced in bacteria fused to different protein tags according to the downstream applications [3]. After their isolation by biopanning against soluble APX, they have been produced and underwent a biophysical characterization in combination with their antigen (APX-Nb complex) to identify the best binders in terms of stability and affinity. The protein complex characterization was supported by Instruct-ERIC and mainly performed at the BIOCEV institute of Prague. Data from Mass Photometry and Dynamic Light scattering evidenced the formation of the protein complexes, whereas the preliminary data of Hydrogen-Deuterium Exchange Mass Spectrometry, performed with the aim of identifying the residues involved in the paratope/epitope interface, were insufficient to clarify the issue and rather suggested that the interaction has low affinity. This indication was then confirmed by ELISA assay. The combination of multiple methods allowed a comprehensive sample characterization which will require further structural analyses to provide a complete picture of the APX-Nb complex.

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Time-resolved structural transitions of the multidrug transporter BmrA using rapid kinetics and time-resolved cryoEM.

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According to the World Health Organization (WHO), antibiotic resistance is a predominant and worrying health problem for humanity due to the emergence of multi-resistant bacteria. The first line of defence of these bacteria is the overexpression of ABC (ATP-Binding Cassette) efflux pumps that expel antibiotics out of the bacteria below their cytotoxicity threshold. The drug is thus inactive, and the bacteria can develop, and with this low exposition to the drug can start to acquire resistance by mutating the target. The Bacillus subtilis ABC transporter BmrA confers resistance to cervimycin C, an antibiotic secreted by Streptomyces tendæ, a natural competitor for the same biotope. BmrA has a broad specificity for a variety of structurally and chemically unrelated molecules conferring to the pump a multidrug efflux capacity.

To better understand the details of the mechanism carried out by BmrA, the team solved its X-ray and cryo-EM structures, the latter in presence of a substrate in pre-release state, never isolated before. These data shed light on how BmrA handles its ligands and expels them. The team recently showed that BmrA structurally deforms in specific regions to accommodate its ligand rhodamine 6G and proposed a new mechanism of structural adaptation to the substrate using intrinsic structural plasticity [1]. In the present work, we aim at defining and characterizing the biomechanical parameters of the drug efflux mechanism in 4D. I first identified the conditions allowing me to identify the conformational changes biochemically and enzymatically. Then I moved on and characterized structurally the conformational changes using time-resolved cryoEM. This multidisciplinary and integrative approach will give us access to the structural dynamics of the pump in the millisecond to second time-scale, and will further our understanding of drug handling by these transporters in 4D.

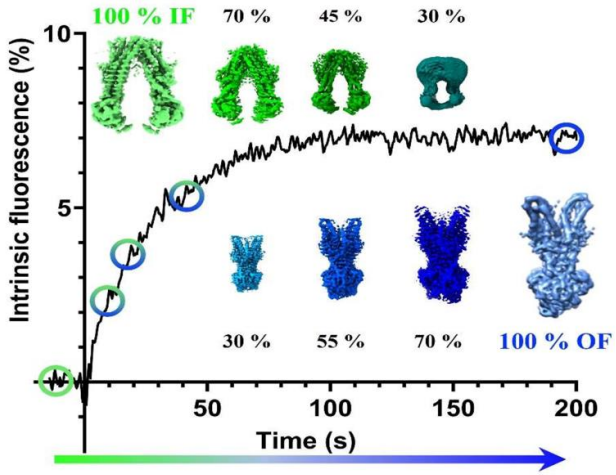

Fig. 1. Observation of the transition inward-facing to outward-facing conformation of BmrA thanks to biochemical and structural approaches in time-resolved.

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Molecular insights into the biogenesis of box H/ACA snoRNPs

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The Box H/ACA snoRNPs are protein-RNA complexes composed of NOP10, GAR1, and the catalytically active DKC1. The latter is known to interact with small non-coding RNAs containing the Box H/ACA motif. Guided by the RNA, the complex finds its target RNAs and converts the nuclear base uridine to pseudouridine, resulting in increased stability of the target RNA due to an additional hydrogen bond. Pseudouridines are vital for various cellular functions, such as protein translation, as they undergo frequent post-transcriptional modifications in ribosomal RNAs.

However, it is still poorly understood how the Box H/ACA snoRNP complexes and their individual protein precursors are assembled. SHQ1, a chaperone-like protein, is proposed to prevent DKC1 from binding to nonspecific ribonucleases during its assembly in the cytosol and subsequently guide the complex to the nucleus, where it interacts with the AAA-ATPase complex RuvBL1/2. These ATPases are known to interact with PIH1D1 and RPAP3 proteins to form the R2TP complex, which is described to play a role in the assembly of multiple macromolecular complexes. Thus, the R2TP complex could be responsible for sequestering SHQ1 from DKC1, allowing DKC1 to interact with Box H/ACA snoRNAs and ultimately assemble the mature snoRNP.

The aim of this work is to elucidate the maturation of the Box H/ACA snoRNP by characterizing its precursor complexes composed of SHQ1, DKC1, and RuvBL1/2. This was achieved using size exclusion chromatography, dynamic light scattering, differential scanning fluorimetry, surface plasmon resonance, mass photometry, cross-linking mass spectrometry, and cryo-EM.

We were able to confirm the interaction between RuvBL1/2 and DKC1 or SHQ1. Additionally, we purified and stabilized the SHQ1:RuvBL1/2 complex and identified multiple complex species. Revealing the interaction site between the AAA ATPases and SHQ1 led to the initial 3D reconstruction of the complex's structure using Cryo- EM

Exploiting the micro-crystal toolbox to generate a mechanistic understanding of GmhA heptose biosynthesis in *Burkholderia pseudomallei.*

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Antimicrobial resistance is one of the greatest threats to global health. Gram-negative bacteria are leading the way with many species possessing multi-drug resistance resulting in plummeting patient prognosis. Gram-negative bacteria possess a unique cell wall which is integral to their virulence and viability, making it an attractive therapeutic target. Sugar synthesis across gram-negatives is highly conserved, making this a target for a broad-spectrum antibiotic that can be used in mixed species infections, such as cystic fibrosis. Our target organism, *Burkholderia pseudomallei*, uses sedoheptulose-7-phosphate isomerase (GmhA) to form sugars in the LPS of the bacterium and by inhibiting this, the membrane will be more susceptible to current therapeutics. We know the protein converts these sugars in a 2-step fashion but the exact mechanism is unknown.

We are applying a multi-faceted approach by utilising micro/nanocrystal techniques available at both Diamond and ESRF to build a molecular movie of GmhA activity. Through this journey we have learnt how to manipulate our protein to form crystals ranging from 200 nm to 0.3 mm in size. Additionally, we have optimised sample preparation pathways to obtain 1 to>1000 crystals per 0.8 µL microbatch drop. Thus far we have built a library of samples for VMXm, serial crystallography, MicroED and single crystal methods. Each of these methods have come with their own advantages and challenges to overcome. Combining these techniques has led to the deepened understanding of GmhA negative and positive co-operativity and how we can possibly exploit mechanistic steps in inhibitor design.

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Using NMR spectroscopy to study the pathological linkage in alpha-1-antitrypsin polymers

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The glycoprotein alpha1-antitrypsin (AAT) is a 52 kDa serine protease inhibitor found at high concentrations in human plasma. The Z mutation (E342K) occurs in 1 in 1700 Northern Europeans and promotes ordered aggregation ('polymerisation') leading to liver cirrhosis and early-onset emphysema. Solution NMR investigations of the monomeric states of the wild-type and Z variants, using 2D ^{1}H,^{13}C experiments at natural abundance on AAT samples purified from patient donors have allowed us to probe structural and dynamic features at the earliest stages of misfolding and polymerisation (Jagger, Nat Commun 2020). However, the structure of the polymer itself is currently unknown, yet critical to a full understanding of the polymerisation mechanism and application to ongoing drug development efforts.

Polymerisation of isotopically enriched, methyl-labelled AAT was induced artificially at elevated temperature and followed in real-time by 1D ^{1}H NMR, translational diffusion and ^{1}H, ^{13}C HMQC experiments. This analysis yielded the first high-resolution ^{1}H, ^{13}C spectrum of AAT polymers. These spectra overlaid closely with a proteolytically cleaved, monomeric form of the protein, from which we could obtain and transfer methyl resonance assignments. As it is not certain that recombinant samples accurately recapitulate what is found within patients, we purified pathological polymers from liver explant for comparison. These were fractionated into varying polymer chain lengths for characterisation by ^{1}H NMR. Using a methyl-selective Ernst angle excitation to improve sensitivity, we compared the high-field methyl resonances of ex vivo polymers with the artificial polymer standards. These showed resonance equivalence that could only be explained by a mechanism in which there is full insertion of the RCL and intermolecular domain swap of the C-terminus of the molecule. These data explain the mechanism of aberrant polymer formation in Z AAT deficiency.

Here, we have used NMR techniques to perform the first direct elucidation of the internal structure of the AAT polymer subunit. Further work is being completed on 2D CH solid state spectrum of the liver-derived AAT polymers at natural isotopic abundance.

The molecular architecture of a 2MDa Plastid-Encoded RNA Polymerase complex in a unicellular photosynthetic eukaryote

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The plastid-encoded RNA polymerase (PEP) complex, an eubacterial-like multisubunit RNA polymerase, is found in all chloroplasts. In the land plant *Arabidopsis thaliana*, PEP primarily regulates the transcription of photosynthetic genes, whereas a separate nuclear-encoded phage-type polymerase is responsible for the transcription of the other chloroplast genes. Conversely, in the unicellular green alga *Chlamydomonas reinhardtii*, PEP transcribes all chloroplast genes. Despite this essential function, the molecular components and architecture of the PEP complex in this alga remain elusive.

Here, we developed an affinity purification method that enabled us to isolate the Chlamydomonas PEP complex in high yield and purity. Our findings reveal that the Chlamydomonas PEP is a large complex of about 2 MDa —twofold larger than its land plant counterpart— and includes 12 additional nuclear-encoded subunits, beyond the anticipated chloroplast-encoded bacterial-like core subunits. We named these newly identified PEP subunits PEPS1-12. We subjected the purified complex to single particle analysis and obtained a global resolution of 2.7 Å. Our structural model shows that the PEPSs act in concert to stabilize the subunits forming the catalytic core, giving this complex a singular structure compared to other RNAPs. Furthermore, akin to recent findings regarding the land plant PEP-Associated Protein 12, our structure reveals that PEPS12 mirrors the 3D organization and spatial positioning of the omega subunit in eubacterial RNA polymerase, despite lacking sequence similarity to both the omega subunit and PAP12. Using phylogenetic analysis, we also identified PEPS2 and PEPS3 as homologs of the land plant PEP-Associated Protein 1 and 11, respectively. However, we found that many other PEPSs have a scattered distribution across the green lineage and that three of them are restricted to Chlamydomonas.

Overall, our study challenges a previously held assumption regarding the functional evolution of nuclear-encoded PEP subunits, which were thought to be adaptations associated with plant terrestrialization. Instead, it strongly suggests that structural adaptations have shaped the complexity of the PEP within the green lineage via convergent evolution. As such, our findings lay the groundwork for deepening our understanding of the evolutionary adaptations and fundamental principles governing chloroplast gene expression in diverse photosynthetic organisms.

Protein phosphorylation and its impact on structural studies of SLX4- associated nuclease complexes

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All cells in the human body protect themselves from the accumulation of deleterious and often pre-malignant mutations by multiple mechanisms of DNA repair. Failure of DNA repair is associated with increased risk of cancer, neurodegeneration, immune system dysfunction and accelerated ageing, as observed in a number of devastating inherited DNA repair defective diseases. The SLX4 protein is a highly-conserved multi-domain platform protein that provides a molecular scaffold for the assembly of multiple protein complexes required for the maintenance of genome stability. Amongst many other factors, SLX4 binds three structure-selective nucleases (XPF-ERCC1, MUS81-EME1 and SLX1) to form the 'SMX' tri-nuclease complex. SLX4 interactions with its protein partners are regulated by kinases and recent work has described the action of SLX4 and its associated nucleases at the biochemical, genetic and cellular level. However, our understanding of their molecular mechanism remains incomplete due to limited structural information. We wanted to address the mechanistic basis of SMX biology using structural methods, initially focussing on a subcomplex containing the C-terminal region of SLX4 in association with MUS81-EME1 and SLX1, which we refer to as 'SLX4C'. We have simultaneously co-expressed all four SLX4C subunits in insect cells and developed a highly reproducible protocol for the over-expression and purification of the SLX4C complex. The purified SLX4C complex shows endonuclease activity on appropriate DNA substrates. The quantity and quality of this pure material allowed us to identify distinct particles of SLX4C in complex with Holliday junction (HJ) substrates using negative stain electron microscopy (EM) and an initial structural model using Small-Angle Synchrotron X-ray scattering (SAXS) and EM were achieved. The intrinsic flexibility of the SLX4C complex remains a significant problem for its structural analysis, therefore *in vitro* phosphorylation assays have been performed to evaluate the impact on its stability, activity and substrate interactions. Together, this will provide new insight into the structure and mechanism of one of the most important, yet poorly understood DNA repair complexes.

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Disruption of the Mitochondrial Network in a Mouse Model of Huntington's Disease Visualized by In-Tissue Multiscale 3D Electron Microscopy

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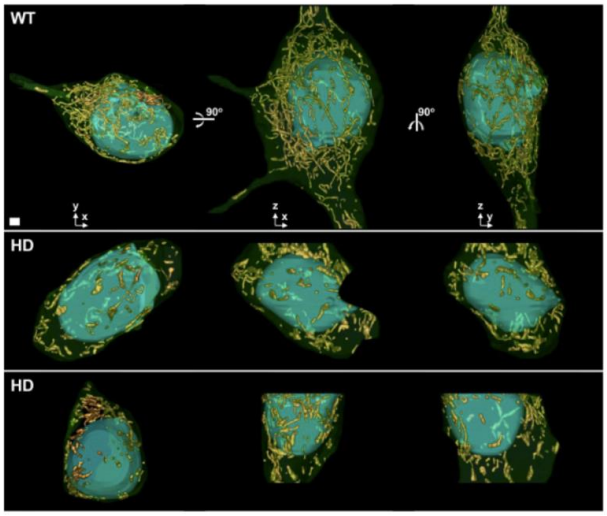
Huntington's disease (HD) is an inherited neurodegenerative disorder caused by an expanded CAG repeat in the coding sequence of huntingtin protein. Initially, it predominantly affects medium-sized spiny neurons (MSSNs) of the corpus striatum. No effective treatment is still available, thus urging the identification of potential therapeutic targets. While evidence of mitochondrial structural alterations in HD exists, previous studies mainly employed 2D approaches and were performed outside the strictly native brain context. In this study, we adopted a novel multiscale approach to conduct a comprehensive 3D in situ structural analysis of mitochondrial disturbances in a mouse model of HD. We investigated MSSNs within brain tissue under optimal structural conditions utilizing state-of-the-art 3D imaging technologies, specifically FIB/SEM for the complete imaging of neuronal somas and Electron Tomography for detailed morphological examination, and image processing-based quantitative analysis. Our findings suggest a disruption of the mitochondrial network towards fragmentation in HD. The network of interlaced, slim and long mitochondria observed in healthy conditions transforms into isolated, swollen and short entities, with internal cristae disorganization, cavities and abnormally large matrix granules.



3D visualization of the FIB/SEM volumes. Three different views of MSSNs are presented in the top (WT), middle (HD) and bottom (HD) rows, respectively. The leftmost views show the volumes with their Z axis running through the depth, a 90° rotation around the horizontal axis results in the views at the central panels, and a subsequent 90° rotation around the vertical axis produces the rightmost views. Segmented mitochondria are depicted with isosurface representation in gold color. Plasma and nuclear membranes are displayed in 85% transparent green and 50% transparent cyan, respectively, allowing visualization of the mitochondria behind the nucleus. The missing wedge in the volume shown in the middle row (central panel) is caused by a technical drift while FIB/SEM acquisition. Bar: 1 µm.

Mapping the interactions between SARS-Cov-2 spike and neuronal cell adhesion protein CNTN1

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SARS-Cov-2 has made a significant impact on our society in recent years. COVID-19, caused by SARS-Cov-2, has not only resulted many deaths globally, but also caused a sub-group of patients to experience neurological symptoms[1]. Recently, a new interaction has been found between the fusion spike protein of SARS-Cov-2 and CNTN1, a cell adhesion molecule found to regulate various processes in the nervous system, such as neurite extension and myelination[2,3]. In our studies, we aim to gain insight into this interaction, using various techniques such as cryo-EM, mass photometry and *in vitro* binding assays, to provide a basis for future therapeutic development against neurological implications of COVID-19.

CNTN-1 is a monomeric protein tethered to the membrane, comprised of 4 fibronectin type-III domains and 6 Ig-like domains, of which 4 form a horseshoe-like structure. We have identified the interaction of ectodomain-spike with CNTN1 to be in the nM range and described the importance of the 6Ig-domains of CNTN1 for this interaction, but challenges remain to narrow down specific areas and domains that may be relevant. We have recently developed nanobodies against CNTN1 derived from llamas, which can be used as tools to further gain insight in how SARS-Cov-2 spike interacts with CNTN1, and cryo-EM analysis of spike-cntn1 complex is ongoing.

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Investigating the physiology and pharmacology of SK channels using a dual biological and structural strategy

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Small conductance calcium-activated potassium (SK / KCa2) channels are selective for K+ ions and are gated by Ca2+ via calmodulin molecules. Three isoforms (SK1-3 ; encoded by KCNN1-3) have been identified, showing different but overlapping tissue expression, notably in the heart and the central nervous system (CNS). For example, SK1 and SK2 proteins display considerable overlap in regions including cortex and hippocampus while SK3 expression is higher in the monoaminergic cell regions.

SK channels are known to play an important role in neuronal excitability by modulating the firing rate and firing pattern of neurons. As a result of their physiological roles and distribution, they represent potential targets for the treatment of disorders such as schizophrenia, depression, Alzheimer's and Parkinson's disease or atrial fibrillation. Blockers of these channels such as apamin or UCL-1684 exist but can't be used as a therapeutical tool due to their narrow therapeutic window or their lack of selectivity. Therefore, the development of new non-peptidic blockers combining high affinity and selectivity towards SK2 or SK3 channels is crucial and requires a better knowledge of the structural features essential to the affinity of these ligands.

In this project, we aim to better understand the interaction between SK channels and the archetypical blocker apamin by studying the 3D structures of SK proteins and their activity. From models obtained using AlphaFold, we observed a particular conformation of the S3S4 loop in SK1, 2 and 3, which does not seem to be present in SK4 (or IK – Intermediate conductance potassium channel). Furthermore, in the first three subtypes, we observed the presence of a phenylalanine residue in this loop that appears to be located just outside the channel pore and could play a key role in interaction with apamin. To validate this hypothesis, we generated different mutants of this phenylalanine in SK2 and SK3 channels and studied their activity and sensitivity to apamin and UCL-1684 by coupling molecular docking to in vitro patch-clamp experiments.

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The First Structure of a Ferric-Siderophore Reductase, FHUF from *E. coli* K-12, reveals a novel 2FE-2S Ferredoxin – Cluster Coordination and Hints at Unanticipated Functional Versatility

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Iron is a vital element for life. However, after the Great Oxidation Event, the bioavailability of this element became limited. To overcome iron shortage and to scavenge this essential nutrient, microorganisms use siderophores, secondary metabolites that have some of the highest affinities for ferric iron. The crucial step of iron release from these compounds to be subsequently integrated into cellular components is mediated by Siderophore-Interacting Proteins (SIPs) or Ferric-siderophore reductases (FSRs).

We will present the structure of an FSR for the first time. FhuF from laboratory strain *Escherichia coli* K-12 is the archetypical FSR, known for its atypical 2Fe-2S cluster with the binding motif C-C-X_{10}-C-X_2-C. The 1.9 Å resolution crystallographic structure of FhuF shows it to be the only 2Fe-2S protein known to date with two consecutive cysteines binding different Fe atoms. This novel coordination provides a rationale for the unusual spectroscopic properties of FhuF. Furthermore, FhuF shows an impressive ability to reduce hydroxamate-type siderophores at very high rates when compared to flavin-based SIPs, but like SIPs it appears to use the redox-Bohr effect to achieve catalytic efficiency.

Overall, this work closes the knowledge gap regarding the structural properties of ferric-siderophore reductases and simultaneously opens the door for further understanding of the diverse mechanistic abilities of these proteins in the siderophore recycling pathway.

Unraveling *Deinococcus indicus* arsenic resistance

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Extremophiles are an unexploited reservoir of genetic and metabolic diversity with high potential application for novel biotechnologies [1]. Deinococcus genus is renowned to harbors extremely resistant species to high levels of γ/UV radiation [1]. Deinococcus indicus is a gram-negative bacteria that besides this remarkable resilience to radiation, is endowed with the ability to resist arsenic [2]. Arsenic is a wildly distributed heavy metal with the most prevalent inorganic forms being trivalent arsenite [As(III)] and pentavalent arsenate [As(V)], both being highly toxic affecting water systems Worldwide [3]. Our objective is to characterize the resistance to arsenic by D. indicus at the cellular level and shed light in its mechanism of resistance at the protein level. First we characterize its resistance to As(V) in two culture conditions. We also performed Cryo-stem EDX and reported for the first time the presence of polyphosphate granules in this specie. At the protein level we studied arsenate reductase (ArsC2) a protein that catalyzes the reduction of As(V). In order to complement our studies, the D. indicus ArsC2 was structural characterized, in apo-form and bound to arsenate ArsC2-As, revealing the structural evidence for a catalytic cysteine triple redox system for As(V) reduction.

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Characterization of SARS-CoV2 Nsp1 as metal-dependent DNA and RNA endonuclease

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Coronaviruses are a group of viruses that infect many animals, including humans and have been studied for more than 50 years. Recently, we lived under a pandemic caused by the rapid spread of the Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV2), and the disease was recognized as COVID-19. Originating in Wuhan city, this pandemic has affected over 695 million individuals, and killed over 6.9 million people worldwide. One of the key virulence factors of Coronaviruses is the Non-structural protein 1 (Nsp1), known to suppress the host cell's protein translation machinery by binding to the 40S ribosome, allowing the virus to produce its own proteins. To understand the molecular mechanisms of SARS-CoV2 Nsp1, we have addressed its biochemical and biophysical properties in the presence divalent metals (calcium, magnesium, and manganese). Ours assays indicate that the protein in solution is a monomer, and it binds to both manganese and calcium, with high affinity, inducing a change in its conformation. Surprisingly, our results demonstrate that SARS-CoV2 Nsp1 alone displays metal-dependent endonucleolytic activity towards both RNA and DNA, revealing Nsp1 as a novel nuclease within the coronavirus family. Furthermore, the Nsp1R124A/K125A presents no nuclease activity for RNA, although it retains activity for DNA, suggesting distinct binding sites or affinity for DNA and RNA. Thus, we present evidence that the activities of Nsp1 are modulated by the presence of different metals, which are proposed to play an important role during viral infection. This research contributes significantly to enhance our understanding of Coronavirus mechanisms.

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Hydrogen-Deuterium Exchange coupled with Mass Spectrometry (HDX-MS) to characterize multiple conformational SARS-CoV-2 Spike epitopes recognized by polyclonal antibodies.

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Following the COVID-19 pandemic, the research for the development of new tools to characterize epitopes recognized by antibodies is in even greater demand. Epitope mapping is essential for understanding adaptive immunity and has potential applications for the development of diagnostic tools, therapeutic antibodies, and vaccines. It is particularly important to better understand the immune response following SARS-CoV-2 infection and vaccination, and to facilitate the clinical treatment for people with immune disorder and to combat SARS-CoV-2 variants. Epitope mapping by HDX-MS is now a tool of choice for studying protein interaction, such as antigen-antibody interaction. Although this technique is perfectly suited to characterize the interaction between an antigen and a monoclonal antibody, it does not provide a global view of the immune response, which require the study of the response to polyclonal antibodies. However, epitope mapping using polyclonal antibodies is more challenging since the concentration of antigen-specific antibodies is unknow, the binding occurs at different rates, and they may be cross-reactive. Here, we describe how we address the challenging use of polyclonal antibodies with HDX-MS, in the context of characterizing the immune response of SARS-CoV-2 infected and vaccinated individuals. We show how our data allows to visualize the epitopes recognized by individuals' polyclonal antibodies.

Measurement of binding-induced structural changes in nucleic acids and proteins using switchSENSE®

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Structural changes induced by ligand binding have pivotal influence on the function of proteins and nucleic acids. Consequentially, these structural changes are increasingly exploited in structure-based drug discovery. Successful examples include the antibiotic small molecule ribocil, which exerts its function by causing a change in RNA structure. Another example is the innovative antineoplastic drug rebastinib, which inhibits a conformational switch of its target protein, retaining it in an inactive state. These examples show how the combined characterization of molecular interactions as well as the structural changes they induce can advance the drug discovery process. Here, we present switchSENSE®, an automated biosensor technology, that enables the simultaneous measurement of real-time binding kinetics and the detection of induced structural changes. It is a fluorescence-based technology that employs electrically actuated DNA nanolevers on a sensor chip surface. We demonstrate the feasibility with two different molecule types. In the first example, we show the measurement of conformational changes in a protein induced by the binding of a small molecule. The protein is immobilized to the sensor surface using DNA origami nanostructures and changes in hydrodynamic friction allow conclusions on its conformation. In the second example, we examine structural rearrangements in a DNA aptamer upon small molecule binding. Using fluorescence resonance energy transfer, we can monitor the conformational change of the aptamer and can conclude that binding and restructuring of the aptamer appear as a single process. The presented application examples show how switchSENSE® can be used to comprehensively characterize binding-induced structural dynamics in different molecule types.

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The cryoWriter – a new and reliable way of automated cryo-EM grid preparation

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Compared to X-ray crystallography and NMR spectroscopy, cryogenic electron microscopy (cryo-EM) is still an upcoming technology to resolve protein structures. One of the bottlenecks of cryo-EM is sample preparation. Conventionally, microliter sized samples are applied on a grid, of which >99% is removed by blotting. The blotting step has limited reproducibility and can damage the sample. Here, we present our solution to tackle this challenge, the cryoWriter. The cryoWriter is a robot for automated and reproducible sample preparation in cryo-EM (Fig.1a). Using microcapillaries, the cryoWriter replaces manual pipetting tasks for grid preparation in an automated way. It applies (writes) nanoliter sized samples on a grid and vitrifies them for subsequent cryo-EM analysis. The system has been optimized for single particle analysis. Such particles can be globular, but also membrane, or filamentous proteins. The gripper and glass capillary are at the core of the system and are key to create a flexible, automated liquid-handling system. The gripper automatically transports the transmission electron microscope (TEM) grid to all stations of the preparation workflow: It carries grids from a grid box via the on-board glow discharge unit to the writing platform. After the writing, the gripper plunges the sample into liquid ethane and puts the grid into a cryo-puck, stored for subsequent analysis by cryo-EM. It takes less than 200ms to vitrify the sample after writing. The whole procedure from grid gripping to storing takes less than 2 minutes, whereas the glow discharge step takes up most of this time.

The pipette unit handles loading the sample and writing it to the grid. Inside the pipette there is no air-water interface, leading to more reliable grid writing. It only writes a few nanoliters of sample and the conventional blotting step can be omitted. In addition, the use of pipettes provides the basis for the unprecedented modularity of the cryoWriter. For example, an inline purification step can be performed in the pipette. Importantly, the environment is precisely controlled during the entire process. A newly implemented feature allows for full access to both the relative humidity in the cryoWriter as well as the temperature of the pipette, the sample and the grid holder. As a result, the writing happens at a temperature that can be set close to the dew point. This feature has immensely enhanced the reproducibility of the process resulting in repeatedly well written grids, with tens to partially over 100 grid squares with well vitrified sample. An example of a written grid is shown in Fig.1b.

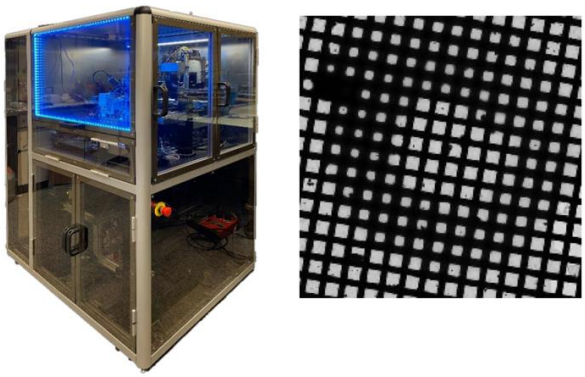

Fig.1. (a) Photograph of the cryoWriter – a fully automated sample preparation robot for cryo-EM. (b) TEM image of a grid written with the cryoWriter with high reproducibility in quality.

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The effect of the CPVT1-associated mutation S616L on the structure, dynamics and dantrolene binding of the human cardiac ryanodine receptor 2

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Ryanodine receptors are the largest presently known ion channels. Found in the membrane of the sarcoplasmic reticulum, their main physiological role is to release Ca2+ ions into the cytoplasm, which triggers a cascade of reactions resulting in muscle contraction [1, 2]. Three isoforms of RyR have been identified in mammals: RyR1 is predominantly expressed in skeletal muscle, RyR2 appears most often in the myocardium, and RyR3 appears in a variety of tissues. Dysfunction of RyR1 causes myopathies [2, 3], of RyR2 causes tachycardias and arrhythmias [2, 4, 5] and of RyR3 has been linked to Alzheimer's disease [6]. Dantrolene, a postsynaptic muscle relaxant that decreases excitation-contraction coupling in muscle cells, is used to treat several of the myopathies associated with RyR1 dysfunction and is potentially useful for treating RyR2 dysfunction [7].

Although it is known that dantrolene binds to RyR1 and RyR2 [7], little is known about the molecular basis of this interaction. We have combined *in vitro* and *in silico* approaches to better characterize the binding of dantrolene to human RyR2 (hRyR2) and the effect of a disease-associated mutation, S616L [5], which is part of the dantrolene binding site (DBS). Our results show that the S616L mutation increases the thermal stability of the hRyR2 NTD without affecting its fold, but does not increase the affinity of dantrolene for the hRyR2 NTD. Molecular dynamics shows that the S616L mutation decreases the overall flexibility of the NTD hRyR2 and alters the dynamics of a loop next to the DBS, which may potentially alter the interdomain interactions between the NTD and the long helical domain, which is thought to bind a number of regulators. This work helps to better understand the effect of the CPVT1-associated S616L mutant on the structure and function of hRyR2 and to characterize the binding of dantrolene to hRyR2 at the molecular level.

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NMR study of the 60 kDa myristoylated Src using selective methionine labelling

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Src kinase is a non-receptor tyrosine kinase which belongs to the Src family of kinases (SFKs) and plays important roles in diverse cellular processes, including proliferation, differentiation, migration and adhesion. Its overexpression or overactivation is associated with many human cancers such as colorectal, breast and prostate cancer.

The domain architecture of the 60 kDa Src is shared among the SFKs and consists of an N- terminal disordered region, including the SH4 and Unique domain (UD), followed by three consecutive folded domains: SH3, SH2 and SH1 (kinase domain). The SH4 becomes myristoylated at its N-terminus and directs Src to the inner layer of the plasma membrane with the help of a cluster of positive charged residues. The UD sequence diverges within the SFKs granting a unique function to each of the members. The SH3 and SH2 domains regulate the activity of the SH1 domain by intramolecular interactions or by acting as hubs for intermolecular complexes directing downstream signaling. Src undergoes complex dynamics at different time and length scales, which is crucial for its regulation. The SH1 domain is highly dynamic and can switch between multiple conformations that are either active or inactive through the interaction of the SH3 and SH2 domains. Thus, to understand the dynamic regulation of Src, the process must be studied with the intact 60 kDa myristoylated protein.

This challenging objective has been achieved by optimizing the expression, myristoylation and purification protocols of selectively ^{13}C-methyl methionine labelling, enabling the study of full length Src in vitro by NMR. Src contains ten methionine residues all of them located in strategic regions of the SH1 domain. Methionine methyl chemical shifts are very sensitive to the local microenvironment and probe the various conformational forms available to Src and their interconversion though chemical exchange. Comparing the isolated kinase domain and full-length Src, we observe clear chemical shift perturbations reporting functionally relevant conformational events associated to the transitions between open and closed states, the effect of phosphorylation, and the internal conformational transitions within the SH1 domains.

The detailed characterization of the alternative conformations of Src provides insight into the response to drugs. Src inhibitors represent promising therapeutic agents for various cancers and other diseases associated with aberrant Src activity. Dasatinib and eCF-506 are two known Src inhibitors that block Src in the open and closed states, respectively. Methyl methionine NMR clearly report on these two different action modes and can provide valuable guidance for the design and optimization of Src-targeted therapeutics as insight on the inhibition mechanism can be obtained in a matter of minutes, instead of having to wait for the resolution of the crystal structure of the Src-drug complexes.

In conclusion, our study demonstrates the potential of methionine NMR for the study of SFK and explores for the first time the structure of the myristoylated full length Src and the interactions responsible for their complex regulation in the isolated protein or the effects of drugs. These findings have important implications for the understanding of Src intramolecular interactions and future development of targeted therapies.

NMR structural studies of flagelliform spider silk proteins

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Spider silk is one of nature's most amazing biomaterials. It is composed of proteins called spidroins that aggregate into fibers with a β-sheet secondary structure. Understanding and reproducing the exact mechanism of silk formation is crucial for obtaining artificial spider silk fibers with native-like properties, which could have applications in regenerative medicine, drug delivery, and other fields. Each spider species can produce up to seven different types of silk. Only dragline (major ampullate) silk has been studied in detail, while other silk types have received less attention. Flagelliform silk is used for making an orb web's capture spiral, and is the most elastic among all. To gain a more complete understanding of the protein structure-mechanical property relationships, we have studied flagelliform spidroins and their conversion into fibres using a biomimetic approach. The formation of spider silk occurs in the spider's silk gland, where changes in environmental conditions facilitate the structural conversion of the spidroins into solid fibers. We employ nuclear magnetic resonance (NMR) to simultaneously assess protein structure and stability at the residue or even atomic level. Thus, we have mimicked the process of spider silk formation in the NMR tube and studied the role of individual silk protein domains. Based on our findings, we have proposed a stepwise mechanism of structural changes required to obtain high-performance fibers. Finally, we use magic angle spinning solid-state NMR to characterize the structure of artificial spider silk fibers obtained using a biomimetic approach. These experiments provide valuable information on how the protein structure is affected by the silk protein sequence, spinning technique, and post-spinning treatments.

Unlocking Snai1's Grip: Insights into how Snai1 recognizes E-box

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The Epithelial-to-Mesenchymal Transition (EMT) is a critical biological process involved in embryonic development, wound healing, and the pathogenesis of cancer, characterized by the transformation of epithelial cells into mesenchymal cells. This transformation involves a loss of cell-cell adhesion, a gain in motility, and significant changes in gene expression. Among the transcription factors driving EMT, Snai1 plays a pivotal role by directly repressing E-cadherin expression, facilitating cell detachment, and promoting invasion and metastasis in cancer. Snai1, a member of the Snai1 transcription factor family, binds to E-box motifs in target gene promoters through its zinc finger (ZF) domains, orchestrating the complex regulatory networks underlying EMT. This study delves into the molecular specifics of Snai1 interaction with the E-cadherin promoter's E-box sequence, employing X-ray crystallography and various biophysical assays to unravel the precise nature of this protein-DNA interaction. Our findings underscore the importance of the ZF2-3 pair in Snai1 for the specific recognition of the E-box motif, setting it apart from other EMT-related transcription factors. By providing a detailed view of the Snai1 binding mechanism, our research offers new avenues for targeting the EMT process in cancer therapy, highlighting the potential of disrupting Snai1-DNA interactions as a strategy to inhibit tumor progression and metastasis. This work not only advances our understanding of the transcriptional regulation of EMT but also lays the groundwork for the development of novel therapeutic interventions.

Keywords : EMT, Snai1, Zinc-Fingers, E-Box, X-ray crystallography

Ensemblify: a user-friendly tool for generating ensembles of intrinsically disordered regions of AlphaFold or user defined models

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AlphaFold has emerged as a groundbreaking innovation in protein structure prediction [1]. This artificial intelligence tool predicts protein structures with remarkable accuracy, achieving atomic-level precision for many proteins, as demonstrated in the Critical Assessment of Structure Prediction (CASP) XIV competition [2]. Despite its immense success, AlphaFold has limitations in predicting the structure of multi-domain and intrinsically disordered proteins [3]. This protein class is better represented not by a single protein structure but by a structural ensemble that describes the accessible conformational space. The current state-of-the-art offers low-confidence metrics for these proteins, hindering our understanding of their structure and function. Here, we present Ensemblify, a user-friendly tool capable of generating conformational ensembles for low-confidence regions of AlphaFold or user defined models by applying a Monte Carlo sampling approach, leveraging the widely used PyRosetta Python interface [4]. Exploration of the protein's conformational space is done by sampling dihedral angles from a tripeptide database built from intrinsically disordered regions. Ensemblify allows the user to define a secondary structure sampling bias and to customize the sampled tripeptide database. The input starting structure can be an AlphaFold model, a user defined model, or a protein sequence. Ensemblify can also restrict sampling to regions with AlphaFold quality metrics below a user-defined threshold. The generated ensembles can optionally be reweighted using SAXS experimental data and analysis of several structural metrics is also available through an interactive graphical dashboard. Ensemblify also provides an HTML interface to generate the input parameters file. All its functionalities can be used either directly as a Command Line Interface tool or as a Python library, for more advanced users, highlighting its modular nature. We have created Ensemblify not only for seasoned structural bioinformaticians but also for any researcher in the biomedical field, with or without programming skills, who wants to better understand the structure-function relationship of their protein of interest.

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Elucidating the conformational landscape and oligomerization pathways of the SARS-CoV-2 Nucleocapsid Protein through explicit modelling and construct diversity.

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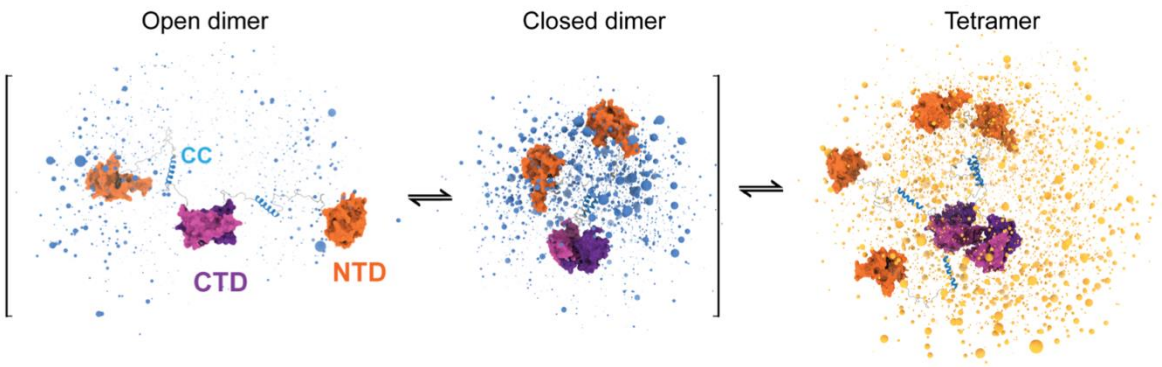
The SARS-CoV-2 N-protein is a highly disordered multidomain protein involved in high-order oligomer formation and liquid-liquid phase separation (LLPS), crucial for virion assembly, RNA packing, and host-cell response modulation. In this study, we investigate the principles governing N-protein-mediated LLPS and its structural ensemble and oligomerization equilibrium. We simultaneously characterize the conformational ensemble and distribution of oligomeric states of N-protein. Our findings reveal a partially structured leucine-rich α-helix within its disordered interdomain linker (IDL), directing coiled-coil interactions necessary for higher-order oligomer formation and influencing protein-mediated LLPS. By integrating Small-angle X-ray scattering, Nuclear Magnetic Resonance spectroscopy, and explicit computational modeling, we elucidate the ensemble structures of N-protein in dynamic, transient, and flexible dimer-tetramer states. The IDL emerged as a key mediator of N-protein's high-order self-assembly, linking dimers via a parallel coiled coil. Our observations highlight dynamic structures and unveil a mechanism for N-protein self-association via an IDL parallel dimer and a second dimerization interface at the C-terminal domain (CTD). We also conducted turbidity assays and confocal microscopy experiments to assess the impact of mutations abolishing the CC site on the stability and kinetics of LLPS. Our results reveal that the CC interdomain helix plays a crucial role in modulating N-protein homotypic phase separation, influencing its stability and kinetics. These functional insights into N-protein's self-assembly mechanism and LLPs shed new light into this crucial player, essential for COVID-19 pathogenicity.



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Structural basis of epitope recognition by anti-alpha-synuclein antibodies MJFR14-6-4-2

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Intraneuronal alpha-synuclein (α-syn) inclusions in the brain are hallmarks of so-called Lewy body diseases - Parkinson's disease and Dementia with Lewy bodies. Lewy bodies are cytoplasmic inclusions, containing mainly aggregated α-syn together with some other proteins including ubiquitin, neurofilament protein, and alpha B crystallin. In its monomeric form, α-syn is predominantly localized in nerve terminals, regulating neuronal transmission and synaptic vesicle trafficking. Monomeric α-syn lacks a well-defined three-dimensional structure and is considered an intrinsically disordered protein. However, in diseased cells α-syn aggregates into oligomeric and fibrillar amyloid species, which can be detected using aggregate-specific antibodies. Here we investigate the aggregate specificity of rabbit monoclonal MJFR14-6-4-2 antibodies, preferentially recognizing aggregated α-syn species. We conclude that partial masking of epitope in unstructured monomer in combination with a high local concentration of epitopes instead of distinct epitope conformation is the main reason for apparent selectivity towards various aggregates, including oligomers, fibrils, and artificial virus-like particle constructs bearing multiple copies of the MJFR14-6-4-2 epitope. Based on the structural insight, we were able to express mutant α-syn that when fibrillated are unable to bind MJFR14-6-4-2. Using these "stealth" fibrils as a tool for seeding cellular α-syn aggregation provides a superior signal/noise ratio for detection of cellular α-syn aggregates by MJFR14-6-4-2 immunocytochemistry. Our data provide a molecular-level understanding of specific recognition of toxic amyloid oligomers, which is critical for the development of inhibitors against synucleinopathies.

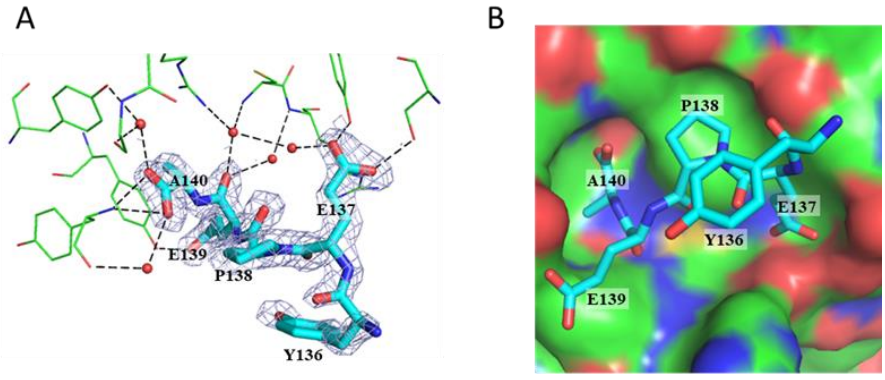

Figure 1. Structure of epitope peptide – Fab fragment complex. (A) Polar interactions between peptide (thick stick model) and Fab fragment residues (thin sticks). H-bonds are shown as dashed lines. Water molecules involved in bridging interaction between Fab fragment and peptide are shown as spheres. (B) Interactions of the peptide with the Fab fragment, are shown as an accessible surface model. Atom colours in both panels – carbon-green (Fab) or magenta (peptide), oxygen -red, nitrogen – blue.

Structural characterization of p97 in complex with the SPRTN protease

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DNA-protein crosslink (DPC) is a type of DNA lesion in which a protein becomes irreversibly covalently bound to DNA when exposed to endogenous or exogenous crosslink inducers. DPCs are common DNA lesions that represent a physical blockage to all DNA transactions: replication, transcription, recombination and repair. If not repaired, DPCs cause genomic instability and adverse phenotypes in humans including premature ageing, neurodegeneration and cancer. Mammalian protease SPRTN initiates the removal of DPCs through proteolytic digestion of cross-linked proteins. Molecular mechanisms and structural knowledge behind the protease-mediated DNA-protein crosslink repair (DPCR) is still lacking. Data from yeast indicate that SPRTN might work in concert with the ATP-dependent AAA family segregase p97, another essential protein linked to DPCR.

Our aim is to characterize the molecular interactions between SPRTN and p97 *in vitro* and to determine the 3D structure of the complex using cryo-EM. To test whether 200 kV cryo-TEM is sufficient, we first after extensive sequence and sample preparation optimization,determined the 3D structure of the wild-type p97 hexamer and its conformationally more rigid mutant. To our knowledge, this is the first time that the structure of p97 has been determined using a 200 kV cryo-electron microscope. We will also describe our attempts to determine the 3D structure of the p97:SPRTN complex, as it is very challenging to obtain a stable complex due to the relatively low affinity between the two components and the intrinsic structural disorder and flexibility of SPRTN.

Structural plasticity of the Notch1-Jagged1 interaction

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Keywords: cell signaling, development, Notch1, Jagged1, protein interactions

One of the most extensively studied regulators of cell differentiation is the Notch signaling pathway. It plays a critical role in controlling cellular fate by restricting equipotent cells and inducing differentiation among adjacent cells within distinct cell populations. Intercellular signaling of the Notch1 receptor is triggered by the Jagged1 ligand through interactions of the 40 and 19-domain extracellular segments of each protein, respectively. Previously, our group uncovered some low-resolution insights into the Notch1-Jagged1 interaction using cross-linking mass spectrometry (XL-MS), revealing the remarkable flexibility of the interaction. However, we still lacked high-resolution structural details. Our objective is to acquire detailed information regarding the interaction between Notch1 and Jagged1, focusing on high-resolution structural analysis. We also aim to investigate the structural plasticity of this interaction in response to changes in the intermembrane distance and, thereby, provide insights into the signaling pathway mechanism. Our strategy involves producing high-quality protein expressed in HEK293 cells and crystallizing the relevant extracellular domains involved in the interaction. Additionally, we plan to analyze trans interactions dependent on membrane distance by cryo-electron microscopy, using the full ectodomain of mouse Notch1 and Jagged1 conjugated on liposomes.

Resolving the structure of sub-80 kDa prolyl oligopeptidase using a CryoEM based biophysical techniques

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University of Nottingham

Chagas disease, caused by the protozoan parasite Trypanosoma cruzi, remains a significant health concern in Latin America. A potential breakthrough in Chagas disease vaccine development lies in targeting Trypanosoma cruzi 80kDa prolyl oligopeptidase (TcPOP), a validated antigen. In this study, TcPOP was expressed in Escherichia coli, purified, and utilized as an immunogen in mice to investigate the resulting immune response.
Recombinant TcPOP was successfully purified to homogeneity and analysed using SEC-MALS and SEC-SAXS. Secondly, the purified TcPOP was used to immunise mice in conjugation with an adjuvant, eliciting a robust immune response. Monoclonal and polyclonal antibodies were purified from immunized mice using hybridoma technology and assessed for their reactivity against TcPOP and exhibited a strong and specific response confirmed using Enzyme-Linked Immunosorbent Assay (ELISA), Biolayer Interferometry (BLI) and Mass photometry. Despite sub-80 kDa size of TcPOP, the antigen was employed on cryoEM grids to be screened under the cryoTEM.
I collected data on Krios 300 KeV and after processing the dataset I was able to resolve the structure of apo-Tc80 at 3.5-4Å resolution. Interesting, I found that the structure exhibits in multiple conformation specifically closed, and open states, therefore, I was able to deduce both the conformation at ~ 4Å resolution and also able determine the secondary structure elements in the electron density. This outcome demonstrated the structural variability and dynamics of not just TcPOP but the family of prolyl oligopeptidases, and the immunogenicity of TcPOP and its potential as a vaccine candidate. Mice immunization with Tc80 yielded promising results.
Future research endeavours will focus on utilizing the information from the TcPOP-mAb complex, to later pin down the epitope that could be crucial for antigen recognition and parasite invasion. These structural insights will provide valuable information for rational vaccine design and therapeutic development against Chagas disease.

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Unveiling O2 damage on Mo/W Formate dehydrogenases and their innate protection mechanism

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The reversible interconversion of CO2 into formate by Mo/W-Formate dehydrogenases (Fdhs) placed these enzymes on the spotlight, probing a promising route not only for green-house gas sequestration but also a sustainable way to produce fuel. Formic acid is a safe option for hydrogen storage/delivery (53g H2/L) in cell power applications [1]. W-FdhAB is a periplasmic heterodimer and the main responsible for CO2 reduction in *D. vulgaris (Dv)* [2]. It comprises two pyranopterin cofactors in the W active site (bisMGD, selenocysteine and a sulfido ligand) and four [4Fe-4S] clusters responsible for electron transfer.

Due to its robustness and high catalytic activity, *Dv*FdhAB is a suitable model for biocatalytic applications for CO2 reduction [3, 4]. However, it is known that metal-dependent Fdhs are oxygen sensitive and easily inactivated when exposed to O2, thus hampering their productive use as a biocatalyst for industrial CO2 reduction to formate. Nonetheless, the exact chemical and structural consequences of O2 damage are unknown but crucial to help devise a protection mechanism and optimize the biocatalyst. Our recent study [5], combining biochemical, spectroscopic, and structural studies of *Dv*FdhAB, when exposed to oxygen, reveals that O2 inactivation is promoted by the presence of either substrate and involves forming a new active site species, reproducibly captured in the crystal structures, where the SeCys ligand is displaced from tungsten coordination and replaced by a dioxygen or peroxide molecule. Furthermore, these results prove that oxidative inactivation does not require reduction of the metal, as widely assumed, as it can also occur in the oxidized state in the presence of CO2 [5].

*Dv*FdhAB is considerably more oxygen-tolerant than other Fdhs and can be purified aerobically in the absence of substrates [3]. In fact, the formation of a conserved disulfide bond, uncovered by our team [6], reduces enzyme activity and protects it from oxidative inactivation. *Dv*FdhAB can protect itself from transient O2 damage when exposed to physiological concentrations of formate (low µM). Our structural studies disclosed the allosteric mechanism responsible for transducing the signal from the surface exposed disulfide bond to the deeply buried active site [6].

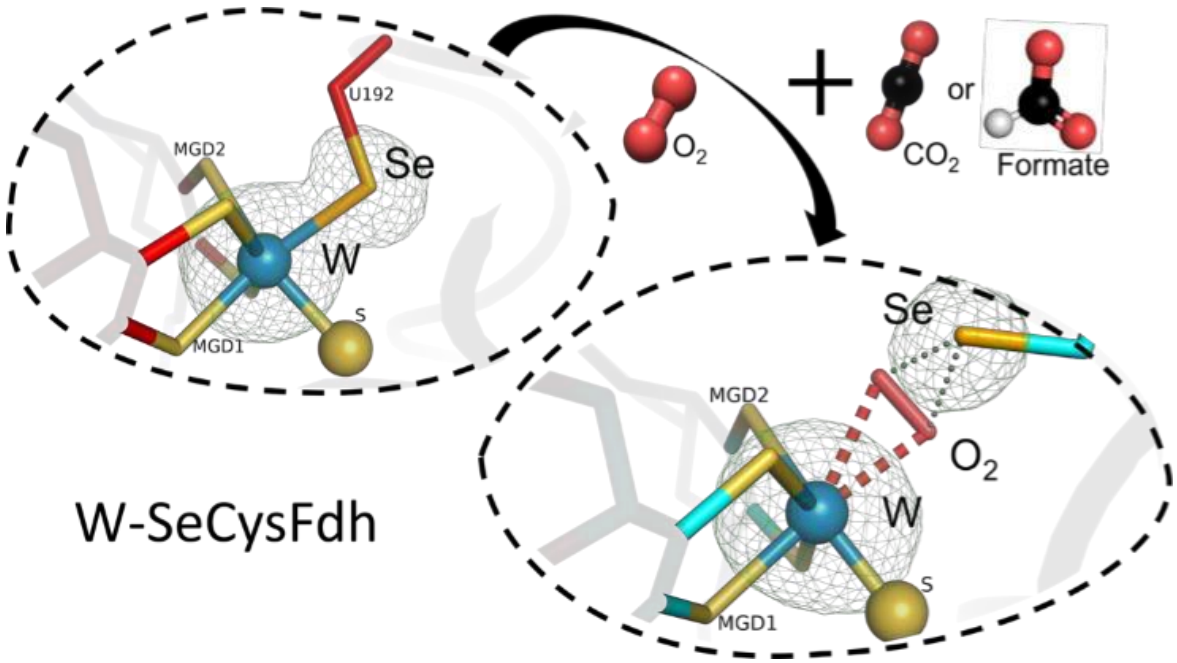

Fig 1. Displacement of the catalytic SeCys in W-FdhAB active site, induced by co-exposure to O_2 and either substrate (formate or CO_2). Anomalous difference map contoured at 5σ (green mesh) [5].

Acknowledgments: This work is financed by national funds from FCT - Fundação para a Ciência e a Tecnologia, I.P., through fellowships. 2023.00286.BD (G.V.-A.), in the scope of the project PTDC/BII-BBF/2050/2020, and UIDP/04378/2020 and UIDB/04378/2020 of the Research Unit on Applied Molecular Biosciences - UCIBIO and LA/P/0140/2020 of the Associate Laboratory Institute for Health and Bioeconomy - i4HB.

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The Regulatory Cortactin-WIP complex: Structure and pharmaceutical implications

Chana G. Sokolik[1], Shams Twafra[2], Hava Gil-Henn[2], **Jordan H. Chill**[1]

Regulatory complexes are by nature of intermediate affinity and tend to challenge structure determination by crystallography or cryo-EM methods, whereas solution NMR is well-suited to handle such structures. A case in point is the complex formed between the C-terminal SH3 domain of cortactin (CortSH3), responsible for actin polymerization and a biomarker for invasive cancers [1], and WASp-interacting protein (WIP), a cellular multitasker regulating actin cytoskeleton remodelling and a 'hub' of protein–protein interactions impacting health and disease [2,3]. While this binding event was previously mapped to the proline rich region of WIP [4], to date no structural evidence has supported this hypothesis. Employing NMR, we have identified the WIP cortactin binding motif and elucidated the structure of the WIP/cortactin complex. Strikingly, WIP binds the cortactin N-terminal SH3 domain in a combined canonical/non-canonical mode that gives rise to a 'fuzzy' complex of intermediate affinity (Figure 1). By scanning mutagenesis, we demonstrate the contribution of the various molecular determinants to the binding energy. This molecular view of the CortSH3/WIP interaction has recently acquired pharmaceutical importance. We have found that the non-receptor tyrosine kinase Pyk2 mediates cortactin involvement in invadopodia formation and the metastatic phase of breast cancer, and a Pyk2-derived peptide inhibits the CortSH3/Pyk2 interaction and greatly reduces formation of metastases [5]. The combined information from both CortSH3/Pyk2 and CortSH3/WIP structures becomes a powerful driving force for developing peptidic inhibitors with pharmaceutical potential for halting the often lethal metastatic progression of breast cancer. Current efforts in developing this research avenue suggest NMR-based methods play an important role in both structural studies and in drug-design efforts.

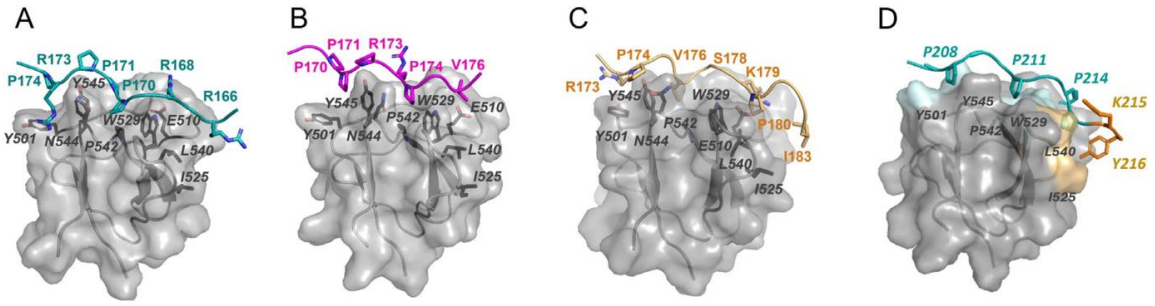

Figure 1. Structures of the cortactin C-terminal SH3 domain (grey, surface representation) with **(A-C)** WIP$^{165\text{-}183}$ in teal/magenta/light orange, **(D)** Pyk2$^{202\text{-}220}$ (in cyan/orange).

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Covalent Binding of Benzensulfonamide Within Active Sites of Human Carbonic Anhydrases

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The CA IX protein, a transmembrane protein, is highly overexpressed in hypoxic solid tumors. It plays a crucial role in cancer cell survival and proliferation by acidifying the tumor microenvironment, thus facilitating invasion and metastasis processes. In this study, we observed a unique covalent binding of an inhibitor within carbonic anhydrase isoforms. Two similar compounds were used in attempts to crystallize with tumor-associated human carbonic anhydrase IX and the widespread off-target isoform II. Although the compounds were nearly identical, their binding modes differed significantly. One of the compounds experienced a change in its tail moiety - the acetic acid fragment separated from the molecule, leading the molecule to form a covalent bond via the His64 amino group. The covalent bond between the compounds and the protein was demonstrated through mass spectrometry and X-ray crystallography. The obtained data clearly demonstrates the presence of a covalent bond and offers valuable insights for future ligand design.

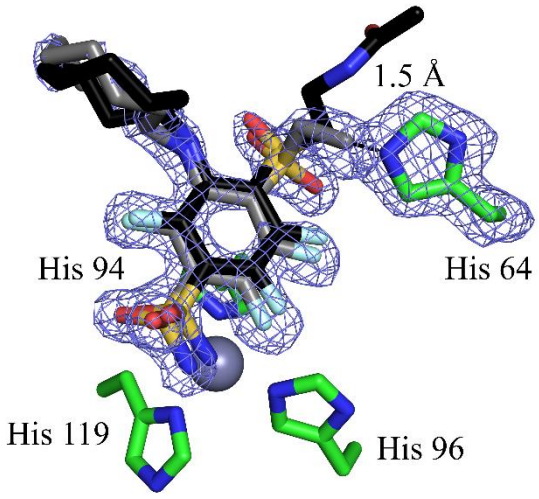

Figure 1. Comparison of binding modes of compound A (gray carbons) within the active site of hCA II and compound B (black carbons) within the active site of hCA IX. The zinc ion is shown as a gray sphere and its coordinating histidines (His94, 96, and 119) are shown in green. Covalent bond is indicated, and the responsible residue is also shown. For clarity, F_o-F_c OMIT electron density is shown only for the compound A and His64 and contoured at 3σ. The figure was prepared using PyMOL (DeLano The PyMOL Molecular Graphics System San Carlos, CA, USA, DeLano Scientific).

Structural insights into MraZ conformation and DNA Binding

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Cell division is a fundamental cellular process and the basis of microorganism survival. Thus, the molecular machinery involved in cell division is exposed to a strong evolutive pressure that in bacteria results in highly conserved gene division clusters and regulatory proteins. The *division cell wall (dcw)* gene cluster of many bacteria is regulated by a sequence-specific DNA-binding protein named MraZ. Whereas the promotor recognition sequence of MraZ is well-known in most organisms, the molecular determinants driving MraZ binding to DNA remain to be elucidated. Here, we study MraZ protein of the model organism *Mycoplasma genitalium* to obtain structural insights into its mode of action and mechanism of DNA interaction. We have solved MraZ structure alone and in complex with DNA. Upon DNA binding, MraZ undergoes a conformational change leading to the disruption of its closed ring-shaped structure, transforming into a helical conformation. Remarkably, MraZ is the unique member of the SpoVT/AbrB DNA-binding proteins that oligomerizes and forms a protein complex with multiple DNA-binding sites. To fully understand the biological role of MraZ, we measured the DNA-binding affinities of MraZ *wild type* and mutants lacking DNA binding capacity or stable oligomeric state. Overall, we expect that exploring MraZ oligomerization and DNA binding may not only shed light into MraZ mechanistic details but also help us describe the biological need that triggered the origin of a supramolecular complex with multiple DNA-binding sites.

Structural and Function of a Human Molecular Machine: The RuvBL1/RuvBL2 Complex and their Role in PAQosome

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RuvBL1 and RuvBL2 are highly conserved eukaryotic proteins of the AAA+ family of ATPases. They are known to participate in large molecular complexes such as INO80, TIP60 or R2TP, and have been associated with many cellular functions including chromatin remodelling, transcription, DNA damage repair and apoptosis, suggesting that they could act as a scaffolding complex. In addition, a link between RuvBLs and cancer was established as these proteins interact with transcription regulators known to be involved in oncogenic pathways and are overexpressed in many cancer types. However, little is known about the disease-related molecular mechanisms. This PhD project aimed to identify partners and client proteins of RuvBL1/RuvBL2 in the context of large cellular complexes. We focused on understanding how these complexes assemble and their overall architecture, all with an eye towards exploring their potential for drug development. To achieve this, we implemented an innovative enzyme-catalyzed proximity labelling method, termed TurboID. This method enabled us to explore the interactome of RuvBLs within their natural cellular environment, identifying their associated partners and client proteins. We successfully detected certain proteins known to be part of the macromolecular complex PAQosome and involved in the snoRNPs biogenesis, with statistically relevant confidence. To validate their interactions with RuvBLs, we employed Surface Plasmon Resonance (SPR) using our new ly developed Extract2Chip methodology. This approach avoids the need for protein purification by utilizing cell lysates enriched in biotinylated targets, allowing us to kinetically characterize their interactions with protein partners or small molecules through SPR. In addition, we conducted comprehensive biochemical-, biophysical- and structural analyses. This approach enabled us to correlate 3D-structures with functional studies, shedding light on the assembly mechanisms and functions of these molecular machines.

Structure of the Ecumicin-bound anti-Tuberculosis drug target ClpC1 provides insights on antibiotic mechanism of action

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The unfoldase ClpC1 is one of the most interesting drug targets against tuberculosis. This AAA+ unfoldase works in cooperation with the ClpP1P2 protease and is the target of four natural product antibiotics: cyclomarin, ecumicin, lassomycin, and rufomycin. Due to their sterilising activity these molecules are promising starting points for anti-tuberculosis drug development. Nevertheless, their mechanism of action remains largely unknown. Taking advantage of a middle domain mutant, we determined the first structure of *Mycobacterium tuberculosis* ClpC1 in its apo, cyclomarin-, and ecumicin-bound states using the Instruct cryo-EM infrastructures at CNB-CSIC Madrid. The obtained structure displays features observed in other members of the AAA+ family and provides a map for further drug development. While the apo and cyclomarin-bound structures are indistinguishable and have N-terminal domains that are invisible in their respective EM maps, around half of the ecumicin-bound ClpC1 particles display three of their six N- terminal domains in an extended conformation. Our structural observations suggest a mechanism where ecumicin functions by mimicking substrate binding, leading to ATPase activation and changes in protein degradation profile.

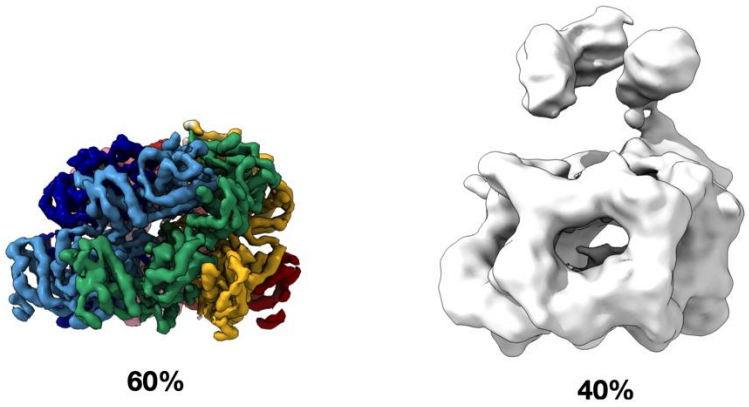

Figure 1. Class 1 (rainbow) and 2 (white) maps of Ecumicin bound ClpC1 with the percentage of particles found in each state. *(Weinhäupl, Katharina et al. "Structure of the drug target ClpC1 unfoldase in action provides insights on antibiotic mechanism of action." The Journal of biological chemistry vol. 298,11 (2022): 102553. doi:10.1016/ j.jbc.2022.102553)*

Targeting the Bacterial c-di-AMP Signaling Pathway: Screening for Small-Molecule Modulators of CdaA

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The diadenylate cyclase CdaA is an important enzyme responsible for the biosynthesis of the bacterial second messenger c-di-AMP. This molecule plays a critical role in regulating a variety of bacterial processes, including cell wall homeostasis, osmotic pressure, K^+ machinery, DNA integrity, fatty acid synthesis, biofilm formation, sporulation and central metabolism. In order to identify small-molecule modulators of the full-length CdaA activity, we established a robust 384- well high-throughput screen (Z'=0.66) using a KinaseGlo^{TM} Luminescent Kinase Assay. A structurally diverse 10,000 compound small-molecule library (Chembridge DiverSet library) was screened by monitoring ATP consumption by CdaA-CdaR liposomes in the presence and absence of screening compounds, and 18 hits were identified, showcasing both inhibitory and activating effects on CdaA activity. The selected compounds were retested by a secondary high- performance liquid chromatography (HPLC) assay and then tested against the isolated DAC domain. Compounds #11 and #7 demonstrated the ability to inhibit the production of c-di-AMP in a concentration-dependent manner both in the full-length protein and isolated catalytic DAC domain. On the other hand, compound #3 increased c-di-AMP synthesis in the full-length complex, but not in the isolated DAC domain, suggesting a unique mechanism of action. Preliminary data from a chemical expansion around selected compounds indicates the possibility of further optimization to increase the potency of these compounds. X-ray crystallography is currently being performed to determine the structures of CdaA in complex with the selected compounds, providing atomic-level insights into the molecular interactions and binding modes. The findings of our study will provide valuable insights into the identification of CdaA activity modulators for the purpose of interrogating c-di-AMP-mediated processes in the bacterial cell and with the potential for the development of novel antibacterial strategies.

Acknowledgements: This work is supported by “La Caixa” Foundation (LCF/PR/HR22/52420004). We acknowledge the support of the i3S BioSciences Screening scientific platform.

Tau liquid-liquid phase separation is regulated by the calcium-binding S100B chaperone

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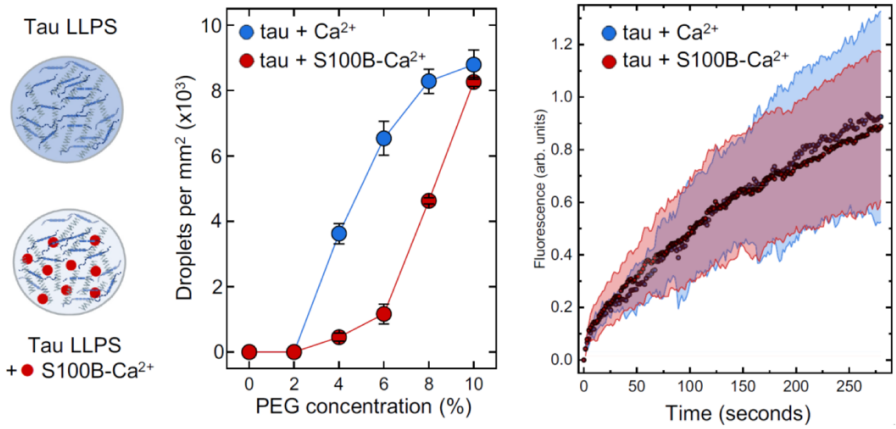
The phenomenon of liquid-liquid phase separation (LLPS) involving tau is increasingly acknowledged as a contributory process in the onset of tau aggregation and the generation of pathogenic conformers within Alzheimer's disease (AD). Neuroinflammation accompanies tau pathology, with late-stage astrocyte-released alarmin exacerbating the condition, while early inflammatory responses encompass protective functions. This applies to the Ca^{2+}-binding protein S100B, which we recently implicated as a proteostasis-regulator that inhibits amyloid-beta (Cristovão et al 2018 Sci Adv) and tau aggregation/seeding (Moreira et al 2021 Nat Commun). These findings suggest a broad holdase-type chaperone function for S100B in counteracting the malformation of protein structures. Our study aims to elucidate S100B's role in tau LLPS. PEG-induced tau LLPS was followed by spectroscopy measurements of light absorbance (400nm) and tau fluorescently labelled. Co-localization of S100B within tau droplets was achieved using fluorescence-labelled proteins and FLIM-FRET. Evaluation of droplet fluidic characteristics encompassed fluorescence recovery after photobleaching (FRAP) and observation of fusion events. Phase diagrams indicate significant suppression of tau droplet formation by Ca^{2+}-S100B, preserving droplet liquid properties. Introduction of Ca^{2+} to PEG-induced LLPS with apo-S100B promptly reduces tau droplet levels, highlighting the dynamic, calcium-triggered nature of Ca^{2+}-S100B's action. Likewise, S100B effectively halts PEG-free Zn^{2+}-induced tau LLPS due to its combined Zn^{2+}-buffering and tau-interaction capabilities. Our results establish S100B as a calcium-dependent suppressor of tau LLPS. Collectively, these findings suggests that S100B, functioning as a chaperone, regulates the formation of various pathological conformers and phase-separated systems, strengthening its pivotal role as a proteostasis regulator in early neurodegeneration. Acknowledgments: EU for funding Twinning Grant EU-TWIN2PIPSA/GA101079147, FCT/MCTES (Portugal) for funding UIDB/04046/2020 and UID/MULTI/04046/2020 (BioISI) and PhD grant 2020.06443.BD (GGM) and Agilebio for funding LabCollector Scientific Award 2021 (CMG).



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Deciphering Protein Misfolding in Inborn Errors of Metabolism: Insights from Structural Biology

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Inborn Errors of Metabolism (IEM) belong to the group of rare diseases and are associated to defects in enzymes involved in different metabolic pathways. Although individually rare, they are collectively quite numerous and affect millions of people worldwide. The vast majority arise from single gene mutations that disrupt cellular metabolism, frequently resulting from faulty protein folding. While the genomic era has facilitated the identification of more cases, establishing a clear correlation between genotype and phenotype remains challenging.

To contribute to a better molecular understanding of IEM in the last years we have been investigating disease-related variants combining biochemical, biophysical and structural methods to establish the effects of point mutations on protein folding, stability and function. Here, we highlight our recent investigations in Multiple Acyl-CoA Dehydrogenase Deficiency (MADD) and Glutaric Aciduria-type I (GA-I).

MADD results from deficiencies in the alpha or beta subunit of electron transfer flavoprotein (ETF), or in the ETF:ubiquinone oxidoreductase (ETF:QO), and is associated to impaired mitochondria beta oxidation. Our biochemical and structural analyses of ETF:QO variants offer a molecular basis for understanding the reduced enzyme activity observed in patients [1, 2].

GA-I is a neurometabolic disorder caused by deficiency of glutaryl-CoA dehydrogenase (GCDH). By conducting studies on two disease variants (GCDH-p.Arg227Pro and -p.Val400Met), we elucidated genotype-phenotype correlations, revealing that both variants maintain the overall protein fold while exhibiting distinct conformational stabilities [3]. Furthermore, compromised enzymatic activity on both proteins depend on different features [3]. Additionally, we have provided a molecular explanation for the potential efficacy of riboflavin supplementation in GA-I patients [4].

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Protein Production facility at the Centre of Molecular Structure

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The Protein Production core facility (CF) at the Centre of Molecular Structure (CMS) offers comprehensive protein production services, covering every step from DNA to the purified protein. These include gene cloning into expression vectors, site-directed mutagenesis, and heterologous expression in *Escherichia coli* expression systems, followed by protein purification.

Our cloning services include both traditional cloning using restriction enzymes and restriction-free (RF) methodologies. You can provide us with your templates, or we can order them from external companies. Subsequently, we can deliver and test a number of our plasmids. Furthermore, we perform small-scale expression and solubility tests using various *E. coli* strains under different conditions. Finally, we offer large-scale production and purification of target proteins.

In protein purification, we employ a range of affinity techniques such as Strep-Tactin XT, and immobilized metal chelate affinity chromatography (IMAC), both on FPLC or in gravity flow setups. Chromatography techniques include ion-exchange chromatography and size exclusion chromatography using Superdex columns (75 or 200, 10/300 increase, or HiLoad 16/600). You can request customization to our standardized protocols or provide your established protocols or implementation.

Moreover, we are expanding our services by introducing eukaryotic production during this year. We will provide protein production using human embryonic kidney cells (HEK) and Sf9 insect cells as an alternative to prokaryotic expression in *E. coli*.

The Biocev Protein Production core facility is a part of CMS operated by the Institute of Biotechnology, Czech Academy of Sciences. The Centre of Molecular Structure is supported by: Czech Infrastructure for Integrative Structural Biology (CIISB), Instruct-CZ Centre of Instruct-ERIC EU consortium, funded by MEYS CR infrastructure project LM2023042), and European Regional Development Fund-Project „UP CIISB" (No. CZ.02.1.01/0.0/0.0/18_046/0015974).

Centre of Molecular Structure in BIOCEV – State of art structural biology facillity

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The Centre of Molecular Structure offers wide range of methods of structural biology. It operates in BIOCEV as part of Institute of Biotechnology, AS CR. CMS consists of facilities devoted to crystallization of macromolecules, X-ray diffraction and scattering, biophysical characterization, advanced mass spectrometry, and infrared and fluorescence spectroscopy. The open access services are provided via the Czech Infrastructure for Integrative Structural Biology (CIISB) and Instruct-ERIC. The essential core equipment consists of 15T-Solarix XR FT-ICR (Bruker Daltonics) for mass spectrometry, D8 Venture (Bruker) diffractometer with MetalJet X-ray source (Excillum), crystallization hotel RI-1000 equipped with SONICC (Formulatrix), Prometheus and two Monoliths (Nanotemper) for protein characterization and affinity measurements, and Chirascan for circular dichroism measurements, MP Two (Refeyn) for single molecule mass estimates. Recently, this instrument portfolio was extended by SAXS Point 2.0 (Anton Paar) with MetalJet X-ray source (Excillum) for small angle X-ray scattering studies of biomolecules in solution, MALDI TOF mass spectrometer, excimer laser for induced protein modification, and room for spectroscopy with Fourier-transformed Infrared (FTIR) spectrometer and a FLS1000 spectrofluorometer. CMS was also extended with Protein Production facility.

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Heterotypic Interactions Drive Anti-Aggregation Activity of Nanobodies Against S100B on Tau Aggregation

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Alzheimer's disease (AD) is a neurodegenerative disorder characterized by the World Health Organization as a public health priority. The pathology is characterized by the aggregation of amyloid-β (Aβ) and Tau protein into amyloid-β plaques and neurofibrillary tangles, respectively. Neuroinflammation is also implicated in AD and is responsible for the secretion of alarmins, which include the S100B protein. S100B is highly studied in the context of AD, and it's known for its dual function as a detrimental pro-inflammatory mediator and a beneficial anti-aggregation chaperone over Aβ and tau, making it an amenable drug target. Since there is still no cure for AD and is highly attractive to target chaperones with an already reported anti-aggregation activity, a library of single-domain antibodies (or nanobodies) targeting S100B was developed to potentiate S100B chaperone activity and modulate tau aggregation. Here, we employed ThT-monitored kinetics of heparin-induced K18 aggregation to study the effect of nanobodies alone and in combination with S100B on K18 aggregation. Moreover, we performed mechanistic analysis to determine which step of the aggregation reaction are targeted by the nanobodies. Several nanobodies potentiated S100B inhibitory effect over K18 (Tau244-372), possibly by harnessing S100B in a more competent conformation to bind K18. Surprisingly, control experiments revealed that some nanobodies alone significantly inhibit K18 aggregation even at sub-stoichiometric ratios. This striking observation is discussed in the context of possible heterotypic interactions between the nanobody CDR3 region and Tau/K18. Further, mechanistic analysis demonstrates that different nanobodies target multiple steps of K18 fibrillation. These findings uncover the therapeutic potential of anti-S100B nanobodies, which can be used as modulators of K18 aggregation or as activators of S100B chaperone activity.

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Exploring Early Extracellular Disease Mechanisms: Cross-Interactions Between Tau and Amyloid β in Alzheimer's Disease

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Since the underlying mechanisms of early Alzheimer's Disease are not well understood, we aimed to explore plausible extracellular cross-interactions between Tau and Amyloid beta (Aβ) that could condition disease development, as well as the effect of the early-stage extracellular chaperone S100B in this context. The effect of Aβ on Tau was assessed following the aggregation of a fixed concentration of the Tau AD core (TADC, Tau306-378) by monitoring X-34 fluorescence intensity in the presence of Aβ42 monomers under cofactor-free conditions. To study the effect of Tau over Aβ, the in vitro aggregation of a fixed concentration of Aβ42 monomers in the presence of increasing proportions of TADC and Tau-K18 (Tau244-372) was monitored by ThT fluorescence. Finally, the influence of S100B on TADC aggregation, with and without heparin, was similarly evaluated with the fragment alone and in the presence of Aβ42 monomers. The toxicity of end-point species was assessed by liposome leakage assays. TADC aggregation was accelerated in the presence of Aβ42 in a concentration-dependent manner, and the end-point species present were fully toxic for liposomes. On the contrary, Aβ42 aggregation was inhibited by both TADC and K18. S100B had a dual-behaviour on TADC aggregation, accelerating it at substoichiometric proportions and fully inhibiting it at equimolar proportions, though this inhibition was not complete when heparin was present. Finally, under mixed conditions with Aβ42, the inhibitory effect of S100B over TADC aggregation was lost. In conclusion, Tau and Aβ have opposite effects on each other's in vitro aggregation. S100B, while able to inhibit TADC aggregation and toxicity, showed a concentration-dependent dual-behaviour, accelerating its aggregation at lower proportions. These results highlight the complex interplay of events in early-disease scenarios in Alzheimer's Disease.

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NMR-based investigation of intrinsically disordered regions of modular proteins for tailored drug-design

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Numerous RNA-binding proteins (RBPs) exhibit modular structures containing folded domains and intrinsically disordered regions (IDRs). Investigating the role of these domains and their potential interaction is essential for understanding protein function and developing intervention strategies. The Nucleocapsid protein (N) of SARS-CoV-2 is a pivotal example of RBP. Its complex structure encompasses two folded domains and three IDRs. In particular, the globular N-terminal domain (NTD) is responsible for the viral RNA interaction and the two flanking IDRs play an important synergic role (1). The aim of my PhD project is to design and synthesize molecules able to interfere with the protein function, monitoring the interaction through solution NMR titrations. In particular, taking into account the structural characteristics of the protein, a first peptide was designed to simulate the main interactions driving the viral RNA binding. Then, a series of different peptides with slight modifications were synthesized to discern residues or motifs essential to interact in the protein target site. This collection of peptides has been tested by NMR titrations to identify the sequence displaying the highest affinity with the protein NTD and also with the NTR construct, comprising also the two flanking IDRs. Different NMR experiments were performed to enable the simultaneous observation of globular and disordered regions both with atomic resolution; in particular the interaction was followed through ^{1}H-^{15}N HSQC experiments but also by exploiting the ^{13}C detection, essential to investigate flexible regions. This study is now being improved with the design and synthesis of a peptide-PNA chimera, replacing certain amino acid residues of the parental peptide sequence with four PNA building blocks, selecting four G as nucleobases (2), aiming to better mimic the RNA nature. This promising molecule was tested with both protein constructs in the same experimental conditions. For now, this study yielded two main and clear results: the comparison among the titrations carried out first with the peptides and then with the peptide-PNA chimera has revealed a significantly greater affinity between the protein and the chimera with the respect to the peptide molecules. Additionally, in both cases, the presence of IDRs in the protein NTR construct has shown visibly more pronounced effects in the HSQC spectra compared to the NTD alone, suggesting an important contribution of these flexible regions in the interaction.

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Combining Solid-State NMR with structural and biophysical techniques to develop and characterize potential protein-drug conjugates

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The development of a suitable drug delivery system is a crucial step in drug design, and the conjugation of drugs to natural or synthetic large polymers is often used to prevent their fast clearance, proteolytic degradation, and to improve solubility by reducing the renal excretion1. Human transthyretin (TTR), a physiological tetrameric protein, has already been identified as a possible carrier protein. TTR can be stabilized through the interaction with moleculeslike Tafamidis. The binding of the two compounds is exploited to anchor cytotoxic molecules on the Tafamidis moiety in order to exploit the TTRTafamidis complex in an anticancer treatment (Figure 1). Tafamidis is covalently attached to the cytotoxic molecule through a linker containing an easily hydrolysed ester bond exploitable for the release of the cytotoxic agent to its pharmacological target. A combination of x-ray crystallography, solution NMR and solid-state NMR allowed us to get aglobal insight of the cytotoxic molecule-Tafamidis interaction derivative to the TTR. The results demonstrate the high sensitivity of solid-state NMRto the effects of ligand binding and the small conformational heterogeneities. This technique resulted to be extremely helpful to characterize the interaction of drug candidates with large carrier proteins as a complementary technique to X-ray and solution NMR, as well as a technique on its own when conventional biophysical techniques are not suitable.

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Exploiting relaxation to study structurally heterogeneous proteins with NMR

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CREB-binding protein (CBP) is a transcriptional coactivator involved in the transcription of several human genes as well as in many signalling pathways[1]. More than 60% of the protein's residues are predicted to be disordered, while the others are organized in seven globular domains. So far, characterization of both folded domains and disordered regions of CBP has been carried out by isolating them one by one. Although this approach greatly simplifies the in-vitro application of structural biology techniques such as NMR spectroscopy and X-ray crystallography, it allows for the study of a single domain at a time.

In this work, we propose a different approach that aims to study two contiguous CBP domains with NMR spectroscopy, the CBP-TAZ4 construct. This construct is formed by the zinc-binding domain TAZ2[3]and the flanking ID4 disordered region[4]. The two domains have very different relaxation properties, that reflect their structural features. Here we show that two- and three-dimensional NMR experiments can be tuned to exploit these differences to enhance spectral quality and obtain clean information about the two domains. Moreover, our data show that the presence of ID4 alters the structural features of TAZ2 and vice versa. NMR spectroscopy, and ^{13}C-direct detected NMR in particular, has been proven to provide clean information about highly flexible disordered regions also when part of complex multidomain proteins[2,5]. With this work we further expand the NMR toolbox to address these complex systems.

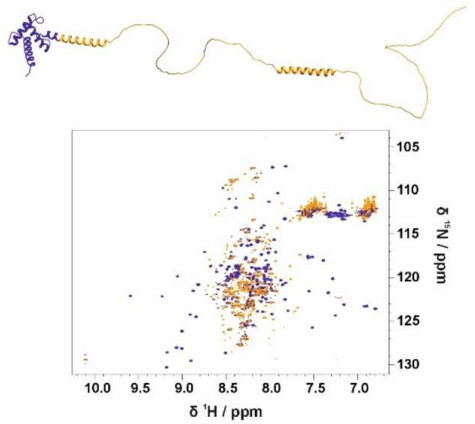

Figure 1. A relaxation-edited ^{1}H-^{15}N HSQC spectrum is reported on the bottom part of the figure. The positive peaks (in blue) belong to the TAZ2 domain, while the negative ones (orange) belong to the ID4 region. The two sets of peaks can be discriminated thanks to the insertion of a relaxation filter in the NMR pulse sequence. A structural model of TAZ4 is reported on top of the figure; the TAZ2 domain is reported in blue while ID4 is reported in orange.

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Specificity of the interaction between Neuraminidase N1 of the avian influenza A virus H1N1 1918 and a2-3 or a2-6 glycan receptors of avian and human cell targets.

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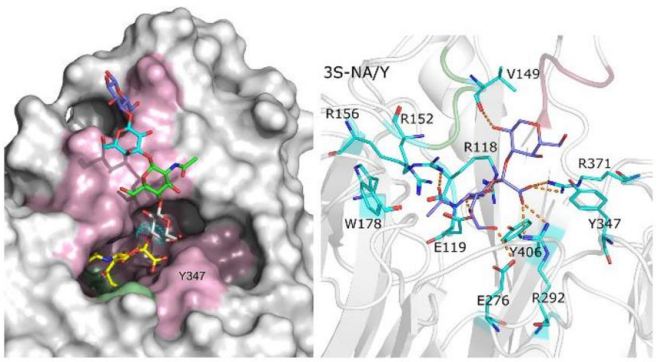
Influenza A virus (IV) is extremely diffused and dangerous in poultry farms and only rarely it infects and is transmitted among humans by aerosol becoming potentially pandemic. IV presents two surface recognition proteins: hemagglutinin (HA) and neuraminidase (NA), the former binds specifically a2-3 (avian) or a2-6 (human) Neu5Ac-Gal terminated glycoconjugates of cell host surface and drives the adhesion of the virus[1]. Neuraminidase hydrolyses the Neu5Ac-Gal linkage removing the Neu5Ac cap of these glycoconjugates. This event is advantageous for the IV life cycle, since it prevents the entrapment of IV by sialylated mucines of the upper respiratory tract[2] or promotes the releases of the newly formed viruses at the end of the replication[3]. Interestingly, an efficient IV life cycle requires a balance between the opposite activities of HA and NA and respect to the population of sialoglycans present in the target host tissues, equilibrium that change for adaptation to a new host species[4]. The specificity of human adapted HA toward a2-6 correlates with its transmissibility between humans by aerosol, while NA presents a less pronounced and known specificity. In our studies we focused on the characterization of the specificity of interaction that allows N1 the H1N1/1918 pandemic IV to bind (and hydrolyse) a2-3 or a2-6 Neu5Ac-Gal. MD simulation reveals multiple molecular details, that help to understand why position 347 (N2 numbering) discriminates the hydrolysis efficiency of N1 toward a2-3 or a2-6 sialyl-capped glycoconjugates. Comparative evolutionary analysis of site 347 suggests that substitutions at NA position 347 occurred during host switches, therefore, position 347 is a marker of host range and adaptive evolution of influenza viruses. Interestingly, few amino acid mutations in the active site of NA (analogously as observed in HA) are required to switch their specificity toward a2-3 or a2-6 Neu5Ac-Gal glycoconjugates, resulting in alteration of the infection efficiency in a selected host. This study will help to design new antiviral strategies[5].



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Isotope labelling of Siglec-7 in HEK293 cells: protein and protein:ligand complex characterisation at atomic levels by NMR spectroscopy

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Siglec-7 is a sialic acid-binding immunoglobulin-like lectin structurally composed by an N-terminal V-set Ig domain linked to two C2-set Ig spacers, a transmembrane region, and a C-terminal tail bearing one immunoreceptor tyrosine-based inhibitory motif (ITIM) and one ITIM-like domain. Siglec-7 preferentially binds glycoconjugates with α(2,8)-linked disialic acids and branched α(2,6)-linked sialic acids, as those found in GD3 and GT1b gangliosides. Mainly expressed by resting and activated natural killer (NK) cells, Siglec-7 is a known negative regulator of NK cell-mediated functions and a promoter of proinflammatory microenvironment, thus making it an attractive target molecule for the development of cancer immunotherapies [1]. We designed and transiently expressed three constructs of Siglec-7: (i) the extracellular domain fused to the Fc fragment of human IgG (Siglec-7_hIgG_Fc), (ii) the extracellular domain (Siglec-7_FED), and (iii) the N-terminal V-set Ig carbohydrate recognition domain (Siglec-7_CRD) in the human HEK293S GnTI- cell line to ensure the appropriate posttranslational processing of Siglec-7, i.e., formation of disulfide bonds and incorporation of N-glycans at the predicted sites. The proteins were analysed by SDS-PAGE in reducing and nonreducing conditions, nanoDSF, DLS, and AUC. Interestingly, the extracellular domain of Siglec-7 was shown to form a stable noncovalent dimer even at low micromolar concentrations - a previously unknown observation with potential implications in our understanding of the ligand binding and signal transduction mechanisms of Siglec-7. The biophysical characterisation of Siglec-7:ligand complexes was performed by sedimentation velocity AUC and STD NMR experiments, where the functionality of the three variants of Siglec-7 was confirmed. We, therefore, decided to proceed towards the structural description of Siglec-7_CRD on its own and complexed with ligands of interest by protein-based NMR experiments. We generated a doxycycline-inducible HEK293S stable cell line for Siglec-7_CRD using the piggyBac system [2] and adapted it to growth as an adherent monolayer to allow for the expression of Siglec-7 in the specialised CGM-6000 isotopically labelled cell culture medium. Determination of the degree of isotopic substitution was performed by MS and confirmed the successful preparation of 15N/13C double labelled Siglec-7_CRD suitable for further protein-based NMR experiments.

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Unravelling Molecular Mechanisms of Leukodystrophies Associated to gGutamyl-tRNA Synthetases (EARS2) Disease Variants

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Mitochondrial diseases (MD) are the most common group of inherited metabolic disorders characterized by dysfunctional mitochondria due to defects in oxidative phosphorylation. In recent years the interest around mitochondrial aminoacyl-tRNA synthetases (mt-aaRS), a family of enzymes that ensure proper translation by adding the appropriate amino acid into the correct tRNA molecule, is emerging due to the increasing number of MD patients with mutations on these enzymes, including in Portugal. Although the knowledge around these disorders has been increasing, there are still open questions including the fact that the characterization of human mt-aaRS at the protein structural, conformational and functional levels is scarce.

Aiming at contributing to the clarification of the molecular mechanism of leukodystrophies associated to mt-aaRS defects here we present our recent work on glutamyl-tRNA synthetase (EARS2) wild-type and three disease variants. We have used protein biochemical and biophysical methodologies to make for the first time, to our knowledge, a structural characterization of human EARS2 protein.
EARS2 wild-type protein was expressed in E. coli cells and purified with the purity yield higher than 90%. Analysis of secondary and tertiary structure of EARS2 wild-type revealed that the purified protein presents a folded conformation, with the expected α- helical structure. Further, the thermal unfolding process of this protein is cooperative, with an apparent Tm of ~45°C. Currently, we are dedicated to the studies on the three disease variants.

We expect our results to provide new information regarding EARS2 molecular defects, and could in the future open new avenues to improve disease prognosis and therapies.

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Mechanistic insights into an alternative - HelD-protected pathway of bacterial transcription initiation

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Protein HelD was originally discovered as a partner of bacterial RNA polymerase interfering with transcription. Our understanding of the role of HelD in bacterial transcription has been significantly enriched by the 3D structure of its complex with RNAP in a previous series of cryo-EM structures (Kouba et al., 2020). What seemed to be a protein factor involved mainly in release of stalled transcription complexes, dissociating RNAP from nucleic acids, is now also understood as a protein involved in a target protection mechanism of antibiotic resistance (Sudzinova et al., 2022).

Our recent functional analyses and series of cryo-EM structures bring evidence for HelD from Mycobacterium smegmatis forming complexes with RNAP associated with the primary sigma factor σ^A and transcription factor RbpA but also participating in complexes of RNAP with promoter DNA at various stages leading towards initiation complex. The structural snapshots describe mechanistic aspects of HelD release from RNAP and its protective effect against rifampicin. Biochemical evidence defines the role of ATP binding and hydrolysis by HelD in the process, and confirms the rifampicin-protective effect of HelD. The details of interactions of HelD with RNAP, the mechanism of rifampicin resistance, of HelD release and of an alternative pathway of transcription initiation will be discussed.

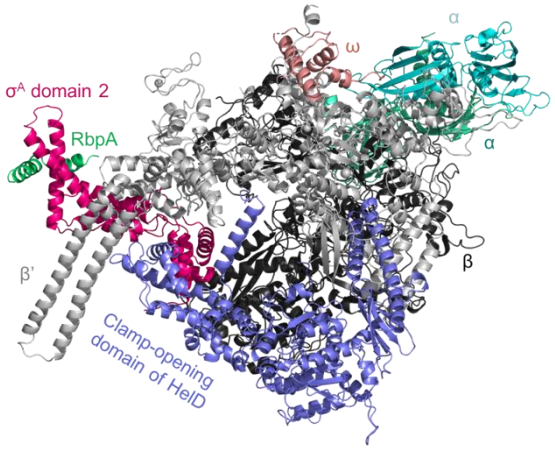

Figure 1. Cryo-EM reconstruction at 3.1 Å of M. smegmatis holoenzyme with RbpA and HelD. HelD in purple, σA in hotpink, RbpA in light green.

We acknowledge support by MEYS for access to CIISB, the Instruct centre (LM2018127043, LM2023042), CSF (23-06295S), Grant Agency of Charles University in Prague (GAUK 236823), Czech Academy of Sciences (86652036) and the project National Institute of virology and bacteriology (Programme EXCELES, LX22NPO5103, Next Generation EU).

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In vitro and In-cell recognition of Quadruplex-duplex hybrids (QDH): conformation, folding, and recognition by drug-like ligand molecule

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Keywords: Nucleic acids, DNA-drug interaction, Solution NMR, In-cell NMR. z

Nucleic acids represent the fundamental biomolecular machinery that transfers genetic information and regulates gene expression. G-quadruplexes (G4) represent one of the crucial non-canonical structures in the genome's non-coding part, such as promoters of (onco-)genes and telomeres having significant biological functions. A quadruplex-duplex hybrid (QDH hereafter) is designed upon the juxtaposition of a duplex stem-loop onto a G4 across the multiple edges of a quadruplex core. QDH shows diverse functionalities ranging from (patho)biological processes to nanotechnology. Under in vitro conditions, the individual conformations can be separated and characterized at the atomic level. However, the identity of the conformation, which is responsible for biological function in vivo, remains obscured due to the lack of suitable technology allowing high- resolution structural studies under physiological conditions in vivo. The biggest challenge is to develop a ligand specific to a bioactive genomic QDH sequence with minimal off-target binding. This work showed the selective recognition and structural elucidation of distinct QDH conformation from the PIM1 gene by two Bis-quinolinium ligands with state-of-the-art solution NMR in conjugation with low-resolution spectroscopic techniques. The structural insights will help to design sequence and scaffold-specific ligands.

On the contrary, we customized "in-cell" NMR strategies to monitor de-novo QDH DNA folding and ligand binding in Xenopus laevis Oocytes (eukaryotic cell model) by using selectively labelled ^{19}F modified QDH sequences. The complexes retain their native folding inside the cellular environment with reduced off-target binding and refolding. Our in vitro observation, coupled with in-cell NMR data, is well-suited for assessing the binding epitome and capability of promising drug-like candidates identified through in vitro screening assays, thereby modulating their further development.

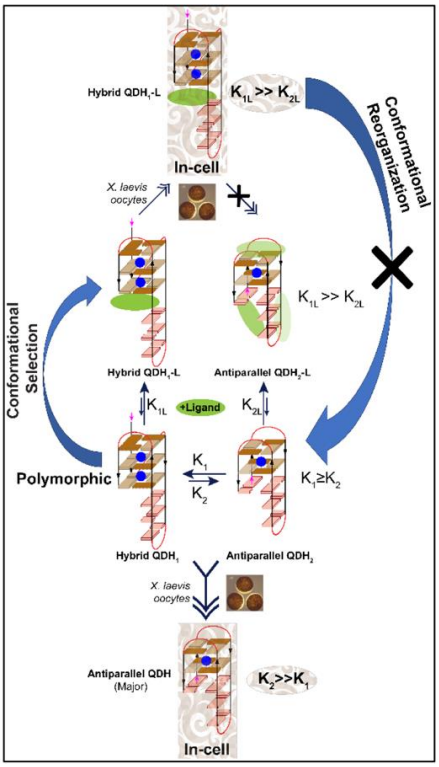

Figure 1. A schematic depiction of conformational polymorphism of QDH topologies and their interactions with ligands under in-vitro and in-cell scenarios

Structural investigation of the muscarinic-acetylcholine receptor M_2R with an intracellular negative allosteric modulator

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G protein-coupled receptors (GPCRs) are the largest family of cell surface receptors in the human genome. As receptors for hormones, neurotransmitters, growth factors and other external stimuli, they play essential roles in almost any aspect of human physiology and disease and represent one of the most important classes of drug targets. Muscarinic acetylcholine receptors (M_1R-M_5R) are a subfamily of GPCRs that regulate many fundamental functions of the central and peripheral nervous systems. However, specific targeting of the M_1R and M_4R subtypes by therapeutic drugs for the treatment of Schizophrenia and Alzheimer's disease has proven difficult due to the highly conserved acetylcholine ligand binding site across members of this family. As a result, non-specific activation of the peripheral M_2R leads to serious side effects.

Here, we developed a small molecule negative allosteric modulator (NAM) that binds to the intracellular side of the M_2R to reduce unwanted cholinergic side effects. To elucidate the molecular determinants of NAM binding and its mechanism of action at the M_2R, we have exploited different fusion protein designs to facilitate cryo-EM structure determination of the receptor alone without signaling protein. High resolution structure determination will allow us to further optimize the NAM and to design an allosteric modulator that could be used clinically to selectively silence signaling through the peripheral M_2R. This could reduce cholinergic side effects while targeting the M_1R with a currently available non-specific orthosteric drug for the treatment of Alzheimer's disease and Schizophrenia. Targeting the intracellular transducer binding site of GPCRs with allosteric ligands represents a novel way to specifically target and silence GPCR signaling. We expect that this concept can be applied to other GPCRs, paving the way for the development of more specific and safer therapeutics for a variety of different diseases.

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The insights into the interaction between Galectin-3 and the human natural killer cell activation receptor NKp30

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Natural killer (NK) cells are lymphocytes that play a crucial role in innate immunity. They can identify and eliminate infected or malignant cells without prior activation. To achieve this, NK cells exhibit a set of activation and inhibition receptors on their surface, including a glycoprotein NKp30, the activation natural cytotoxicity receptor [1]. Among other known ligands of NKp30, there is currently significant interest in specific tumor-related ligands such as B7-H6 [2], BAG-6 [3], and Galectin-3 [4]. Interestingly, Gal-3 can inhibit NK cell functions by binding to the NKp30 receptor. As such, it could be a promising target for new antitumor therapies [5]. It is a challenging task to study the interaction between wild-type Gal-3 and NKp30 due to a few factors. Firstly, wild-type Gal-3 can create dimers through an odd cysteine, which can interfere with measurements. Secondly, NKp30 carries three *N*-glycosylation sites (N42, N68, N121) [6] within its ligand binding domain (LBD), each contributing differently to this interaction. To gain a better understanding of this interaction, a cysteine-less form of the carbohydrate recognition domain of Gal-3 was produced in *E. coli*, and NKp30 LBD *N*-glycosylation mutants were expressed in HEK293T cell line. The stability of *N*-glycosylation mutants was studied using differential scanning fluorimetry, and microscale thermophoresis was used to determine the affinity of the interaction between Gal-3 and NKp30 LBD mutants. Additionally, the most promising *N*-glycosylation mutant (NKp30_LBD_G121) was selected for crystallization trials of the Gal-3:NKp30 complex with the goal of solving its structure using X-ray crystallography.

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Bag1 has a key role in the Hsp70-assisted, proteasome-mediated degradation pathway

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Eukaryotic cells maintain cellular proteostasis through intricate protein quality control systems that orchestrate chaperone-mediated protein folding and protein degradation systems, preventing potential harm from accumulated misfolded proteins. The chaperone system attempts to refold the abnormal proteins and solubilize aggregated proteins; if unsuccessful, these aberrant proteins are removed through the protein degradation system.

In the ubiquitin-proteasome system (UPS), covalent conjugation of poly-ubiquitin chain on substrates guides specific protein degradation by 26S proteasome. The degradation process is initiated by recognition of ubiquitinated substrates by the 19S regulatory particle (RP), which is then followed by unfolding and translocation of substrates through a channel into the catalytic chamber 20S core particle (CP) that executes protein degradation Six AAA+ (ATPases associated with diverse cellular activities) ATPases subunits, Rpt1-6, unfold substrates by processive threading through a central channel driven by sequential ATP hydrolysis. Aside from the canonical subunits, proteasome function is finely tuned by transiently associated cofactors including UBL-UBA proteins which are involved in recruiting polyubiquitinated substrates to the 26S proteasome.

On the other hand, Bag1 has been shown to interact with Hsc70/Hsp70 to modulate the chaperone activities. Through an ATP-driven conformational cycle, HSP70 can recognize misfolded proteins, promote refolding, prevent protein aggregation, and resolubilize protein aggregates. Despite their many different roles, all members of the Hsp70 family contains two highly conserved structural domains: the substrate binding domain (SBD) and the nucleotide binding domain (NBD). The ADP/ATP switch is catalyzed by a group of cochaperones called nucleotide exchange factors (NEF), which bind to the NBD and favor ADP release from the active site and ATP re-uptake. Bag1 is one of such NEFs and contains both UBL and BAG domains and interacts with the 26S proteasome through the UBL domain to degrade unfolded proteins. However, how cochaperone Bag1 bridges between refolding system and protein degradation system, and how Bag1 enhances degradation of unfolded proteins remain unanswered.

In this work, using cryoelectron microscopy (cryoEM) and different biochemical and biophysical techniques, we have revealed that Bag1 plays a key role in Hsp70-mediated, proteasome-dependent protein degradation, not only by physically linking Hsp70 to the proteasome (through its subunit Rpn1), thus facilitating protein delivery to the latter, but also by inducing a series of conformational changes in the proteasome regulatory domain that facilitate the client protein degradation.

Molecular mechanism of caspase-9 CARD oligomerization using cryo-EM and NMR spectroscopy

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Apoptosis, a form of programmed cell death, maintains cellular balance by eliminating damaged cells. The protease caspase-9 (C9) plays a central role in apoptotic signaling by activating downstream caspases. At the molecular level, C9 comprises a caspase activation and recruitment domain (CARD) and a protease domain separated by a flexible linker. The CARD belongs to the death domain fold superfamily, many members of which oligomerize and assemble into filaments. For CARD-containing caspases, activation of the protease domain is often coupled to filament formation. However, only a few caspases have been reported to form filaments, and the molecular mechanisms that regulate filament formation remain unclear. Here, using an integrative structural biology approach, we found that the C9 CARD oligomerizes and forms filaments in vitro at physiological pH and salt concentrations. The propensity to form filaments was enhanced under low-salt or acidic conditions, suggesting a significant role for electrostatic interactions in driving filament formation. Indeed, charge-altering mutations of the lone histidine residue to introduce a positive (H38R) or negative (H38D) charge, or to remove the pH-dependence at this site altogether (H38N), dramatically altered the filament propensity. We solved a 2.4-Å crystal structure of a C9 CARD tetramer and used cryo-election microscopy to determine a 3.4-Å structure of the filament. We observed extensive electrostatic interactions between the CARD subunits within the filament, including an inter-protomer salt bridge involving H38. With the cryo-EM-derived structure of the C9 CARD filament, we aim to rationally design mutations and assay for functional activity and filament formation. Our study will provide a molecular framework for understanding the formation and oligomerization of filamentous death domains.

Characterization of nuclear receptors from parasitic nematodes to overcome anti- infectious drug resistance.

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Resistance to ivermectin (IVM), the most important broad-spectrum antiparasitic drug in use today, is widespread in parasites to the extent that it compromises successful control of human and animal parasites. New therapeutics are urgently needed to overcome resistance to actual treatments. Two nuclear hormone receptors NHR-8 and DAF-12, of parasitic nematodes play key roles in lipid metabolism, reproduction, and longevity of worms as well as in drug detoxification[1]. Due to their central role in development of nematodes and in IVM resistance[2], NHR-8 and DAF-12 are potential therapeutic targets to fight parasitic diseases. However, their mechanism of action needs to be deciphered. Using X-ray crystallography and biophysical characterization (Nano-DSF, Thermal Shift Assay, Isothermal Titration Calorimetry, fluorescence anisotropy assays), new structural information about ligand and transcriptional coregulators binding are acquired and allow a better understanding of NHR-8 and DAF-12 biological functions. In addition, a number of ligands of interest are currently selected to validate their action in cellular assays in order to orient the design of DAF-12 and NHR-8 inhibitors.

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Biomimetic Adhesives: New Adhesive Proteins Inspired in Sea Urchin Nectin Structural Domains

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Currently, there is a great need in biomedicine and biotechnology for biological adhesives that are non-cytotoxic and efficient in wet/humid environments (i.e., surgical adhesives or cellular adhesion promotors for in vitro cultures). It is already known that marine invertebrates are able to attach to several substrates in the presence of sea water (high dielectric and ionic strength similar to physiological fluids) through the production of adhesive secretions. Some of which already inspired the development of new biomimetic adhesives (i.e. Cell-Tak™, a formulation containing proteins from marine mussel). However, successful examples are still scarce and there is still a need for new adhesive systems, with novel capabilities.

Sea urchins bioadhesives are an ideal framework to develop new bioinspired adhesives due to their adhesion strengths up to 0.5 MPa, which are higher than commercially available biological adhesives (e.g., fibrin glue 0.2 MPa). Our group has been studying sea urchins' adhesion, focusing on Paracentrotus lividus. For this species, Nectin was identified as an important adhesive protein present in both adhesive organs and secretions. It has six galactose-binding discoidin-like (DS) domains, which are thought to be important for its adhesive function. Aiming to develop a new synthetic bioadhesive inspired in sea urchins' Nectin, we are currently studying the full-length Nectin protein and several combinations of its DS domains (constructs), to obtain an adhesive, stable protein for large scale production. To do so, several E. coli strains, and growth conditions were tested. The best targets were purified using a combination of chromatographic methodologies, and protein quality was evaluated using biochemical methods. For the top targets, a structural and conformational characterization was obtained through circular dichroism, fluorescence spectroscopy and differential scanning fluorometry. Adhesiveness will be assessed through surface coating analysis and atomic force microscopy.

By now, we were able to express the full-length protein and 4 out of 6 constructs. Furthermore, at least one construct was purified with a 90-95% purity and structurally characterized. Circular dichroism spectra indicate that the purified construct presents a folded conformation. And monitoring loss of secondary structure, or tryptophan fluorescence emission during thermal stress showed a fairly thermal stable protein. Providing a deeper characterization of the first identified sea urchin adhesive protein, this project contributes to the current knowledge on P. lividus adhesion mechanisms and opens new avenues for the development of sea urchin inspired bioadhesives for biomedical/biotechnological applications.

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Unravelling Retinoic Acid Receptor transcriptional regulation and allosteric dynamics controlled by DNA

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The retinoic acid receptors (RARs) mediate retinoid signaling and play multiple roles in vertebrate development, organogenesis, homeostasis, immune functions, and reproduction. Through heterodimerization with retinoid X receptors (RXRs), RAR/RXR complexes bind specific retinoic acid-responsive elements (RAREs) in the regulatory sequences of target genes, inducing specific transcriptional gene regulation. These RAREs are typically composed of two direct repeats of the consensus RGKTCA classically spaced by one, two or five nucleotides (DR1, DR2 or DR5) or are non-classic elements, as DR0 or inverted repeated (IR0). The different RAREs lead to differential transcriptional responses, suggesting a role played by the DNA towards RAR regulation. In the absence of all-*trans*-retinoic acid (ATRA), RAR/RXR forms a repressive complex by recruiting corepressors (CoRs, e.g., NCoR) and histone deacetylases, leading to chromatin condensation and gene silencing. ATRA binding to RAR induces a conformational change in RAR/RXR that allows corepressor dissociation and recruitment of coactivators (CoAs, e.g., TIF2), leading to gene activation. Seeking to dissect the transcriptional regulatory mechanisms played by RAR and to explain how signals coming from the various components are integrated and turned into a particular physiological response, we combined a great set of biophysical, structural and computational methods to describe the structures and dynamics of the repressive and active complexes formed by full-length RAR/RXR heterodimer, DNA and coregulators. Firstly, fluorescent anisotropy experiments confirmed the differential binding of the heterodimer RAR/RXR to the different RAREs with the lowest affinity for DR0 (Fig.1-A). Interestingly, the RAR/RXR-DR0 complex also exhibits a reduced affinity with the coactivator TIF2, which agrees with the observation that RAR/RXR binding to DR0 fails to induce activation of the target genes. The solvent differential accessibility for RAR/RXR-DR0 complexes measured by Hydrogen/Deuterium eXchange Mass Spectrometry (HDX-MS) corroborates these affinities measurements (Fig.1-B). Secondly, we observed a protective effect from the DNA not only in the DNA binding domain (DBD) but also at long-range distances, in the ligand binding domain (LBD) and, surprisingly, until the coregulator molecules. Additionally, HDX-MS data indicated a new interaction region on NCoR (Fig.1-C). By combining Alpha Fold and molecular dynamic (MD) simulations, we showed that this region of NCoR consistently interacts with RXR, confirming the observations from HDX-MS and mutagenesis assays (Fig.1-D). Thirdly, SAXS intensities showed differences in the complexes' conformation in solution, revealing a signature among the RAREs (Fig.1-E). Our MD contact maps and domain distance calculations showed that positive-like RAREs complexes (RAR/RXR bound to DR5 and IR0) are preferentially more elongated and can do numerous intra and intermolecular contacts (Fig.1-F), whereas other RAREs sustain compactness and low contact numbers in the complexes. Overall, our structural integrative data provide a deep understanding of RAR regulatory mechanisms and confirm the DNA effect on the stability, conformation and dynamics of the complexes formed by RAR/RXR, DNA and coregulators. Additionally, we revealed an additional interface between the CoR and RXR, potentially relevant for the RAR repressiveness and for the activity of other nuclear receptors that heterodimerize with RXR (e.g., PPAR, LXR, FXR, VDR, TR).

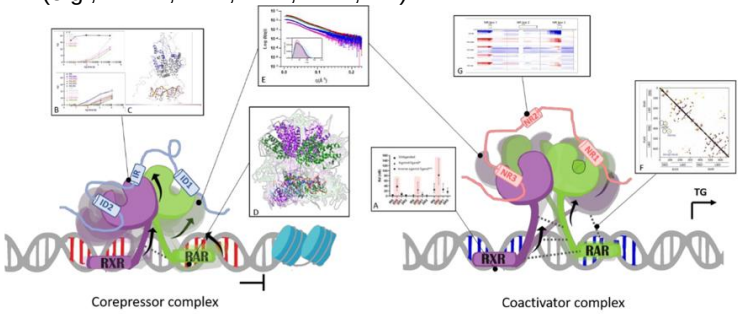

Figure 1. Schematic representation of RAR/RXR regulation mechanisms when complexed with the corepressor NCoR (left) or the coactivator TIF-2 (right). All the techniques and their respective outcomes are represented inside the boxes, and their meaning can be found in more detail in the text.

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Chaperone multimers suppress the generation of Aβ42 neurotoxic oligomers implicated in Alzheimer´s Disease

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Alzheimer's disease (AD) involves extracellular aggregation of Aβ42 into toxic oligomers and fibrils, whose emergence is regulated by molecular chaperones. These include S100B alarmin, a homodimeric EF-hand protein with intra and extracellular functions which acts as a Ca2+- switched Aβ42 anti-aggregation chaperone. However, S100B occurs also as a homotetramer, with uncharacterized neuroprotective roles. Here, we compared the chaperone activities of both S100B multimers and explored their impact on the formation of Aβ42 oligomers (AβO). S100B anti-aggregation activity was evaluated by thioflavin-T (ThT) Aβ42 aggregation assays. Aβ42 conformers targeted by S100B were accessed by computational and structural- biophysical spectroscopies. Aβ42 oligomer distributions were determined through mechanistic analysis of fibril formation and via early detection of Aβ42 species using the X-34 fluorophore. Aβ42 aggregation kinetics revealed that, unlike the dimer, tetrameric S100B delays Aβ42 aggregation and reduces the amounts of fibrils formed at sub/equimolar ratios, an effect that persists even in the absence of Ca^{2+} binding. Structural analysis revealed that this enhanced catalytic efficiency results from a secondary Ca^{2+} independent binding site formed upon tetramerization of S100B, with which monomeric and fibrillar Aβ42 interact (Figueira et al JMB 2022). Kinetic and mechanistic analysis revealed that dimeric and tetrameric S100B preferentially inhibit Aβ42 fibril surface-catalyzed nucleation, decreasing the reactive influx towards oligomers down to <10%. Such results comply with an independent screening of AβO using a combination of the thioflavin-T and X-34 fluorophores (Figueira et al Front. Neurosci. 2023). Our study sheds new insights on the functional landscape of S100B chaperone multimers, suggesting its critical role in the regulation of proteopathic Aβ42 aggregation and oligomerization in AD.

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Genome anchoring, retention, and release by neck proteins of *Herelleviridae* phage 812

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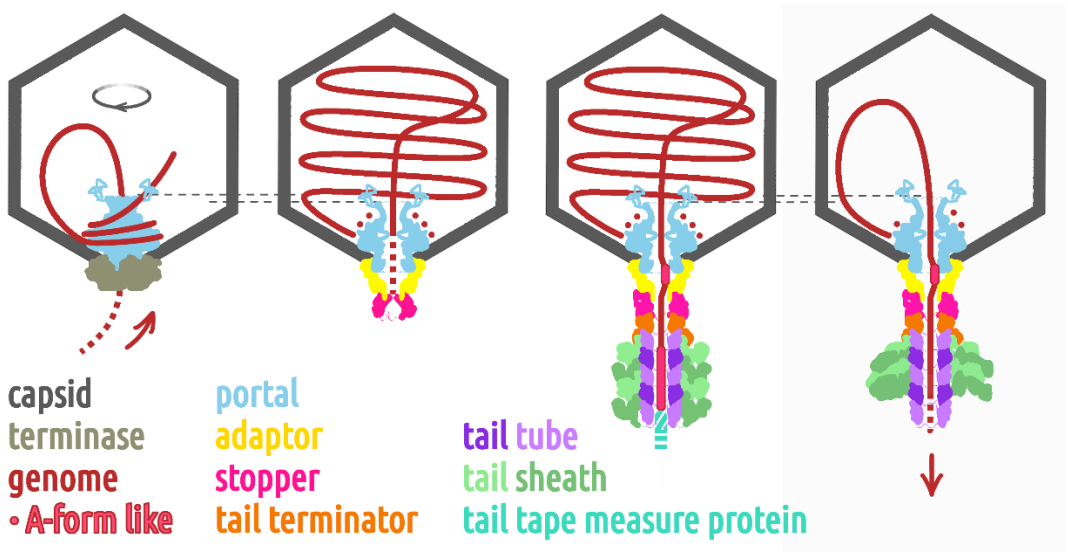
The virion of *Herelleviridae* phage 812 is formed by an icosahedral capsid and a contractile tail joined together by neck proteins. Despite the role of the neck proteins in virion assembly, DNA packaging, and regulation of genome release, their functions are not well characterized. We show that the neck of phage 812 consists of portal, adaptor, stopper, and tail terminator proteins decorated on the outside by two types of cement proteins. A dodecameric DNA-binding site on the portal complex anchors a region of the genome inside the capsid, which directs the spooling of the packaged genome and may prevent an accidental escape of the DNA during the initial stages of genome packaging. The adaptor complex induces a local B-to-A form transition of the DNA in the neck channel that may serve to pause genome translocation. The gating loops of the stopper proteins prevent genome loss from fully packaged proheads by blocking the neck channel prior to the tail attachment. The binding of the tail terminator complex to the stopper complex induces opening of the gating loops and advancement of DNA into the tail. The structure of neck proteins is unchanged by tail sheath contraction. The expulsion of the tail tape measure protein rather than tail sheath rearrangement thus triggers genome release. Our results explain how the active interplay between neck proteins and the genome directs DNA packaging, prevents premature genome release, and enables its ejection into the host cell.



Succinylation's impact on MCAD enzyme - from structure to function

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Metabolic regulation encompasses a complex interplay of genomic, proteomic, and metabolomic adjustments within cells. A particular group of non-enzymatic post-translational modifications (PTMs) known as acylations, such as succinylation and glutarylation, have emerged as important regulators in mitochondrial enzymes [1].

The extent of acylations is closely associated with the accumulation of intermediate metabolites such as succinyl-CoA and glutaryl- CoA that occur under certain conditions such as fasting, caloric restriction or in several metabolic disorders, creating a unique scenario for anomalous protein acylation [2]. It has been recently shown that the enzyme glutaryl-CoA dehydrogenase (GCDH), which participates in amino acid catabolism, is prone to high levels of glutarylation due to an increase in glutaryl-CoA production stimulated by lysine catabolism, and this modification diminish enzyme activity [3]. Moreover, it was demonstrated that this acylation was regulated by sirtuin5 (Sirt5). Exploring this new concept which suggests that enzymes involved in pathways that handle these metabolites are more prone to protein acylation, in this work we combine biochemical and biophysical techniques to address the impact of succinylation on the structure and function of the Medium Chain Acyl-CoA Dehydrogenase (MCAD) enzyme.

Our results show that purified MCAD is easily succinylated and glutarylated. Through spectroscopic techniques such as circular dichroism or fluorescence we were able to demonstrate that succinylation has no major implication on MCAD's structure. Further using thermal denaturation assays, we also access protein thermal stability, and observe no major changes due to succinylation. Nonetheless, this modification induces an increase of enzymatic activity. In addition, we show that SIRT5 incubation reverts succinylation and brings function to similar levels to unmodified MCAD, in agreement with studies in other proteins.

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Asymmetric reconstructions of immature Tick-borne encephalitis virus particles reveal assembly mechanism of flaviviruses

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Tick-borne encephalitis virus (TBEV) is an enveloped virus belonging to the family *Flaviviridae*, which causes severe disease of central nervous system in humans. The smooth virion surface is covered by envelope proteins (E-protein), that are together with the membrane proteins (M-protein) anchored in the virus lipid bilayer. The immature, non-infectious virus goes through a maturation phase during the viral life cycle. Proteolytic cleavage of prM and a significant rearrangement of the envelope proteins on the viral surface are two aspects of this process.

We isolated immature TBEV particles from infected tissue culture cells and visualized them using cryo-electron microscopy in order to determine their structure. The E-protein-prM-protein complex forms the "spiky" surface of the immature particles. We used cryo-electron tomography and single-particle analysis to show that the TBEV immature particles are asymmetric. Defects induced during immature particle assembly frequently disturb the symmetric, icosahedral structure of the E-protein-prM-protein spikes on the particle surface. However, these irregularities do not hinder the subsequent maturation process and produce mature particles with vacant patches in the "herring bone" structure of the mature viral surface. The findings shed additional light on the viral assembly and maturation process, which may be the subject of future antiviral medication development.

This work was supported by the project National Institute of Virology and Bacteriology (Programme EXCELES, ID Project No. LX22NPO5103) - Funded by the European Union - Next Generation EU.

Initial stages of picornavirus infections in vivo

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The propagation of viral infections highly depends on the success of its initial stages. Non-enveloped viruses, such as those from the order Picornavirales, utilize host endocytic machinery to enter the cell. It is known that the binding of specific receptors, along with the acidification of the endosomes, promotes virus activation and subsequent genome release. However, to be delivered to the cytoplasm where replication takes place, the viral genome needs to overcome two barriers: the capsid shell and the endosome membrane.

In our research, we employ *in situ* cryo-electron microscopy to visualize the early stages of picornavirus infection. The obtained results suggest a novel mechanism for the endosomal escape and release of viral RNA into the cell interior across multiple picornaviruses. The demonstrated uniformity of the early infection stages contributes to a better understanding of the infection process of non-enveloped viruses, providing useful information for the development of effective targeted antiviral compounds in the future.

PhikZ Baseplate Structure

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PhiKZ is a bacteriophage that infects *Pseudomonas aeruginosa*, an opportunistic human pathogen. The phage phiKZ is known for its large genome and complex structure, making it a notable subject for structural biology studies. Among all its parts, the baseplate is the most complex. It adheres to the phage prey and triggers the genome ejection. The signal that triggers the ejection travels from the tail fibres to the tail through the baseplate. Here, we used cryo-Electron Microscopy to visualise the structure of the phiKZ tail and the baseplate at high resolution, the first one of a jumbo bacteriophage. Our results reveal an intricate network of proteins organised in six-fold symmetry. Structural comparisons with related systems highlight the universal conservation observed in contractile injection systems. This sheds light on phiKZ's baseplate specificities and suggests a potential mode of action. Its structural analysis enhances our understanding of phiKZ and contributes with valuable knowledge to the broader field of myovirus biology.

Cryo-EM Reveals the Structure and Infection Mechanism of Phage LUZ19

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Infections caused by antibiotic-resistant bacteria are a growing health concern. As antibiotic treatments become less effective, alternative approaches are explored. One of the promising methods is phage therapy. However, our understanding of phage–bacterium interactions is limited, and phage therapy is currently considered an experimental treatment. We use cryo-electron microscopy to study the structure of bacteriophage LUZ19 and the process of LUZ19 infection of PAO1, a clinically relevant strain of P. aeruginosa. The virion particle is composed of an icosahedral capsid and a tail decorated by six long tail fibres. The capsid is built from major capsid, head cement, and flexible head decoration proteins, and encloses a 43.5 kbp-long dsDNA genome and an inner core complex. The symmetry mismatch between the capsid and the tail is mediated by a dodecameric portal complex which occupies one vertex of the capsid. The portal complex interacts with the adaptor dodecamer of the tail through its “stem” helices which interlock with the adaptor C- termini. LUZ19 tail extends to a hexameric tail nozzle which is decorated with six flexible tail fibres. The nozzle and the tail fibres mediate the attachment of phage LUZ19 to the type IV pili of PAO1. The contraction of the pili carries the phages to the cell surface, where they irreversibly attach and infect the host cell. Studying phage LUZ19's structure and its infection mechanism aims to deepen our understanding of phage-bacterium interaction.

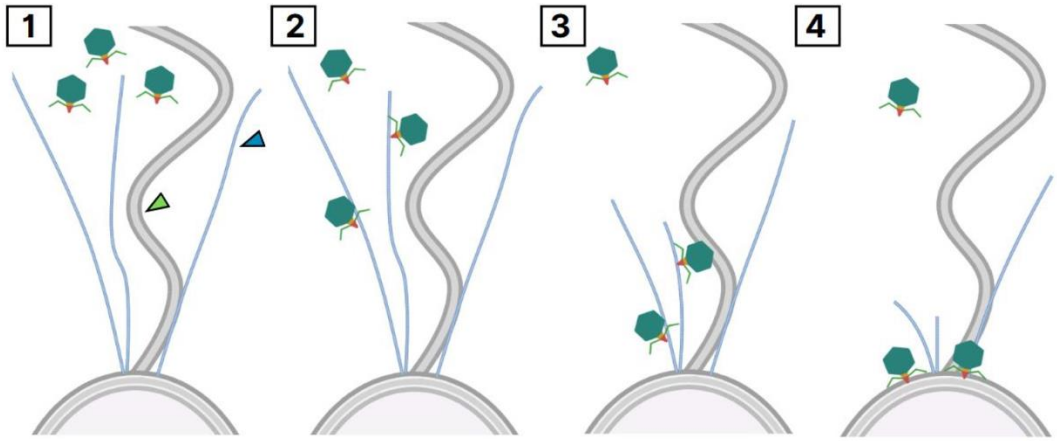

Figure 1. Graphic scheme of the phage attachment process. 1. Free-floating LUZ19 phages. 2. Attachment of LUZ19 to type IV pili of a PAO1 cell. 3. Contraction of pili towards the cell. 4. Irreversible attachment of phages to the cell surface.

Cryo-electron Microscopy and Tomography core facility (CEMCOF) of CEITEC Masaryk University is gratefully acknowledged for obtaining the scientific data presented in this poster. This work was supported by the project “National Institute of Virology and Bacteriology” (Programme EXCELES, ID Project No. LX22NPO5103) - Funded by the European Union - Next Generation EU.

Structural dynamics of botulinum neurotoxin type A from hydrogen/deuterium exchange mass spectrometry

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University of Exeter

Botulinum neurotoxins (BoNT's) produced by Clostridium botulinum, induce muscle paralysis by inhibiting acetylcholine release at the neuromuscular junction. The allosteric changes facilitating translocation of BoNT to the synapse have not been fully identified. Therefore, it is critical to determine the molecular mechanisms of BoNT trafficking to understand the biology of infection and enable rational design of BoNT as a biotherapeutic. BoNT serotype A targets motor neurons by binding to the Synaptic Vesicle Glycoprotein 2C (SV2C). The precise changes that are induced throughout the molecule upon binding to SV2C have not yet been identified. Here, we aim to build a highly resolved model of the BoNT A structural dynamics, showing the allosteric changes induced when BoNT A binds to SV2C.

Hydrogen/deuterium-exchange mass spectrometry (HDX-MS) was used to ascertain the structural dynamics of BoNT A and variants of the binding partner protein. A bottom-up approach was applied with an immobilised pepsin column (Enzymate, Waters). Equilibrium binding experiments with D-labelling times between 0 and 1,667 min were done with BoNT A +/- SV2C. This revealed the local allosteric changes in BoNT A upon binding to the receptor protein. BoNT in its apo state has been shown to be a dynamic molecule with regions of flexibility throughout its structure. When BoNT A is bound to SV2C, significant allosteric changes are induced throughout the BoNT A molecule, alongside the changes observed in the SV2C binding site. These changes were validated as being statistically significant at the peptide level. BoNT A in its apo state and coupled with the SV2C binding partners is amenable to bottom-up HDX-MS study, yielding excellent structural resolution with high reproducibility. This allowed visualisation of the extensive structural changes in BoNT A and binding partners, including allostery. Here, we present the visualization of BoNT A allosteric effects elucidated by the binding partner, thus providing information to design improved therapeutics, and enhancing our ability to target these specific biological processes of BoNT A.

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Novel sample delivery for small nanoparticles and biomolecules for cryo electron microscopy

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Cryo-electron microscopy (Cryo-EM) is one of the key techniques in the field of structural biology. Recent years brought considerable improvements both on the software and hardware of the microscopes and resolving high-resolution structures of proteins has become a standard procedure. However, in terms of sample preparation the majority of cryo-EM grids are still prepared by plunge freezing, a technique that was developed about ~40 years ago. There, the sample is pushed into grid holes to form thin liquid-layers that are subsequently plunged into liquid ethane. During this process, proteins can be exposed to the air-water interface, possibly causing a preferential orientation or damaging their structure.

Here, we present the novel freeze-and-deposit sample delivery approach to deposit particles for cryo-EM using shockfreezing technology. The cooling process produces cold high-density beams of nanoparticles, including biological specimen such as viruses or proteins, which was designed for XFEL single-particle-imaging experiments [1, 2]. In this process, nanoparticles and macromolecules are aerosolized, for instance, by electro-spray ionization, and then rapidly cooled in the gas phase using a cryogenic buffer-gas cell [3, 4]. Adopting this with cryo-EM-grid handling allows for the controlled deposition of already shockfrozen samples on the grid, completely bypassing the need for blotting and the exposure of particles to the air-water interface. This approach will ensure the deposition of particles in a near-native state and overcoming the issue of deposition of preferentially oriented proteins.

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Uncovering the structural dynamics of a predatory lipid transport System

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University of Oxford

Mammalian Cell Entry (MCE) domain-containing proteins have been implicated in glycerophospholipid transport, a key component of the gram-negative OM. The model organism Escherichia coli contains three MCE proteins residing within unique operons; MlaD, PqiB and LetB, each presenting potential novel targets for innovative antibacterial agents. Characterising these proteins and their associated complexes within multiple organisms may provide additional insight into these putative lipid transport systems. Here, we focus on MCE proteins within Bdellovibrio bacteriovorus HD100, a predatory gram-negative bacterium, that alternates between free-living growth and predation upon other gram-negative bacteria. Thus far, we investigated Bd1152, a two-MCE domain containing protein residing at the centre of a tripartite operon. The structural flexibility of Bd1152 posed challenges in achieving a high-resolution structure. Standard cryo-EM data processing approaches using cryoSPARC or Relion were insufficient, leading us to employ the Scipion framework, integrating various software in a single processing pipeline (Instruct-ERIC internship PID2530). This effort yielded the final structure of the Bd1152 top MCE ring at 3.4 Å resolution (Figure 1, left), supplemented by an x-ray structure at 2.6 Å resolution. Both structures reveal hexameric assemblies (MCE rings) featuring a central channel implicated in phospholipid transport. Moreover, we employed the novel Zernike3D algorithm, and cryoDRGN algorithms to compute inherent continuous flexibility. This analysis unveiled Bd1152's significant structural plasticity, a trait not previously reported for MCE domain proteins, offering potential insights into lipid transport mechanisms. Overall, this study has provided the first structural and function insights into the MCE proteins of Bdellovibrio bacteriovorus, including the first structure of 2-MCE domain-containing protein. Furthermore, we introduced non-canonical cryo-EM data processing approaches, which could prove beneficial for solving structures of other 2-MCE domain-containing proteins, or proteins exhibiting similar challenges in their
biophysical characterisation.

Modulation of Viral Factory Formation with Affimers

Monika Olejnik

University of Leeds

Rotavirus infection causes gastroenteritis and contributes to over 200,000 children's deaths annually, primarily in developing countries. In infected cells, rotavirus infection occurs at viral factories, which are droplet-like biomolecular condensates formed by liquid-liquid phase separation. Two key rotavirus non-structural proteins, NSP2 and NSP5, are recognised to mediate the formation of viral factories, however the precise mechanism by which they do so remains unclear. We have employed structural mass spectrometry methodologies to characterise the interaction between octameric NSP2 and intrinsically disordered NSP5, uncovering key regions on the two proteins that mediate the interactions facilitating condensate assembly. We are also investigating how affimers, which are small (~12 kDa) binding proteins, that have been selected via phage display to bind to NSP2, influence the propensity of NSP2/5 to phase separate in vitro. Through functional assays (nephelometry, protein-protein affinity), Differential interference contrast (DIC) Microscopy, and Native Mass Spectrometry, we have identified affimer candidates which disrupt the formation of NSP2/5 droplets and begun work to map the interaction interfaces that they target. This work provides insight into viral factory assembly and dynamics, and could be a starting point to identifying ways to modulate viral factory formation. Such information is key for the development of novel antivirals against rotaviruses and the many viral infections which exploit similar mechanisms of replication

Characterizing predatosome proteins in Bdellovibrio bacteriovorus

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Bdellovibrio bacteriovorus (Bdellovibrio) is a Gram-negative predatory bacteria which preys on other Gram-negative bacteria. Due to a broad range of Gram-negative bacteria it preys upon, there is potential for Bdellovibrio to be used as living antibiotics. In its life cycle, Bdellovibrio swims freely in search of prey, invades into the prey, and consumes it from within, generating new progenies in the process. Upon prey contact, a group of invasion-specific predatosome genes are upregulated. Some of these proteins are predicted to contain a conserved Bdellovibrio-specific novel ß-sandwich domain, followed by a regularly-spaced repeated motif. Currently nothing is known about the actual structure nor function of these cryptic proteins. Structure prediction using AlphaFold2 followed by modelling showed the possibility of these proteins interacting with itself, forming a multimer. Currently there are challenges in obtaining the predatosome proteins, therefore a homologous Bdellovibrio protein Bd1369, which also contains the ß-sandwich domain followed by a repeating motif, is being investigated. Purified Bd1369 was observed to either form a viscous solution at low concentrations or a clear solid gel at higher concentrations. To understand the peculiar behaviour of this protein, mutations targeting the interaction interface of the Bd1369 were introduced, theoretically disabling it from selfinteracting. Data from dynamic light scattering and binding studies showed evidence that Bd1369 is indeed forming a higher-order structure with itself.So far, crystals of the Bd1369 ß-sandwich domain and crystals of an engineered Bd1369 double mutant were obtained. Unravelling the structure of these proteins using X-ray crystallography will give insights to their function, and potentially give clues to the characterization of Bdellovibrio predatosome proteins.

Structural and functional analysis of the unique broad-range phospholipase C from Listeria monocytogenes

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The intracellular Gram-positive bacterium Listeria monocytogenes (Lm) is a causative agent of the food-borne disease listeriosis. Listeriosis poses a significant health risk, especially for vulnerable groups such as pregnant women, newborns and immunocompromised individuals. Infection by Lm is facilitated by a variety of specialized bacterial effectors. Upon entry into the host cell, a pore-forming protein, listeriolysin O (LLO), the most important Lm virulence factor, facilitates the breakdown of the vacuolar (phagosomal) membrane and thus the release of the trapped Lm into the cytosol. In addition, two listerial phospholipases C (PLCs), phosphatidyl-inositolspecific (LmPI-PLC or PlcA) and broad-range (LmPC-PLC or PlcB), facilitate the vacuolar escape of Lm by cooperating with LLO in vacuolar membrane disruption. In our recent study (Petrišič et al., https://doi.org/10.1038/s41467-023-42134-4), we determined the crystal structure of LmPC-PLC and complemented it with the functional analysis of this enzyme. Our results show that LmPC- PLC has evolved several structural features to regulate its activity, including the invariable position of the N-terminal tryptophan (W1), the structurally plastic active site, the Zn2+-dependent activity, and the tendency to form oligomers with impaired enzymatic activity. We also demonstrate that the enzymatic activity of LmPC-PLC can be specifically inhibited by its propeptide added in trans. Furthermore, using biochemical approaches, we show that the phospholipase activity of LmPC-PLC facilitates the pore-forming activity of LLO, while the cryo-EM imaging revealed how the enzyme affects the morphology of LLO oligomerization on lipid membranes. Our insights into the atomic structures and molecular mechanisms of action of virulence factors reveal their multifaceted synergy and serve as a prerequisite for understanding disease progression and developing alternative solutions for their containment.

Polyphosphate metabolism in the radiation resistant bacteria Deinococcus radiodurans

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ITQB NOVA

Inorganic polyphosphate plays a crucial role in various organisms. In bacteria, the polyP polymers are stored in granules. Our research focuses on understanding the functions of nano-compartments, known as electron dense granules, in the radiation resistant bacteria, Deinococcus radiodurans. These PolyP nano-compartments are vital in D.radiodurans‘s stress response and contain metals like potassium, calcium, manganese, as confirmed by X-ray fluorescence imaging. To complement our studies, we determined the level of polyphosphates in D.radiodurans cellular extracts by NMR, using a 31P-NMR. Additionally, we have focused our studies on the polyphosphate kinases: PPK1 and PPK2 to elucidate the molecular mechanisms involved in the polyphosphate formation. PPK2 was successfully purified, crystallized and its X-ray crystallographic structures were determined in apo form, as well as in complex with ADP, and ATP bound to polyphosphates. The X-ray structures diffracted to resolutions of 2.06 Å, 2.08 Å and 1.64 Å. At the cellular level, we subjected D.radiodurans PPKs knockout mutants to various conditions, including exposure to hydrogen peroxide (H2O2), methyl viologen (MV) and UV-C radiation. Although, the molecular mechanisms underlying PolyP formation and degradation remain incompletely understood. Our findings contribute to a deeper understanding of the role of polyphosphate in D.radiodurans’ stress response.

Structural basis for postfusion-specific binding to Respiratory Syncytial Virus F protein by the antigenic site-I antibody 131-2a

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The Respiratory Syncytial Virus (RSV) Fusion (F) protein is a major target of antiviral antibodies following natural infection and vaccination and responsible for mediating fusion between the viral envelope and the host membrane. The fusion process is driven by a large-scale conformational change in F, switching irreversibly from the metastable prefusion state to the stable post-fusion conformation. Previous research has identified six distinct antigenic sites in RSV-F, termed sites Ø, I, II, III, IV, and V. Of these, only antigenic site I is fully specific to the post-fusion conformation of F. A monoclonal antibody 131-2a that targets post-fusion F specifically has been widely employed as a research tool to probe for post-fusion F and to define antigenic site I in serological studies, yet the sequence and epitope of the antibody remained unknown. We used mass spectrometry-based de novo sequencing to reverse engineer 131-2a. Reverse engineered 131-2a was then used to investigate 131-2a epitope and to define antigenic site I by single particle cryo-electron microscopy. This elucidated the structural basis for the antibody binding to the post-fusion RSV-F.

Visualising Enterovirus 71 Genome Replication In Situ

Lucie Nepovímová

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Enterovirus 71 (EV71), belonging to the Picornaviridae family, presents a significant concern as it is a causative agent of hand-foot-and-mouth disease (HFMD), predominantly affecting small children. The potential for severe neurological complications, leading to mortality rates of up to 19%, underscores the urgency for research endeavours to counter this pathogen [1]. With escalating outbreaks in the Asia-Pacific region, there is a pressing need to explore novel therapeutic approaches. While current vaccines target specific serotypes, their efficacy lacks evidence of cross-protection. This prompts investigation into targeted antiviral therapies[1,2]. However, this pursuit is hindered by gaps in understanding EV71's replication cycle.The core objective of this study is to explore uncharted aspects of Enterovirus RNA replication, with a particular emphasis on its interaction with genome packaging and viral assembly. A pivotal aspect of unravelling the molecular intricacies of the replication process involves the development of a fluorescent reporter system to visualise viral replication sites. This poses a challenge as direct modification of the viral genome is risky since it may affect the viral life cycle or infectivity. Hence, here we present the establishment of a fluorescent reporter system which will enable the specific labelling of viral replication sites without altering the viral genome. The system will serve as a crucial tool in guiding subsequent research phases, including correlative light and electron microscopy for precise lamella preparation and cryo-electron tomography data collection.

Macromomolecular Crystallography (MX) facilities at MAXIV

Swati Aggarwal[1], Oskar Aurelius[2], Monika Bjelcic[2], Cecilia Casadei[2], Manoop Chenchiliyan[1], Ana Gonzalez[1], Ishkhan Gorgisyan[1], Tobias Krojer[3], Mirko Milas[2], Jie Nan[1], Thomas Ursby[2]

[1]BioMAX beamline, MAXIV laboratory, Lund University
[2]MicroMAX beamline, MAXIV laboratory, [3]FragMAX platform, MAXIV Laboratory, Lund University

MAXIV has two MX beamlines: BioMAX and MicroMAX along with fragment-based drug discovery platform being formally attached to BioMAX. BioMAX was the first beamline at MAX IV to open to users and started operations in 2017. The beamline was designed to be very versatile, with a large energy and beam size range; catering to the needs of the user community, interested in MX projects ranging from standard cryo-data collection to challenging projects involving large unit cells, room temperature data collection, energy scans and serial synchrotron crystallography (SSX) experiments. While BioMAX beamline supports high-throughput manual and unattended rotational data collection on single crystals, MicroMAX on the other hand will be dedicated to SSX, microfocus and time-resolved experiments.

MicroMAX has a similar energy range to BioMAX, providing a focused beam size of around 10 x 5 µm2 and will be 1x 1 µm2 when the focusing mirrors are added in 2025. It has two monochromators: a double crystal Si(111) monochromator like at BioMAX, as well as a Multi-Layer Monochromator (MLM) that can achieve a flux about 50 times higher than what is available at BioMAX. To be able to cope with the increased diffraction intensity, MicroMAX will be equipped with two detectors: a Jungfrau integrating detector, with single photon accuracy for low pixel values, and photon-counting EIGER detector. It will also be equipped with a tunable laser source for pump and probe experiments and a chopper for complex sample exposure regimes and X-ray pulses down to 10 µs.

The FragMAX facility supports structure-based drug and chemical tool compound discovery at MAX IV Laboratory. Designed as a platform for crystal-based fragment screening, the underlying workflows apply to all medium- to large-scale protein-ligand studies. The platform is comprised of three primary elements: (i) a high-throughput crystal preparation facility, which includes access to fragment libraries; (ii) automated diffraction data collection at the BioMAX beamline; and (iii) software tools for large-scale data processing.

LEAPS facilities capabilities supporting structural biology research

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[2]ALBA Synchrotron, Spain

LEAPS (League of European Accelerator-based Photon Sources) stands as a vital hub for scientific collaboration, spanning over 10 members and associated states and providing access to over 35,000 researchers from academia and industry across diverse scientific disciplines. Established in 2017, LEAPS has fostered cooperation among 19 cutting-edge user facilities across Europe, leveraging their collective expertise in photon science, infrastructure management, and service provision. Guided by the visionary European Strategy for Accelerator-based Photon Sources (ESAPS) 2022[1], a comprehensive pan-European plan, LEAPS is dedicated to advancing Europe to the forefront of global technological innovation.

In the realm of structural biology, advancements in X-ray crystallography, synchrotron radiation, and other imaging techniques available at LEAPS facilities enable scientists to examine the detailed makeup of biomolecules with near-atomic precision. X-ray crystallography has contributed data for over 212,000 macromolecular structures, deposited in the protein structure database PDB, unveiling biomolecular structures with exceptional detail. Moreover, LEAPS facilities are founded on the three pillars of Structural Biology: X-FEL, Synchrotron, and Cryo-EM. Each pillar offers distinct advantages, ranging from high-throughput data collection and high-resolution imaging to the study of dynamic processes in biological systems.

Additionally, LEAPS plays a fundamental role in modern biology, with Life Sciences accounting for about 30% of all research conducted at LEAPS facilities. The COVID-19 pandemic underscored the high relevance of the LEAPS network, as facilities swiftly offered their capacities to the biomedical community, ranging from imaging patient samples to structurally analyzing COVID-related proteins. Rapid access calls were opened to provide beamtime for COVID-19 related projects, and new mail-in services and remote access tools and procedures were quickly implemented. This timely response minimized the time from proposal to publication of scientific results, facilitating rapid sharing of new molecular insights and contributing to the development of vaccines and therapeutic approaches. In collaboration with strategic partners such as the European Synchrotron and Free Electron Laser User Organisation (ESUO), LEAPS envisions strategic collaboration with leading European entities to accelerate research in key areas such as public health challenges, rational catalyst design, green hydrogen, water-based technologies, and quantum technologies.

Ancestral sequence reconstruction (ASR) is a promising tool for elucidation of ferroptosis pivotal actor: FSP1.

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Ferroptosis is a unique iron-dependent form of programmed cell death, which is driven by lipid peroxidation of membrane bilayers through the action of reactive iron ions producing reactive oxygen species. ROS are highly reactive species due to the presence of unpaired electrons in their valence shell or unstable bonds that spread oxidative stress by the excess production of phospholipid hydroperoxides (PLOOH), which are a type of lipid-based ROS [1, 2]. Recent studies have highlighted the relationship between ferroptosis processes and Ferroptosis Suppressor Protein 1 (FSP1) [3]. FSP1 is a type II nicotinamide adenine dinucleotide-H (NADH): quinone oxidoreductase (NDH-2). By utilizing NAD(P)H, and non-covalently bound flavin it effectively reduces ubiquinone (coenzyme Q10) to ubiquinol within the plasma membrane [4] (Fig.1). This enzymatic process serves to shuttle reducing equivalents from NAD(P)H into the lipid bilayer, thereby acting as a potent inhibitor of lipid peroxidation and contributing to the regulation of oxidative stress. The sensitivity of the cells to ferroptosis can elucidate the complex mechanism of cancer growth and may help the suppression of therapy-resistant tumours. Assessing the supplementary factors that drive the ferroptosis resistance, to overcome the sensitivity to inhibitors of Glutathione Peroxidase 4 (GPX4) of cell lines implicated in tumorigenesis, FSP1 has been detected as a valuable pharmacological target [5]. Emphasizing the pivotal role of this protein, a deep comprehension of its biochemistry and structure has served as a cornerstone in scientific research. In pursuit of these objectives, ancestralsequence reconstruction (ASR) was undertaken. This strategic approach was chosen based on findings suggesting that ancestral proteins frequently exhibit greater thermostability compared to their present-day counterparts. ASR has shown great success in multiple projects conducted in our laboratory. Ancestral FSP1 was purified from E. coli with higher thermostability and yields compared to the human protein. Subsequently, enzymatic characterization, analysis of its cofactor binding properties, enzyme kinetics, crystallization, high-resolution structure determination of various enzyme complexes have been achieved and will be presented.

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The molecular architecture of a 2MDa Plastid-Encoded RNA Polymerase complex in a unicellular photosynthetic eukaryote

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The plastid-encoded RNA polymerase (PEP) complex, an eubacterial-like multisubunit RNA polymerase, is found in all chloroplasts. In the land plant Arabidopsis thaliana, PEP primarily regulates the transcription of photosynthetic genes, whereas a separate nuclear-encoded phage-type polymerase is responsible for the transcription of the other chloroplast genes. Conversely, in the unicellular green alga Chlamydomonas reinhardtii, PEP transcribes all chloroplast genes. Despite this essential function, the molecular components and architecture of the PEP complex in this alga remain elusive.

Here, we developed an affinity purification method that enabled us to isolate the Chlamydomonas PEP complex in high yield and purity. Our findings reveal that the Chlamydomonas PEP is a large complex of about 2 MDa —twofold larger than its land plant counterpart— and includes 12 additional nuclear-encoded subunits, beyond the anticipated chloroplast-encoded bacterial-like core subunits. We named these newly identified PEP subunits PEPS1-12. We subjected the purified complex to single particle analysis and obtained a global resolution of 2.7 Å. Our structural model shows that the PEPSs act in concert to stabilize the subunits forming the catalytic core, giving this complex a singular structure compared to other RNAPs. Furthermore, akin to recent findings regarding the land plant PEP-Associated Protein 12, our structure reveals that PEPS12 mirrors the 3D organization and spatial positioning of the omega subunit in eubacterial RNA polymerase, despite lacking sequence similarity to both the omega subunit and PAP12. Using phylogenetic analysis, we also identified PEPS2 and PEPS3 as homologs of the land plant PEP-Associated Protein 1 and 11, respectively. However, we found that many other PEPSs have a scattered distribution across the green lineage and that three of them are restricted to Chlamydomonas. Overall, our study challenges a previously held assumption regarding the functional evolution of nuclear-encoded PEP subunits, which were thought to be adaptations associated with plant terrestrialization. Instead, it strongly suggests that structural adaptations have shaped the complexity of the PEP within the green lineage via convergent evolution. As such, our findings lay the groundwork for deepening our understanding of the evolutionary adaptations and fundamental principles governing chloroplast gene expression in diverse photosynthetic organisms.

SPC core facility for biophysics at EMBL Hamburg

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The Sample Preparation and Characterization Core Facility (SPC) at the European Molecular Biology Laboratory (EMBL) Hamburg serves as a comprehensive resource for biophysical analysis of biomolecules and their interactions with binding partners. Located within the Centre for Structural Systems Biology (CSSB) in Hamburg, Germany, our facility caters to both academic and industrial researchers, providing a diverse array of services including consulting, training, equipment access, sample handling, and data analysis. Affiliated with prominent transnational research organizations such as Instruct - ERIC, MOSBRI and HALRIC, we facilitate access to our facilities for academic researchers worldwide. Continuously expanding, our data analysis web-server, accessible at spc.embl-hamburg.de, is equipped with cutting-edge tools for biophysical data analysis, with recent updates including modules dedicated to dynamic light scattering (DLS) data analysis. Additionally, we actively contribute to method development in biophysics, exemplified by our work on deriving binding affinities from thermal shift data and developing a pipeline for the characterization and optimization of membrane proteins prior to cryo-electron microscopy (cryo-EM) experiments.

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Macromolecular Crystallography Beamlines at EMBL-Hamburg

Isabel Bento

European Molecular Biology Laboratory (EMBL), Hamburg

The European Molecular Biology Laboratory (EMBL) operates an Integrated Facility for Structural Biology at PETRA III storage ring in Hamburg. This facility houses two Macromolecular Crystallography Beamlines, namely P13 and P14, which offer versatile capabilities ranging from serial crystallography to rapid fragment screening experiments. P13 delivers high photon fluxes (5 x 10^12 ph/sec at 12.7 keV) and operates within the energy range of 4 to16 keV keV. Optimized anomalous scattering experiments on the K-edges of calcium, potassium and heavier elements can be performed routinely for identification of ions with 5 min data collections. The beamline is equipped with an EIGER 16M detector and the beam size can be adjusted down to 15µ to accommodate diverse experimental needs.P14 is equipped with a CdTe 16M Eiger2 detector and offers two distinctive beam modes: a collimated and a micro-focusing mode (user can toggle between both modes within 20 seconds). In collimated mode, CRLs are used to create a top-hat beam, which can be shaped to any size between 20 and 300 µm at the sample position. This homogeneous beam ensures superior data quality, proving to be especially crucial in structural studies of large macromolecular complexes [2], or in ultra-high resolution studies of enzymatic mechanisms under precise dose control [3]. The micro-focusing mode, enables successful data acquisition by serial synchrotron crystallography methods using micro crystals or in situ data collection with CrystalDirectTM crystallization plates [4]. Such type of data collection is integrated into a highly automated workflow with a high level of user control and interactivity. The beamline software and the associated IT infrastructure supports the efficient handling of huge data flows and real-time data evaluation. P14 beamline is also equipped with a second experimental end-station (the 'T-REXX' end-station), devoted to pump-probe time-resolved experiments [5].

For further details, please refer to: www.embl-hamburg.de/services/mx

The beamlines are part of the Instruct Centre EMBL (https://instruct-eric.org/centre/embl-hamburg/) and Instruct supported access is available.

Mapping nanobody binding epitopes on integral membrane proteins by hydrogen deuterium exchange mass spectrometry

Peter Harrison

Diamond Light Source

In structural biology, the use of nanobodies has been transformative in trapping protein conformations and enabling structural analysis of complex macromolecules, particularly integral membrane proteins (IMPs). With cryo-EM of IMP-nanobody complexes becoming the method of choice, identifying the most potent binders and mapping binding sites becomes crucial in guiding the selection of nanobodies. Existing biophysical approaches enable rapid assessment of nanobody potency, but a systematic workflow for mapping their binding to membrane proteins does not exist. Hydrogen-deuterium exchange mass spectrometry (HDX-MS) enables fine mapping of solvent exposed surfaces on proteins as well as secondary structure changes. Therefore HDX-MS can be used to map the diversity of binding sites and conformational changes for various nanobodies, by comparing spectra from isolated antigens and antigen-nanobody complexes. We have developed a standard workflow combining nanobody and membrane protein production with native and HDX-MS analysis to guide the identification of nanobodies as structural chaperones and provide important structural information such as epitope mapping. We show a proof of concept of our workflow on two sub 100 kDa membrane proteins.

Lysine in Transmembrane Helix 5: The Wind Hook in the Inner Gate of L-Amino Acid Transporters

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BasC is a bacterial LAT transporter with a known structure in an inward-facing conformation. In this study, single-molecule Fluorescence Resonance Energy Transfer (smFRET) measurements were conducted on the cytoplasmic gate of BasC by attaching pairs of dyes at different positions in the inner gate to measure differences in distances and dynamics. Under apo conditions, BasC demonstrated an inner gate in equilibrium between open and closed states. Incubation with substrates induced a shift in the equilibrium towards a more closed state, and this movement was blocked by nanobodies that interact directly with transmembrane helix (TM) 1a, as described by the cryo-EM structure of the complex. Molecular dynamics and functional analysis support the notion that the fully conserved lysine in TM 5 of the LAT is key for the stability of TM1 and the closure of the cytoplasmic gate. Overall, these findings provide insights into the transport mechanism of BasC and underscore the importance of the lysine in transmembrane helix 5 in the cytosolic gating of LAT transporters.

A Hybrid NMR approach to study oxidase substrate binding in directed evolution

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McoA is a hyperthermostable multicopper oxidase from Aquifex aeolicus, characterized by a highly flexible 29-residue long methionine rich loop (Met-loop). The Metloop is spatially located over the T1-Cu site and binding pockets and undergoes through transient open-to-close transitions that either expose or occlude the T1-Cu site. An evolved variant with enhanced catalysis for ABTS oxidation, a standard model substrate for laccases, was obtained through directed evolution. Laccases are in high demand as innovative solutions in green chemistry and sustainability since their only byproduct is water. A hybrid NMR strategy was devised to decouple enzyme-substrate interaction from catalysis. We combine Saturation Transfer Difference (STD)- with methyl-NMR to gain valuable insights into the binding interactions between bulky aromatic substrates and McoA, evolved variant 2F4 and loop truncated variants.

From the substrate viewpoint, we identified the ideal NMR conditions that allowed us to assign the resonances for ABTS. Despite displaying slightly stronger STD effects with 2F4 variant, the binding substrate-epitope remains unchanged by the directed evolution. Notably, ABTS binding to 2F4 exhibited increased promiscuity, aligning with improved catalysis. This distinct characteristic can be attributed to the positioning of the Met-loop over the active T1 Cu center in this enzyme. Variants with looptruncated demonstrated reduced non-specific binding. The application of methyl-NMR allowed us to scrutinize the direct interaction between the Met-loop and the aromatic substrate, providing further insights into the molecular mechanisms governing substrate binding in the evolved enzyme. This comprehensive NMR strategy contributes to a nuanced understanding of the intricate interplay between enzyme and substrate, paving the way for the targeted evolution of enzymes with improved catalytic properties.

Unusual RNA structures - G-quadruplexes are present within the influenza A virus genome and are important during viral replication

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Influenza virus is an interesting research subject due to its serious threat to global public health. A comprehensive understanding of the viral RNA (vRNA) structure provides important insights into its biological function. It is known that various structural motifs from the influenza A virus (IAV) genome are highly conserved among strains. Herein, we focused on the noncanonical structures called G-quadruplexes (G4s) formed within the G-rich sequences. More recently, it has been revealed that G4s are present in the Zika virus, Zaire ebolavirus, and also in SARS-CoV-2 genomes. These findings encouraged us to inspect the IAV genome for the presence of G-rich sequences.

Firstly, we investigated the influenza A/California/07/2009(H1N1) genome for the occurrence of potential G-quadruplex forming sequences (PQS). Based on our bioinformatics analysis we identified twelve PQS motifs within the influenza A/California/07/2009(H1N1) and determined that some of them are highly conserved among the IAV strains. Subsequently, we studied PQS ability to fold into a G-quadruplex structure by various biophysical methods. To this end, the UV-melting measurements, NMR, and CD spectroscopy, as well as thioflavin T (ThT) fluorescence measurements were performed. To determine the folding topology and molecularity in G4s, native polyacrylamide gel electrophoresis (PAGE) experiments were conducted.

Our results from biophysical experiments provided direct evidence for the formation of RNA G4s by several PQS oligomers. Moreover, the resultant CD spectra of PQS displayed the profile typical for parallel G-quadruplexes. The PAGE experiments revealed differences in G4s folding molecularity, suggesting bimolecular or tetramolecular structures. To sum up this part, we revealed that six PQS can form RNA G4s, however, three of them were confirmed by all utilized methods. Additionally, we showed that some PQS motifs are present within segments encoding polymerase complex proteins indicating their possible role in the virus biology.

These findings encouraged us to investigate their potential biological function(s). We hypothesize that G-quadruplex structures are formed in the IAV genome and can regulate viral replication. We assessed whether G4-specific ligands (TMPyP4 and BRACO-19) affect the vRNA G-quadruplex formation using reverse transcription (RT) stop assay. Our RT stop assays showed that this process was hindered by both compounds for all wild-type G4 variants. However, the most noticeable effect was observed for one of them after adding TMPyP4. Finally, we investigated the effect of the TMPyP4 compound on virus replication using a minireplicon system. Our analysis revealed that the ligand addition results in the significant inhibition of IAV replication. Overall, for the first time, we show that G4-specific ligands bind to the investigated IAV G4s and suggest that selected G4s can be potential novel anti-influenza drug targets.

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Solution Structure of Tubuliform Spidroin N-Terminal Domain and Implications for pH Dependent Dimerization

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Latvian Biomedical Research and Study Center

Spider silk is a marvel of natural engineering, renowned for its extraordinary strength and versatility. Orb weaving can produce up to seven different types of silk, each tailored for a specific function. The main components of the spider silk threads are large proteins called spidroins, and most of the spidroins are comprised of three distinct domain types. The conditionally high solubility and regulation of pH dependent spider silk formation is mediated by two conserved spidroin terminal domains– the N-terminal domain (NT) and the C-terminal domain (CT), whereas the highly variable central repetitive domain (Rep), is responsible for the silk properties. The NT forms a monomeric five-helix bundle at neutral pH and dimerizes at lowered pH, thereby firmly interconnecting the spidroins. Mechanistic studies with the NTs from major ampullate, minor ampullate, and flagelliform spidroins (MaSp, MiSp, and FlSp) have shown that the pH dependency is conserved between different silk types, although the residues that mediate this process can differ. Here we study the tubuliform spidroin (TuSp) NT from Argiope argentata, which lacks several well conserved residues involved in the dimerization of other NTs. We solve its structure at low pH revealing an antiparallel dimer of two five-α-helix bundles, which contrasts with a previously determined Nephila antipodiana TuSp NT monomer structure. Further, we study a set of mutants and find that the residues participating in the protonation events during dimerization are different from MaSp and MiSp NT. Charge reversal of one of these residues (R117 in TuSp) results in significantly altered electrostatic interactions between monomer subunits. Altogether, the structure and mutant studies suggest that TuSp NT monomers assemble by elimination of intramolecular repulsive charge interactions, which could lead to slight tilting of α-helices

Cryo-EM Structure of Potato Virus M

Rebeka Ludviga

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Potato virus M (PVM) is a filamentous ssRNA virus from the Betaflexiviridae virus family which infects plants of the nightshade family (Solanaceae), including many potato cultivars. Despite being an important pathogen, PVM is relatively less studied compared to other potato viruses such as Potato virus X (PVX) or Potato virus Y (PVY). Notably, no three-dimensional structures of any betaflexiviruses have been determined, and it is unclear how similar or distinctive their particles are compared to the much better studied alphaflexiviruses such as PVX. Like several other plant viruses, recombinant PVM particles could also be used as antigen carriers for vaccine development, however, these efforts require detailed knowledge of the particle structure. To address these issues, we set out to determine the high-resolution structure of PVM.

To produce the particles, the PVM coat protein (CP) gene was cloned and expressed in Escherichia coli which resulted in assembly of filamentous structures that, except for their variable length, were otherwise morphologically identical to the native virus. The particles were purified using density gradient centrifugation and used for structure determination using cryogenic electron microscopy (cryo-EM). From approximately 530 000 helical segments that were semi-automatically picked from ~5 800 micrographs, the PVM structure was reconstructed to a resolution of 2.3 Å (FSC 0.143 criterion) using the helical processing pipeline in the Relion software suite.

The overall structure of the PMV particles shows clear similarities to alphaflexiviruses and can be regarded as a hollow cylinder with an outer diameter of about 130 Å and a narrow, approximately 10-Å wide central lumen. In the particle, each subunit is translated by 3.78 Å and rotated by 40.8 degrees relative to the previous molecule, resulting in a left-handed helix with a pitch of 33.3 Å and approximately 8.8 subunits per turn. Except for a small β-hairpin, the CP is entirely α-helical and consists of a compact central globular domain and extended N- and C-terminal regions; the C-termini line the internal lumen while the long, pronounced N-termini make contact with several surrounding CP subunits on the particle exterior. The structure also reveals an RNA strand interacting with the CP, with five nucleotides bound to each subunit. Overall, the PVM structure helps to better understand the structural diversity of plant viruses and provides the foundation for structure-guided design of recombinant
PVM-derived vaccines

The Biochemistry and Structural Biology Unit at IEO, a platform for protein production and structural characterization

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The Biochemistry and Structural Biology Unit (BSU) at the European Institute of Oncology (IEO – Milan, IT) offers a comprehensive range of services in protein biochemistry, biophysics, and structural biology. We work closely with researchers to facilitate the production and structural characterization of protein macromolecular complexes. The BSU employs optimized protocols and SLIC-based plasmid libraries for recombinant protein expression in various hosts, including E. coli, insect cells (Sf9 and Hi5), and suspension HEK293 cells. Together with multiple AKTA systems, this forms a robust platform for protein expression and purification. Our biophysical instruments encompass SEC-SLS, mass-photometry, micro-ITC, BLITz, and FP plate readers. We have a mosquito nanoliter dispenser for crystallization screenings, all the necessary equipment for hit optimization, regular access to synchrotron facilities and computational resources for structure determination. Additionally, we provide training and support in cryoEM, from sample preparation to data processing. We recently established a cryoEM lab, including a BioComp gradient maker, a glow discharger and a Vitrobot Mark IV. We have regular access to 200 kV and 300 kV cryEM for screening and data collection. For data processing, we operate a multi-GPU workstation that will be soon integrated with a multi-GPU high-performance cluster.

Allostery through DNA drives phenotype switching

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Weizmann Institute of Science

Allostery is a pervasive principle to regulate protein function. Here, we show that DNA also transmits allosteric signals over long distances to boost the binding cooperativity of transcription factors. Phenotype switching in Bacillus subtilis requires an all-or-none promoter binding of multiple ComK proteins. Using single-molecule FRET, we find that ComK-binding at one promoter site increases affinity at a distant site. Cryo-EM structures of the complex between ComK and its promoter demonstrate that this coupling is due to subtle dynamic DNA structural changes. Modifications of the spacer between sites tune cooperativity and show how to control allostery, which paves new ways to design the dynamic properties of genetic circuits.

The effect of phosphoserine 324 on the paired helical filaments of tau protein

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Intrinsically disordered tau protein belongs to the family of microtubule-associated proteins whose primary function is the stabilization and regulation of the microtubules. The properties and function of tau are heavily dependent on its post-translational modifications, including phosphorylation, glycosylation, acetylation, truncation, and others. Especially phosphorylation and truncation are closely associated with tau protein aggregation, which is a common tau pathology present in Alzheimer's disease and other neurodegenerative diseases. Each of them is characterized by a specific type of tau fibrils. Neurofibrillary tangles composed of paired helical filaments and straight filaments are typical for Alzheimer's disease. The study of the tau aggregation mechanism experimentally is still a challenging task. Molecular dynamics simulations provide us with a helpful insight into the dynamics of the fibrils. It has already been published that certain phosphorylations can enhance the stability of the paired helical filaments of tau protein. In our study, we explore the effect of the phosphorylated Ser324 on the free energy profile of the dissociation of the paired helical filaments. This phosphorylation position is highly relevant for Alzheimer's disease because it is frequently present in the neurofibrillary tangles.

Carpe pili! Structure and infection mechanism of Casadabanvirus JBD30 revealed by the combination of cryo-electron and fluorescence microscopy

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Increasing numbers of infections caused by multi-drug resistant bacteria have renewed the interest in bacteriophages. However, our understanding of many aspects of phage lifecycles is incomplete. Here, we used the combination of cryo-electron tomography, single particle analysis and super-resolution fluorescence microscopy to resolve the replication mechanism of Pseudomonas aeruginosa siphophage Casadabanvirus JBD30. JBD30 binds to P. aeruginosa pili type IV by embracing it with its tail fibres. The specificity of the bond orients the phage baseplate towards the cell membrane and ensures that as the pili retracts, the tripod of the receptor binding proteins reach the lipopolysaccharides of the outer bacterial membrane. The tripod and baseplate tip open and three copies of the tape measure protein are released forming a channel through the bacterial cell wall. The release of the tail tape measure proteins triggers DNA ejection. For replication, phage DNA redistributes throughout the cytoplasm. JBD30 major capsid proteins assemble into procapsids that expand 7% in diameter upon filling with phage dsDNA. The capsid expansion enables the attachment of minor capsid proteins that stabilize the capsids under adverse conditions. The DNA-filled capsids are joined with 180-nm-long tails, which bend easily because contacts between the successive discs of major tail proteins are mediated by flexible loops. The newly assembled virions are released 85 minutes post-infection. Due to sequence conservation, it is likely that the structural features and replication mechanisms described here for bacteriophage JBD30 are conserved among all Mulike siphophages that utilize pili type IV as an initial receptor.

Identifying tetraspanin-partner interactions in the function of the nervous system

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Tetraspanins form a group of thirty-three proteins in humans that are pivotal in a diverse array of functions. These functions span from supporting membrane stability and facilitating cellular adhesion to regulating proliferation and proteolysis. Within the context of the nervous system, tetraspanins undertake a crucial role in the formation of paranodes and in facilitating the clustering of the fast excitatory AMPA receptors, alongside other functions. This versatility arises from the ability of tetraspanins to interact with a wide spectrum of partner proteins, that are often disparate in evolutionary lineage and protein structure. This study aims to elucidate the details of the protein-protein interactions of tetraspanins with their partner proteins in the nervous system. Two structure predictiontoaals, Alphafold Multimer and AlphaPulldown are used to screen a large number of protein-protein interactions that would otherwise require substantial investment to test in the lab. Predicted high-scoring interactions are validated using biophysical techniques such as high-performance liquid chromatography, microscale thermophoresis and surface plasmon resonance. Once interactions are experimentally verified we aim to structurally characterize these complexes by protein crystallography and cryo-electron microscopy. This work will guide the design and interpretation of cellular studies into the function of tetraspanins in the organization of the nervous system.

Resolving the structure of sub-80 kDa prolyl oligopeptidase using a CryoEM based biophysical techniques.

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The RNA-induced silencing complex (RISC) is a key player in the regulation of eukaryotic gene expression at the post-transcriptional level. In this process small RNA molecules such as those involved in RNA interference (RNAi), interact with mRNA, either preventing its translation into protein or triggering its degradation. Argonaute (AGO) is the key protein component of the complex, and indeed, the minimal RISC comprises AGO and a tightly bound small guide RNA (~20-30 nt). Additionally, several chaperones, including Hsc70, facilitate and control the assembly of the mature RISC complex.

In this study we carried out the structural characterization of *Arabidopsis thaliana* AGO1 RISC complex in the presence of Hsc70 using cryogenic electron microscopy (cryo EM). An AGO1 mutant in an essential tryptophan (AGO1^{W785A}) was used to stabilize the AGO1-Hsc70 complex in the small RNA-bound state. Despite the lack of high resolution, our current findings indicate that the overall arrangement of AGO1 closely resembles that of previously described AGO10 from *Arabidopsis thaliana* and human AGO1. Notably, AGO1^{W785A} exhibits significant flexibility, particularly evident in the b-finger when compared to wild type AGO1, in which it appears as a rigid element, projecting away from the main body of the protein. The intrinsic flexibility of AGO1^{W785A} at the b-finger may play a pivotal role in the RNA loading process and would serve as a mechanical latch, facilitating a rapidly reversible push-to-bind-to-release mode of Hsc70-AGO interaction during RISC formation.

Additionally, It is noteworthy the presence of a large loop in the C-terminal part of the PIWI domain containing a conserved phosphocluster involved in RISC dissociation. This loop, not previously observed in eukaryotic AGO structures, likely due to disorder, forms a prominent protrusion in the same region as the b-finger. Because the presence of a Piwi-loop is recurrent in all eukaryotic Ago proteins, these results raise the intriguing possibility that Ago proteins use the Piwi loop to exert mechanical on Hsc70 to achieve reversible association.