The 6th CryoNET Symposium

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Program & Book of Abstracts

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Book of Abstracts

Structure Characterization of a Thyroid Hormone Transporter

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Through the iodination of tyrosine, hormones form that orchestrate countless fundamental cellular processes: thyroid hormones, known also as iodothyronines, take part in essential bodily functions such as neurodevelopment, metabolic homeostasis, and growth. Dysregulation of the cellular levels of iodothyronines leads to serious consequences, whose severity and treatability vary depending on different parameters, such as the stage of life and the efficiency of hormone transport. Owing to their hydrophobic nature, thyroid hormones were thought to passively diffuse into cells. Recent studies have shown that this occurs via transporters. Our target transporter is a mammalian membrane protein that facilitates the uptake and efflux of iodothyronines. Loss-of-function mutations in its gene lead to serious consequences which manifest in psychomotor retardation and severe intellectual impairment. Therapeutic options are still inadequate and limited to the alleviation of thyrotoxicity with no apparent neurological improvement – failing to compensate for the defective transporter function. Despite its significance, the structure of this transporter remains experimentally undefined. Therefore, elucidating the structure could assist in a more in-depth understanding of its mechanism of action and its interaction with other proteins, and may contribute to the creation of a better targeted therapy. For these reasons, we have rationally designed a construct to express and produce our target in HEK293 and Spodoptera frugiperda (Sf9) cell lines, while being able to monitor and optimize the process using the GFP fusion partner. We succeeded in developing a good purification profile using an affinity tag. We have collected 2D classes using cryo-EM and have used this structural feedback to introduce more optimal conditions to resolve the 3D structure. We hope that unearthing the structure of our transporter will contribute to a better understanding of the transport of thyroid hormones, and consequently, to a selective drug candidate with real therapeutic potential. We also hope that this research will be the building block for scientists working on the structural

determination of other transporters, paving the way for fresh perspectives in the field of structural biology.

Single Particle Cryo-EM Analysis of the Transient Complex Between Human Aquaporin-4 and Calmodulin

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Membrane integral water channels known as Aquaporins (AQPs) are found in diverse cell types of our bodies, and are critical for body water homeostasis (1). Their activity can be controlled by changes in subcellular localization, a process that is dependent on protein-protein interactions (2). Aquaporin-4 (AQP4) is abundantly expressed in astrocytes in the CNS (3) and its sub-cellular localization is controlled by interactions with calmodulin (CaM). Pathological water flow through AQP4 was shown to play a critical role in CNS edema and targeting its CaM-dependent relocalisation has been shown to be a viable target for CNS edema treatment (4). Here, we aim to elucidate the structural mechanism behind how AQP4 cellular localization is controlled by interactions with regulatory protein CaM by structurally characterizing the transient complex of AQP4 and CaM using single particle cryo-electron microscopy (cryo-EM). A 4 Å Cryo-EM structure of the complex has been solved and we are working on data processing to reach an even higher resolution of the complex. Our work shows the first cryo-EM structures of an aquaporin in complex with its regulatory protein, establishes an excellent tool for structure determination of a protein-complex between membrane protein AQP4 and soluble protein CaM in detergent by cryo-EM, and paves the way for structure-based drug design targeting AQP complexes.

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CryoEM Structure Determination of a Human Zinc Transporter

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The solute carrier (SLC) transporter superfamily is home to more than 400 transporters, of which a growing number have been implicated in Mendelian diseases (monogenic diseases) as mutations in substrate specific transporters give rise to disease phenotypes (1).

Our 80 kDa dimeric zinc transporter is part of the SLC30-family. Our target, specifically, has been shown in KO mice studies to be responsible for accumulating zinc into the synapses of glutamatergic circuits in the hippocampus, the amygdala, and the cortex (2). Understanding the structural changes in this transporter that give rise to cognitive impairment, febrile seizures, Alzheimer's disease, and gendered schizophrenia is impeded by a lack of high-resolution structural images of the transporter (3).

Our aim is to capture the structure of the zinc transporter, as well as its clinically relevant mutations, at atomic resolution with single particle cryoEM.

The transporter constructs were engineered with N-terminal FLAG-tag and C-terminal GFP and 10x-His-tag. Utilizing the Bac-to-Bac system, the transporters were expressed in *Spodoptera frugiperda* (*Sf9*) and purified using affinity chromatography followed by size exclusion chromatography. Purification parameters, tag placement, and yield were optimised and purified protein with and without addition of ligand was vitrified and sent for cryoEM screening at SciLifeLab Stockholm with a Talos microscope, after which high-resolution structural data was collected using a Titan Krios.

We are currently underway structurally determining this target to expand our understanding of zinc transport in zinc-carrying synapses and their role in neurocognitive disorders and to facilitate future therapeutic drug design.

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Structural characterization of pH- and calcium-modulated closed and open states in a pentameric ligand-gated ion channel DeCLIC

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Pentameric ligand-gated ion channels (pLGICs) are critical mediators of electrochemical signal transduction in a variety of excitable cells. Bacterial pLGICs can provide valuable insight into fundamental mechanisms of gating, as they often desensitize to a lesser extent than eukaryotic channels, while retaining sensitivity to modulatory conditions such as pH and environmental calcium. A new prokaryotic family member (DeCLIC) from a Desulfofustis deltaproteobacterium was recently identified, including a previously uncharacterized N-terminal domain, and was shown to be modulated by calcium. Here we used cryo-electron microscopy (cryo-EM) to identify multiple conformations of DeCLIC under acidic conditions. We identified a novel open state in both the presence and absence of calcium, notably divergent from the open X-ray structure. Under calcium-containing conditions, a closed conformation was also identified, reminiscent of previous neutral-pH structures. Conversely, calcium-depleted conditions yielded an alternative closed conformation showing evidence of dynamic rearrangements in the N-terminal lobes. These data provide new insight into pH and calcium modulation and the under-characterized open state of pentameric channels, as well as a novel mechanism of dynamic ion-channel regulation via an N-terminal module.

Structural and functional characterization of streptococcal SpnA-host interactions

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Every major health organization has identified infectious diseases and the concomitant increased resistance to antibiotics as serious global threats. We address these problems by studying the *Streptococcus pyogenes* bacterium; an important pathogen, ranking globally among the top ten causes of mortality from infectious diseases with an estimated >150 000 annual deaths. During an infection, *S. pyogenes* encodes for a variety of virulence factors; many of these virulence factors have not been thoroughly studied. One of these virulence factors is the *S. pyogenes* nuclease A, SpnA, originally described as a cell-wall associated DNAse degrading host neutrophil extracellular traps (NETs) via its C-terminal endo/exonuclease domain.

We study the structure and function of SpnA by combining quantitative and structural proteomics mass spectrometry with integrative structural biology methods. Our results combining AlphaFold modeling, single-particle cryoEM and hydrogen-deuterium exchange mass spectrometry demonstrate that SpnA harbors disordered regions in its N-terminus. We have demonstrated by combining affinity-purification and bacterial surface adsorption mass spectrometry, that in human blood plasma SpnA binds the complement system membrane attack complex (MAC). By combining affinity-purification with crosslinking mass spectrometry we have further been able to determine that SpnA specifically targets the MAC assembly intermediate C5b-C7. We hypothesize that SpnA acts like another streptococcal virulence factor SIC, streptococcal inhibitor of complement, which targets the C5b-C7 complex preventing its insertion into the streptococcal membranes, hence preventing bacterial lysis. Our results indicate that in addition to degrading host DNA in NETs, SpnA mediates other central functions in host immune evasion in streptococcal pathogenesis.

VitroJet: Moving Cryo-EM Sample Preparation into the New Era

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Cryo-electron microscopy (cryo-EM) is increasing in popularity as a method for biomolecular structure determination. [1,2] Once a good quality grid is prepared for the microscope, data collection and biomolecular processing are streamlined. Within the cryo-EM workflow, the main bottleneck is sample preparation. Optimizing the combination of biochemistry and grid preparation needs many and long iterations. The number of required iterations on the microscope can be reduced significantly with reproducible grids of which the quality can be determined before usage. This makes the cryo-EM infrastructures more efficient, enabling structure determination in a shorter timeframe. For this reason, the VitroJet was developed. The VitroJet focuses on controlling the grid preparation process, enabling to investigate protein behavior in a structured manner.[3] An integrated plasma treatment makes grids reproducibly hydrophilic. Sample deposition through pin-printing decreases shear forces in comparison to the traditional blotting method and reduces the required volume extensively. Furthermore, it allows for reproducible control of layer thickness, on which the obtainable resolution of the protein of interest is highly dependent.[4] Visual feedback from two implemented cameras enables sample quality inspection and layer thickness estimation on a nanoscale before electron microscope screening. The visual feedback of the grid camera correlates with the grid atlas taken on the electron microscope, giving a clear indication of the number of usable holes before electron microscopy imaging. Jet vitrification enables vitrification of autogrids, reducing manual handling and removing the need for the tedious postclipping process. Since any grid type and only sub-nanoliter volumes are used, the VitroJet can be used for all single particle analysis applications. In this manner, the electron microscopes can be fed with high quality samples to investigate protein behavior. In collaboration with different labs over the world, we have obtained results varying from protein complexes, membrane

proteins, and cellular like applications. By adapting the parameter settings of the VitroJet, ice thickness and gradients can be adjusted. Measuring of ice thicknesses with the implemented VitroJet camera saves microscope screening time. Ice thicknesses were determined by using the energy filter method in the electron microscope [5], and the outcome was correlated with VitroJet settings and camera feedback. We observed that by changing the velocity, as well as the distance between the grid and the pin during writing, the layer thickness can be adapted in a controlled manner. We show high reproducibility when using the same protocol settings. Furthermore, a high number of different samples have been processed together with our customers, of which some results will be shown. Overall, the VitroJet enables high control and reproducibility of a broad range of cryo-EM samples.

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Structural Understanding of Pore Formation by Mitilysin Toxin

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Cholesterol-dependent cytolysins (CDCs) are a group of pore-forming proteins produced by gram positive genus of *Bacillus*, *Clostridium*, *Listeria*, *Arcanobacerium* and *Listeria* (1). They are responsible for membrane permeabilization and thereby aid in bacterial virulence or pathogenesis. Many CDC-producing bacteria are pathogenic in nature and cause diseases, such as pneumonia, meningitis and necrotizing fasciitis (2). CDCs are secreted as soluble monomer and binds to cholesterol in target membranes. Membrane-bound monomers subsequently oligomerize and undergo large conformational changes to form membrane-inserted pores (3). The mechanism of pore formation is studied for some representative class of CDCs but the key pore forming residues in CDCs are not conserved hence the mechanism cannot be generalized for all CDCs.

Major outstanding questions are thus, how does this class of CDCs align their neighboring b-strands to mediate pre-pore formation, subsequent pore formation and membrane permeabilization, and specifically, what residues are involved in bringing the conformational changes? Here in the current study, we are using toxin called Mitilysin (Mly) secreted by *Streptococcus mitis* to study the pore formation process. This study will answer these critical questions by characterizing pore-pore and pore forms of Mly assembled on cholesterol membranes by cryo-electron microscopy (cryo-EM). Further, we will determine whether altered lipid compositions of model membranes influence Mly pore assembly and lysis. Revealing the mechanisms that underpin the activity of these atypical CDCs will aid in our understanding of pathogenesis.

We have successfully formed the Mly pores on liposomes containing cholesterol. Mly pore were extracted by detergent screening and identified DDM as best detergent.

Initial cryo-EM analysis indicate that Mly can form bigger pores of size 30-35 nm and have preferred orientation on the cryo-EM grid.

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Nematocida displodere mechanosensitive ion channel of small conductance 2 assembles into a unique six-channel super-structure *in vitro*

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Mechanosensitive ion channels play an essential role in reacting to environmental signals and sustaining cell integrity by facilitating ion flux across membranes (1). For obligate intracellular pathogens like microsporidia, adapting to changes in host environment is crucial for survival and propagation (2,3). Despite representing a eukaryote of extreme genome reduction (4), microsporidia have expanded the gene family of mechanosensitive ion channels of small conductance (MscS) through repeated gene duplication and horizontal gene transfer. At least five copies of MscS are present in each to-date characterized microsporidian genome: One subfamily related to eukaryotic MscS and the other of bacterial origin. A single copy of the bacterially derived mechanosensitive ion channel of small conductance 2 (MscS2) is highly conserved amongst all microsporidian species sequenced to date. However, compared to its bacterial counterpart it is extremely reduced and it is unclear if MscS2 forms a channel protein and if so, what role it plays in microsporidia (2).

Here, we investigated the cryo-electron microscopy structure of MscS2 from *Nematocida displodere*, an intracellular pathogen of *Caenorhabditis elegans*. We purified MscS2 and used size exclusion chromatography, negative-stain transmission electron microscopy, cryo-electron microscopy, and mass photometry to analyze size and structure.

Nematocida displodere MscS2 assembles into a unique superstructure *in vitro* with six heptameric MscS2 complexes interacting through their transmembrane domains. Individual MscS2 channels are oriented in a heterogeneous manner to one another, resembling an asymmetric, flexible six-way cross joint. This highly unusual assembly provides a novel basis to design oligomers that interact through hydrophobic interfaces.

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CEllulose SynthAse in its Native Environment (CESANE)

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The main component of the plant primary cell wall (PCW), cellulose, plays a pivotal role in facilitating plant growth and morphology and is a major renewable resource with potentially widespread industrial applications. The cellulose synthase (CESA) complex (CSC) is the major complex synthesizing the PCW (1). The CSC is likely build as hexamer of CESA trimers along with other binding partners (2, 3). These individual CESAs are grouped into distinct clades of which CESA1, CESA3, CESA6 and CESA4, CESA7, CESA8 are involved in synthesis of the primary- and secondary cell wall, respectively. Co-immunoprecipitation studies have revealed a CESA1:CESA3:CESA6 ratio of 1:1:1 suggesting a heterotrimeric arrangement. Conversely, homotrimeric cryogenic electron microscopy (cryo-EM) structures have been determined of recombinantly produced PttCesA8 (8G2J, 8G27) and GhCESA7 (7D5K) (4, 5). It remains a subject of speculation whether the CSC comprises homo- and/or heterotrimers and the overall architecture of the CSC and its native environment within the plant plasma membrane remains uncharacterized. The CSC profoundly influences plant biology as it produces the structural framework that supports plant stature and defines the fundamental aspects of plant morphology. Despite its paramount importance, our understanding of the fundamental mechanisms of the CSC remains limited due to challenges associated with high-resolution visualization of the complex in its native environment. Cryo-EM tomography (cryo-ET) represents a promising avenue to determine central structural details about the CSC, In this project, we aim to use cryo-ET to gain unprecedented insights into the intricate molecular machinery responsible for cellulose production by plant cells, ultimately bridging our current knowledge gap and unveiling the secrets of cellulose synthesis in planta. Consequently, gaining a comprehensive understanding of the molecular architecture of the CSC, responsible for cellulose biosynthesis, holds the key to advancing both fundamental plant biology and practical biotechnological applications.

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Dynamic inter-domain transformations mediate the allosteric regulation of human 5, 10 - methylenetetrahydrofolate reductase

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One-carbon metabolism drives an extensive network of signaling and biosynthetic pathways essential for cell growth and redox regulation[1]. In all domains of life, 5,10-methylenetetrahydrofolate reductase (MTHFR) signals the commitment of folate-derived one-carbon units into biosynthesis of the global donor of trans-methylation, S-adenosyl-L-methionine (SAM)[2]. Eukaryotic MTHFR appends to its conserved TIM-barrel catalytic domain (CD) a unique regulatory domain (RD) that confers feedback inhibition by SAM[3]. We determined, through cryo-electron microscopy, multiple structures (2.8-3.1 Å) of human MTHFR bound to SAM, and its demethylated product S-adenosyl-L-homocysteine (SAH) that relieves SAM inhibition. In the active SAH-bound state, the CDs are flexible and accessible for catalysis. SAM binding in the inhibited state substantially rearranges the inter-domain linker, which remodels the RD pocket, reorients the CD and inserts a hydrophobic plug into the active site. The switch between the active and inhibited states is facilitated by binding of either one SAH or two SAM molecules to the RD, respectively. Occupation of both SAM sites is required for inhibition, but the second site is occluded in the presence of SAH. Together our data explain the long-distance inhibitory mechanism of MTHFR employed by dual SAM binding, demonstrate how a single bound SAH confers a dis-inhibitory signal, and paves the way for rational design of novel therapeutics targeting MTHFR.

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Microsecond time-resolved cryo-EM on a bacterial phytochrome

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One of the most important environmental factors living organisms need to adapt to is radiation from the Sun. The ability to respond to ever-changing light conditions is made possible by a variety of photosensitive proteins, the most well-known examples being opsins, the receptors responsible for vision in animals. In algae, fungi and even plants, the role of light is more complex: photosensitive proteins control cell division, rearrangement of organelles and supracellular coordination. Although most light receptors have been identified, and there are a number of solved low to mid resolution structures for these proteins, atomic resolution is rarely achieved due to the combination of their small size and flexibility. For this reason, dynamic structure and signal transduction is little understood in most cases. In my project, I aim to solve the dynamic structure of the bacterial light receptor P. aeruginosa bacteriophytochrome at high resolution. Since crystallization of the full-length proteins is not feasible, my method of choice is single-particle cryo-electron microscopy. I have recorded single-particle micrographs of the protein on a Titan Krios microscope and am currently working on reconstructing both the resting and the light-activated states of the protein.

Structural characterization of FZD_7 unravel the importance of a water network and cholesterol in Frizzled function

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Frizzleds (ten isoforms: FZD_{1-10}) belong to class F of G protein-coupled receptors (GPCRs), which remains poorly understood despite its crucial role in multiple key biological functions, including embryonic development, stem cell regulation and homeostasis in the adult.

FZD_7 – one of the most studied members of the family – is more specifically involved in migration of mesendoderm cells during development [1] and renewal of intestinal stem cells in adults among others[2]. Moreover, FZD_7 has been highlighted for its particular involvement in tumor development predominantly in the gastrointestinal tract[3].

This study reports the structure of inactive FZD_7, without any stabilizing mutations, determined by cryo-EM at 2Å resolution. We characterized an internal water network within the core of the receptor important for FZD_7 folding and receptor dynamics. Molecular dynamic simulation was then used to investigate temporal distribution of those water molecules and their involvement in FZD_7 protein dynamics. Moreover, we identified multiple lipids interacting with the receptor core. Among them, a conserved cholesterol-binding site that is currently being characterized employing mutagenesis combined with bioluminescence resonance energy transfer-based assays to highlight the key role of cholesterol in FZD protein dynamics and initiation of downstream signaling.

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Automatically sample membrane proteins in cryo - electron tomograms with TomoCHAMPS

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Cryo-electron tomography (cryo-ET) allows visualisation of molecular structures in their native states and context at near-atomic resolution. Due to technical restrictions in image acquisition such as the low intrinsic contrast of biological materials and minimised electron dose to prevent radiation damage in exposed sample, sophisticated computational methods are required to accurately extract and enhance signal of target objects. In particular, small membrane proteins (< 150 kDa) exhibit weak signal. To obtain highresolution structural information of such proteins, increasingly large datasets are acquired and mined. While existing softwares demand extensive manual curation in handling membrane protein cryo-ET data, we present TomoCHAMPS (tomography-based characterisation and analysis of membranes for protein sampling): an automatic image processing workflow dedicated to cryo-ET based characterisation and analysis of membranes for resolving membrane protein structures. By integrating improved opensource tools/methods and in-house scripts in a configurable manner, TomoCHAMPS is expected to support efficient processing of large in vitro and cellular transmembrane and membrane-binding protein cryo-ET datasets up until subtomogram averaging with minimised manual input required. Main features of TomoCHAMPS include pre-processing, tilt series alignment, 3D reconstruction, membrane segmentation within user-defined regions of interest, geometric analysis of membranes, and membrane protein sampling. Here, we showcase the application of TomoCHAMPS to three in vitro reconstituted membrane contact site cryo-ET datasets for resolving small membrane protein structures (~30-40 kDa).

Three-dimensional organization of the crystalloid organelle and its role in malaria transmission as revealed by Volume Electron Microscopy and Functional Genomic Analyses

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Due to the richness in molecular toolkits and the global effort toward elimination, the malaria parasite has become a powerful model for studying how vector-borne pathogens establish themselves for transmission in mosquitoes. Successfully transmitted pathogens must evade the innate immunity system and must endure the environment inside the mosquito. This climate-sensitive issue is of utmost importance, considering the rise of emerging and re-emerging vector-borne diseases around the world. Malarial ookinete is the stage formed after fertilization in the mosquito bloodmeal. Motile ookinetes transverse through the mosquito midgut to establish the oocyst stage at the gut lining. Biologically, ookinetes must achieve a plethora of tasks including meiosis, gut invasion, immune evasion and oocyst formation. This transition creates a population bottleneck in malaria life cycle, making it one of the most vulnerable targets for elimination.

Here we employed FIB-SEM and Electron Tomography to study the three-dimensional organellar organization of ookinetes from rodent malaria parasite model Plasmodium berghei. The overall structure reveals the invasion and motile machineries necessary for mosquito invasion. We found that an enigmatic organelle of honeycomb-like structure, the so-called crystalloid, is surrounded by large vesicular and organellar networks. This unique network consists of heme-containing vesicles, reticulate putative mitochondria with membrane stacks and a perinuclear structure. Using a machine learning approach, 4-5 crystalloid structures were found throughout the ookinete, unlike a classical perception based on TEM of having only two crystalloids per cell. Overall, the crystalloid network occupies approximately one-fourth of the

ookinete volume. The tomography data showed that crystalloid is, in fact, an ordered array structure with small oval-shape units, similar to the prolamellar body in plants. Analyses of known crystalloid components in sexual-stage genome-wide genetic screening showed that the components of crystalloid are often highly expressed in female gametes but do not function in fertilization. They are instead necessary for readying the parasite for successful propagations to make infectious parasites after fertilization.

Overall, this study shows how volume electron microscopy analyses complement ongoing efforts to transfer the technology of genome-scale functional genetic screens to the poorly studied ookinete and oocyst stages.

A deep dive into the mechanisms of P4B-ATPases

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Correct lipid composition of cellular membranes is crucial for cellular function across organisms and is controlled by specialized groups of lipid transport proteins. One of these are the lipid flippases, which are responsible for translocation of specific lipids towards the cytosolic membrane leaflet. The majority of lipid flippases are P4-ATPases, a subgroup of P-type ATPase in complex with an accessory subunit, of which P-type ATPases are classically ion transporters.
Since the revolution of Cryo-EM and the first structures in 2019, the knowledge of structure, function and regulation of P4-ATPases have expanded quickly[1].

However, a distinct subgroup, the P4B-ATPases, function without an accessory subunit and remain mysterious. The first structure of *Saccharomyces cerevisiae* P4B-ATPase Neo1p revealed high structural similarity to the main P4-ATPases and indicated flippase activity[2]. Later on truncations of mammalian ortholog ATP9A have been linked to severe neurodevelopmental phenotypes[3,4].

Through a combined approach of biochemical studies, multiple high-resolution Cryo-EM structures and mass spectrometry we have found clues to likely regulatory elements and native arrested states for Neo1p. Together these data suggests that P4B-ATPases as Neo1p accumulate in an tightly regulated autoinhibited state and is activated upon need, while still leaving the question of substrate specificity unanswered for now.

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Structure and dynamics of differential ligand binding in the human rho-type GABAA receptor

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The neurotransmitter gamma-aminobutyric acid (GABA) drives critical inhibitory processes in and beyond the nervous system, partly via ionotropic type-A receptors (GABA A Rs). Pharmacological properties of rho-type GABA A Rs are particularly distinctive, yet the structural basis for their specialization remains unclear. Here, we present cryo-EM structures of a lipid-embedded human rho1 GABA A R, including a partial intracellular domain, under apo, inhibited, and desensitized conditions. An apparent resting state, determined first in the absence of modulators, was recapitulated with the specific inhibitor (1,2,5,6-tetrahydropyridin-4-yl) methylphosphinic acid and blocker picrotoxin and provided a rationale for bicuculline insensitivity. Comparative structures, mutant recordings, and molecular simulations with and without GABA further explained the sensitized but slower activation of rho1 relative to canonical subtypes. Combining GABA with picrotoxin also captured an apparent uncoupled intermediate state. This work reveals structural mechanisms of gating and modulation with applications to rho-specific pharmaceutical design and to our biophysical understanding of ligand-gated ion channels.

Exploring Flavivirus replication organelles and viral assembly by cryo-electron tomography

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Tick-borne encephalitis virus (TBEV) is part of the flavivirus family that includes Dengue, Zika, Yellow Fever, and others. These enveloped, single-stranded positive-sense RNA viruses replicate within specialized intracellular structures called replication organelles, closely associated with the endoplasmic reticulum (ER). Using classical electron microscopy method, previous studies have shown that TBEV replicates its genome in negatively curved ER membrane spherules, but the mechanisms of such rearrangements, are poorly understood. Here, using the Langat virus, an attenuated version of TBEV, we sought to analyze the structure of the flavivirus replication organelles in their native host cellular environment by cryo-electron tomography. Our findings reveal a transformed ER with clustered replication organelles housing viral RNA replication, characterized by luminal filament-like structures. Notably, these viral organelles exhibit varying sizes, from small to mature, empty or filled, indicating diverse assembly stages. Intriguingly, these organelles possess thicker membranes compared to the ER, suggesting that specific membrane-bound proteins help these structures take shape, potentially contributing to the stabilization of ER membrane curvature. Moreover, our observations include the presence of both immature and mature viral particles surrounding these replication organelles, along with instances of viral budding events occurring in their proximity. This spatial connection between replication organelles and different stages of virus assembly challenges the conventional notion that flavivirus assembly predominantly occurs within the Golgi complex, implying a more intricate interplay between these processes within close cellular proximity.

Structural basis of proteasomal recognition of a branched ubiquitin chain

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Ubiquitin is involved in virtually every aspect of cell biology due to its ability to form a spectrum of conjugates of different architecture and functions. In addition to the well-established functional role of homotypic linear Ub conjugates, the functional importance of heterotypically branched Ub chains is emerging[1]. In the Proteasome Ubiquitin Pathway, the Ub chain branching has been shown to accelerate substrate degradation playing essential roles in cell cycle progression and response to proteotoxic stress[2]. Despite their proven importance little is known about the mechanism by which the branched Ub chains achieve their distinct cellular functions. Herein we report the cryo-EM structure of the human 26S proteasome in a complex with a branched Ub chain. The structure unveiled the molecular basis of how the proteasome recognizes the branching point, and how the branching provides avidity to enhance substrate binding compared to the canonical linear Ub chains. In binding to the branched Ub chain, the regulatory particle of the proteasome undergoes a series of hitherto uncharacterized conformational changes pertinent to substrate recognition. Our findings therefore provide new insights into the mechanism by which the proteasome decodes the complex Ub chain topologies beyond the poly-K48 Ub chains.

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Structure of the reduced microsporidian proteasome bound by PI31-like peptides in dormant spores

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Proteasomes are crucial for degrading misfolded and unwanted proteins in all known eukaryotes. For intracellular pathogens with extracellular stages efficient and tightly regulated proteolysis is vital due to limited available resources. However, in microsporidia, unicellular divergent parasites with extraordinarily streamlined genomes, the proteasome complexity and structure have been unknown which hindered our understanding of how these unique pathogens adapt and compact essential eukaryotic complexes.

Here we present the cryo-electron microscopy structures of the microsporidian 20S and 26S proteasome isolated from dormant or germinated *Vairimorpha necatrix* spores. The discovery of PI31-like peptides, known to inhibit proteasome activity, bound simultaneously to all six active sites within the central cavity of the dormant spore proteasome, suggests reduced activity in the environmental stage. In contrast, the absence of the PI31-like peptides and the existence of 26S particles post-germination in the presence of ATP indicates that proteasomes are reactivated in nutrient-rich conditions. Structural and phylogenetic analyses reveal that microsporidian proteasomes have undergone extensive reductive evolution, lost at least two regulatory proteins, and compacted nearly every subunit.

The highly derived structure of the microsporidian proteasome, along with the minimized version of PI31 presented in this study, highlights the potential for developing specific inhibitors and provides insight into the unique evolution and biology of these medically and economically important pathogens.

Structural characterization of a novel injection system in *Salmonella enterica*

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Salmonella enterica are Gram-negative, flagellated, rod-shaped enterobacteria. Some subspecies of the genus, isolated from cold-blooded animals, and are considered non-typhoid strains, resulting instead in salmonellosis and gastroenteritis upon successful infections in humans, which are rare, though deadly in immunocompromised patients. Typically, infections from *S. enterica* subspecies are passed on from reptiles, marine animals, and soil [1]. As microorganisms evolve under fierce competition with each other, evolving 'weapons', like the contractile injection systems (CIS), such as type six secretion systems (TSSS), pyocins/tailocins extracellular contractile injection systems (eCIS), like the antifeeding prophage (Afp), all specializing in piercing host cell membranes, while some additionally deliver protein payloads [2]. These CIS are related to the contractile tails of bacteriophages, commandeered by bacteria over evolutionary time, to help obtain a survival edge over other microbes. *S. enterica* code for a posited eCIS on their genome [2]. The novel *Salmonella* eCIS cassette codes for an injection system and effector proteins that are most likely injected into bacterial host cells, furthermore these eCIS genes code for an injection system that assembles into unique particles [3]. In this study, we propose the structural elucidation and eventual manipulation of this injection system, as it holds vast potential for biomedical engineering.

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Structural characterisation of AAA+ disaggregase ClpG

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Proteostasis describes a regulated process in which proteins' fate are determined to keep the cell functioning. Components of this process include a variety of proteins that can unfold, degrade, and/or re-fold other target aggregated proteins. One AAA+ protein family involved in these processes is the Clp protein family, which often require adaptor proteins to perform their functions1. However, one member of the Clp protein family, ClpG, has been described as a stand-alone protein that can unfold protein aggregates 2,3. This project aims to structurally characterize ClpG to understand how ClpG is regulated through the organization of various oligomeric states.

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New developments on chameleon: multihole grids as a screening tool and modified grid surfaces

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The chameleon® system automates the sample preparation workflow for single-particle cryo-EM and utilises blot-free technology with nanowire grids. In this poster, we will describe the expansion of our nanowire grid range, highlighting new developments including graphene oxide modified grids and our new multihole grids.

Modifying grids with a continuous layer of carbon, graphene or graphene oxide has been shown to help with issues such as orientation bias, as well as particle distribution and denaturation at the air-water interface. We show it is possible to apply a graphene oxide layer to Quantifoil® Active grids for chameleon and the effects this has on the grid preparation workflow. A range of samples was used to analyse what effects the addition of graphene oxide to Quantifoil Active sample supports has on particle orientation and distribution, and the obtainable resolution.

Quantifoil Active multihole grids are made of a copper mesh with self-wicking nanowires and either a holey carbon or gold support film. They contain four different hole sizes on each grid square, arranged in a regular pattern (Figure 1). They are an ideal screening tool to identify a sample's preference for a specific film geometry. Proteins of various sample types and shapes were tested to study the effect of hole size and spacing on particle distribution and orientation.

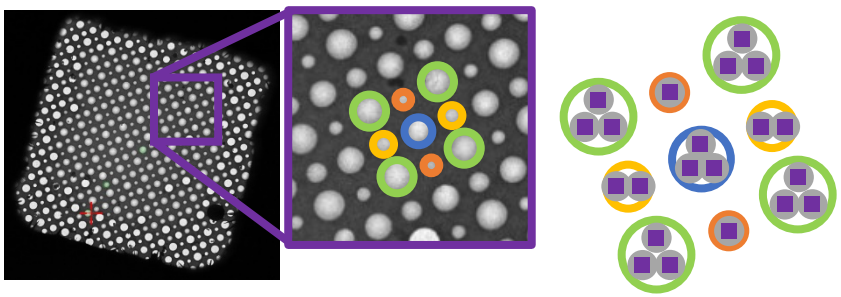

Figure 1. TEM image of a Quantifoil Active multihole grid square. The inset shows a close-up of an area in the gridsquare to highlight the four different hole sizes. The arrangement of image acquisition areas used for high-magnification imaging is shown on the right.

OccuPy: Heterogeneity- and occupancy-aware cryo-EM analysis based on spatial filtering

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Cryo-EM reconstruction averages images of individual particles into a reconstruction, to cancel noise and permit confident molecular modeling in 3D. Flexibility and heterogeneity in the averaged set of images however limit the fidelity of the reconstruction. We have developed a fast and simple algorithm based on spatial filtering to estimate the local heterogeneity of the reconstruction. In the absence of flexibility, this estimate approximates macromolecular component occupancy. We demonstrate the utility of this method for cryo-EM map interpretation, particle-image signal subtraction, and to promote divergence of clusters during conventionally employed maximum-likelihood methods. This method affords both visualization and algorithms the ability to focus on regions of variance in an unbiased way, which can be employed in a number of different capacities. It can e.g. be used to emulate homogeneous data, simplify hierarchical data clustering schemes, and perform accurate signal subtraction. Importantly, this quantifies heterogeneity from the start of processing, makes current processing procedures more reproducible, and makes it easier for structural biologists to interpret their data reliably.

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Structural Studies of Cyanobacterial Carboxysomes

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Rubisco, the enzyme fixing inorganic carbon from CO2 into carbohydrates, evolved at a time when the Earth's atmosphere contained mainly CO2. The Great Oxygenation Event, caused by the first photosynthetic organisms releasing oxygen into the atmosphere, posed a challenge for Rubisco, as it poorly discriminates between CO2 and O2 [1]. Cyanobacteria responded to this change in atmospheric composition by evolving a CO2 concentration mechanism made of bicarbonate importers and carboxysomes [2]. Carboxysomes are large bacterial microcompartments consisting of a protein shell encapsulating Rubisco and carbonic anhydrase (CA). The shell is differentially permeable to small molecules: it keeps O2 out but allows bicarbonate to enter, which is turned into CO2 by the CA, increasing the local concentration of CO2 around Rubisco. Thus, carboxysomes help overcome both the low catalytic efficiency of Rubisco and its poor specificity towards CO2. Understanding the structure and assembly principles of carboxysomes is important not only from a basic research standpoint, but also because it enables the rational engineering of nano bioreactors designed to encapsulate other biochemical reactions of interest. To understand how carboxysomes assemble, our goal is to determine structures of individual complexes reconstituted in vitro from recombinantly expressed components using single-particle cryoEM [3], and of entire carboxysomes purified from their native cyanobacterial sources [4], or in situ in intact cells, using cryoET.

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Towards the molecular architecture of the *tcp* DNA transfer machinery

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Antibiotic resistance genes are spreading worldwide and represent a major health problem to be addressed in this century[1]. The genes encoding antibiotic resistance are often carried by plasmids for which conjugation plays a key role in their spread in bacterial populations[2]. Conjugation is driven by type 4 secretion systems (T4SS), which are able to transfer a DNA strand from a donor bacterium to a recipient bacterium through their respective membranes. The structures of the assemblies that form the channel, known as mating pair formation, have been published for several systems in Gram-negative bacteria such as *E. coli*[3] or *H. pylori*[4,5]. However, no homologous structures have yet been published in Gram-positive systems[6]. One of the more studied T4SS in Gram-positive bacteria is the tcp system of *Clostridium perfringens*[7]. The proteins TcpA, TcpH, TcpC and TcpG have been shown to interact with each other and are involved in the mating pair formation[8].

The aim of this project was to recombinantly co-express these four proteins in E. coli, purify the complex formed in vivo and perform studies. The stoichiometry of the complex was determined using mass photometry, while the structure of the complex was studied using cryo-EM.

In addition to the complex, a hexameric ATPase of the system TcpF was studied. A truncation of the protein was recombinantly expressed in E. coli, its ATPase activity was characterised and the structure was studied by X-ray crystallography and cryo-EM.

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Cryo-EM structures of Staphylococcus aureus 70S ribosomes in complex with elongation factor G and fusidic acid

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Fusidic acid (FA) is an antibiotic effective mainly against gram-positive bacteria and it is commonly used against Staphylococcus aureus infections. FA stalls protein synthesis by binding to elongation factor G (EF-G) after translocation and preventing its release from the ribosome. EF-G is a GTPase that is essential in the translocation of the mRNA and tRNA during translation. There are structures of FA-locked ribosomal complexes in Escherichia coli (cryoEM) (1) and Thermus thermophilus (X-ray) (2). However, no structures of the clinical target are available. There are three main types of FA resistance in S. aureus: fusA, involving mutations on EF-G (3); fusB, mediated by the expression of a resistance protein (4); and fusE, mutations on ribosomal protein L6 (5). To fully understand these mechanisms, a structure of the clinically relevant system is crucial, as it allows for a deeper understanding of the effects of mutations, which might involve specific interactions with the ribosome or the antibiotic. Furthermore, such a structure would allow for target-directed drug design of FA analogues. We have obtained high-resolution cryo-EM structures of the S. aureus FA complex, formed by locking EF-G to the 70S ribosome with FA. Furthermore, we have produced a structure of the same complex with the FA derivative FA cyclopentane (6). We aim to understand why this drug variant is less sensitive to some fusA-type resistance mutants. We also provide the highest resolution S. aureus 70S structure to date, which allows identification of additional rRNA modifications as well as detailed interactions between EF-G and the antibiotic in its clinical target.

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Ion selectivity and rotor coupling of the Vibrio flagellar sodium-driven stator unit

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Bacteria swim using a flagellar motor that is powered by stator units. *Vibrio spp.* are highly motile bacteria responsible for various human diseases, the polar flagella of which are exclusively driven by sodium-dependent stator units (PomAB). However, how ion selectivity is attained, how ion transport triggers the directional rotation of the stator unit, and how the stator unit is incorporated into the flagellar rotor remained largely unclear. Here we have determined by cryo-electron microscopy the structure of *Vibrio* PomAB. The electrostatic potential map uncovers sodium binding sites, which together with functional experiments and molecular dynamics simulations, reveal a mechanism for ion translocation and selectivity. Bulky hydrophobic residues from PomA, prime PomA for clockwise rotation. We propose that a dynamic helical motif in PomA regulates the distance between PomA subunit cytoplasmic domains, stator unit activation, and torque transmission. Together, our study provides mechanistic insights for understanding ion selectivity and rotor incorporation of the stator unit of the bacterial flagellum[1–3].

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Structural and spectroscopic investigations of cable bacteria

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By using long-distance electron transfer (LDET), cable bacteria couple the oxidation of sulfide in anoxic sediment layers to oxygen[1] or nitrate reduction[2] at the sediment surface, however, the mechanism of LDET is largely unknown despite several structural, compositional, and functional studies on cable bacteria. So far, it is known that conductive fibers residing in the shared periplasm of the cable bacterium filaments are responsible for electron transport[3] and that a NiS-rich protein appears essential for the conductive properties of the fiber[4]. In this study, scanning transmission electron microscopy with energy-dispersive x-ray spectroscopy (STEM-EDX) was used to study the elemental composition of cable bacteria filaments, and cryo-electron tomography (cryo-ET) was used to study the so-far unknown cellular structures of cable bacteria. The results show localization of iron, nickel, and sulfur to the periplasmic fibers, while nitrogen is homogeneously distributed throughout the cell and periplasm. While nickel and sulfur signals likely represent the nickel-sulfur rick core of the conductive fiber, the iron signal likely stems from cytochromes that are integral to or associated with the fiber. The elemental composition of several strains of cable bacteria were tested and were shown to contain significant amounts of nickel, indicating that the role of nickel might be universal across cable bacteria species. The cryo-ET revealed several new and previously unknown cellular structures in cable bacteria that together with this new spatially resolved elemental information sheds new light on cable bacteria's metabolism, lifestyle, and the mechanisms of LDET.

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Protease Inhibition Through Progressive Complexation by ITIH4

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Inter-alpha-trypsin (IαI) inhibitor heavy chain 4 (ITIH4) presents a new mechanism of protease inhibition by complex formation with N-terminal fragments on the protease. This has been shown to inhibit the cleavage by mannan-binding lectin-associated serine protease 2 (MASP-2) of its native substrate, C4, but allows continued cleavage of small substrates. To understand the underlying protein chemistry enabling this mechanism, the cleavage of ITIH4 by a truncated, catalytic fragment of MASP-1 is tested in varying ratios and with/without Mg^{2+}. The stoichiometry distribution of complexes resulting from cleavage in Mg^{2+}-containing buffer is determined using native PAGE and mass photometry, and this identification is used to separate individual stoichiometries for analysis of both the ITIH4 monomer and the predominant 2:1 complex by cryo-EM-SPA. For the monomer, data pathologies lead to stagnation at low-resolution reconstructions, although it supports the Alphafold prediction of the protein, placing the novel C-terminal fragment that is missing from the mature paralogues in the IαI family. In the case of the complexes, a clear envelope of the 2:1 complex is obtained by negative stain electron microscopy. To identify the specific residues involved in the complexation for each interface, cryo-fixation is necessary, in which case prohibitive dissociation occurs on the grids prior to plunging. To alleviate this, a small set of detergent additives were tested without much improvement, while a small dataset of 300 micrographs from graphene oxide-covered grids showed features resembling those observed in negative stain. As an alternative approach, high-affinity F(ab) and nanobodies are prepared for target modification.

Improved performance with CRYO ARM II series

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Introduction

According to EMDB and PDB data bases the number of data entries (solved structures) in the last 10 years (2011 – 2021) has been grown almost in an exponential manner by 23-fold. This is an enormous increase which is a result of several advances in cryo electron microscopy technology. Camera models with higher sensitivity and speed, more robust and user-friendly cryo transmission electron microscopes (cryo TEM), refined sample preparation tools and constantly improving analysis software paved the way to this development.

Objectives

Recent developments and achievements with the CRYO ARM™ series from JEOL will be presented along selected application examples.

Materials & methods

JEOL is one of the largest manufacturers of electron microscopes worldwide and from early on contributed to the current success in the field of cryo electron microscopy. Since the early 1980th JEOL has released the 8th generation of cryo TEM's the CRYO ARM™ series. Basically, two version are available comprising unique features like a cold field emission source which was pioneered by the CRYO ARM series for the cryo electron microscopy community as well as in-column energy filters and outstanding resolution performance without sophisticated and expensive add-ons like correctors and monochromators.

Results

Higher throughput in terms of moves per hour were achieved by more efficient data collections routines and microscope alignments. Multi images per hole routines together with "Koehler mode" set up resulted in > 500 images per hour depending on the detector used. New direct detector camera generations were evaluated and gave rise to high resolution data sets for tomography, single particle (SPA) and MicroED applications.

Conclusion

All together these data demonstrate the high flexibility and robustness of the latest generation CRYO ARM™ II series for the needs of current and future applications in the field of structural biology.

Structural and functional insight into the interaction of *Clostridioides difficile toxin B and FZD$_7$*

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The G protein-coupled receptors (GPCR's) of the Frizzled family, in particular the Frizzled paralogs FZD$_{1,2,7}$ belong to a group of receptors exploited by *Clostridioides difficile* toxin B (TcdB), the major virulence factor responsible for pathogenesis of the gastrointestinal tract. Here we employ a functional live-cell assay examining the affinity between full-length FZDs and TcdB. Moreover, we present two cryo-EM structures revealing large structural rearrangements implicated in receptor mediated pathogenesis. Altogether, we aim to elucidate the molecular basis of TcdB-FZD binding with structural and pharmacological assays with the capacity to investigate strategies towards combatting TcdB pathogenesis in vitro.

PI-(3,5)P_2 mediated oligomerization of the endosomal sodium/proton exchanger NHE9

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NHE9, a crucial component found in all cells, plays a pivotal role in maintaining intracellular pH, sodium levels, and cell volume regulation.NHE9 (SLC9A9), intricately fine-tunes the pH within the endosomes of hippocampal neurons. Its activity has been closely associated with a range of health conditions, including glioblastoma, autism spectrum disorders, epilepsy, and attention deficit hyperactivity disorder (ADHD). We present cryo-electron microscopy (cryo-EM) structures of the NHE9 homodimer at impressive resolutions of 3.2 Å and 3.6 Å. These newly revealed structures uncover previously unknown features of NHE9. Notably, we unveil a loop domain, comprising two β-hairpin strands, positioned approximately 15 Å above the dimerization interface. This domain interacts with the endosome-specific lipid PI-(3,5)P2. Our findings are substantiated through a combination of thermal-shift assays, solid-state membrane (SSM) electrophysiology, and molecular dynamics (MD) simulations, all of which confirm the specific binding of PI-(3,5)P2 to NHE9. Furthermore, we establish that this lipid enhances sodium binding.In light of our findings, we propose a model in which the activity of the late endosomal exchanger NHE9 is tightly regulated by the PI-(3,5)P2 lipid. This model suggests an 'activation-upon-arrival' mechanism driven by lipids, aligning seamlessly with the well-established requirement for both NHE9 and PI-(3,5)P2 in ensuring the correct trafficking of epidermal growth factor receptor (EGFR)."

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Cryo-EM for studying the structural aspects of ribozyme catalyzed RNA replication

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The biophysical properties of RNA allow formation of ribozymes, i.e., catalytic RNAs, that possess abilities including self-cleavage, ligation, peptidyl-transfer and for controlling essential cellular functions. These properties underline RNAs role in the hypothesized RNA world in which RNA self-replication likely constituted life's first hereditary system. Even though RNA self-replication is not found in extant biology, in vitro selection has led to the development of RNA polymerase ribozymes able to copy RNA templates - however with low efficiency. We use cryo-EM single particle analysis to study the structural basis for ribozyme catalyzed RNA replication, exploring both the ribozymes and their RNA substrates. Our investigations have revealed the structure and functional landscape of a 97.5 kDa RNA polymerase ribozyme hetero-dimer (4.9 Å). Our investigations have also led to the identification of a new type of RNA complexes, formed by small circular RNA templates that - due to the persistent length of the RNA duplex - are too small to fully hybridize with their complimentary strands as monomers and instead form dimers. Interestingly, these dimeric RNA complexes can be extended by the ribozyme polymerase and thus outline a new type of rolling circle synthesis, that because of its generality may have played a role during primordial replication. Together, our findings provide a foundation for better understanding of the core requirements for RNA self-replication.

Cryo-EM structures and gating mechanism of the eukaryotic magnesium channel Mrs2

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Magnesium (Mg^{2+}) is essential in physiology, and the most abundant cation in all organisms. The CorA/Mrs2 family of proteins are cardinal for influx of Mg^{2+} across cellular membranes. Mrs2 imports the cation to mitochondria in eukaryotes including human. While it is established that CorA/Mrs2 members form symmetrical cone shaped homo-pentamers with a central pore in the closed state, the conducting and regulation mechanisms of permeation remain elusive, particularly for the eukaryotic Mrs2 members. Here, we report closed and open Mrs2 cryo-EM structures determined at overall resolutions of 2.6 and 3.2 Å, accompanied by functional characterization. In the open configuration, fully hydrated Mg^{2+} is likely concentrated at the pore entry, and partially hydrated Mg^{2+} is delivered to the GMN selectivity motif, and flux permitted by a narrow hydrophilic pore, as driven by electrochemical gradients. Permeation is regulated by positively charged and hydrophobic residues as observed in the closed state. The conductance is orchestrated by conserved sensor-serving Mg^{2+}-binding sites in-between monomers in the soluble domains that are situated in the matrix, stabilizing the closed conformation. At lower levels of Mg^{2+}, the ions are stripped permitting an alternative yet symmetrical shape, maintained by the RDLR-motif that replaces one of the sensor sites. Collectively, our findings thus establish the molecular basis for selective Mg^{2+} influx of the eukaryotic Mrs2 channel and an auto-ligand-gating regulation mechanism. Similarities and differences between CorA proteins present in prokaryotes and Mrs2 are also highlighted that may have bearing for drug-discovery efforts.

Activity modulation in anaerobic ribonucleotide reductases: nucleotide binding to the ATP-cone modulates long-range order-disorder transitions in the active site

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The enzyme ribonucleotide reductase (RNR) is essential for all free-living organisms by virtue of its critical role in the biosynthesis of the building blocks of DNA. All classes of RNR are dependent on radical chemistry. A small nucleotide-binding domain, the ATP-cone, is found at the N-terminus of most RNR catalytic subunits. Binding of ATP to this domain up-regulates and binding of dATP down-regulates the enzymatic activity of all classes of RNR (aerobic, anaerobic or oxygen-indifferent). Functional and structural work on class I, aerobic RNRs has revealed a plethora of ways in which dATP inhibits activity by inducing RNR oligomerization and preventing a productive long-range radical transfer between two subunits to the active site. The structural basis for this is increasingly well understood. In contrast, anaerobic RNRs store a stable glycyl radical in direct proximity to the active site and the structural basis for their dATP-dependent inhibition is completely unknown.

We have determined seven high resolution cryo-EM structures of the anaerobic RNR from *Prevotella copri* (PcNrdD) which, together with extensive biochemical and biophysical information, give the first insights into the effects of ATP and dATP on anaerobic RNR activity. The structures implicate a complex network of interactions in activity regulation that involve the glycyl radical domain (GRD) more than 30 Å away from the dATP/ATP molecules in the ATP-cone, the allosteric substrate specificity site and a conserved but previously unseen flap over the active site. In the presence of ATP, dimeric PcNrdD is active and has a fully ordered (GRD) in one monomer. In contrast, binding of dATP to the ATP-cone results in loss of activity that correlates with

disordering of the flap and GRD. Interestingly, the glycyl radical is formed even in the dATP-bound form. Taken together, the results suggest that dATP inhibition in anaerobic RNRs acts by disordering of the flap and GRD, thereby preventing both substrate binding and radical mobilisation.

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Structural and functional characterization of contractile injection systems in *Photorhabdus luminescens*

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Cell membranes are the main physical barriers limiting the delivery of macromolecules to cells. To overcome this, nature has evolved nanoscale syringes, the extracellular contractile injection systems (eCISs)1,2. Within the eCIS family, Photorhabdus Virulence Cassettes (PVCs), produced by bacterial pathogens of human and insects, are well investigated systems3,4,5. However, several aspects of their mechanism of action are still unknown. The PVC structure is related to contractile tails of bacteriophages6, and can pierce membranes and inject large toxins into the target cell, presenting an eminent opportunity to be reengineered as a targeted drug delivery system7. The genome of *Photorhabdus luminescens* strain DJC encodes six PVCs, some carrying toxin-like effectors of different sizes. This project aims for the large-scale production and purification of *P. luminescens* PVCs, and to characterize their structure by single-particle cryo-electron microscopy. The high-resolution structural information is combined with functional assays over prokaryotic and eukaryotic organisms, to obtain insight into the mechanism of action of PVCs and their putative toxins. The results of this study will provide structural and functional information about PVCs in *P. luminescens* and their payload, increasing the understanding to exploit PVCs as targeted drug delivery tools.

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Protein design for tools to elucidate protein function and signalling

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Protein design allows researchers to reach into the "dark matter" and create new protein structures and functions.[1–3] Many pieces of software[4–6] and designed protein modules[7–10] allow us to alter and control native systems. We are using this to give us new insights into the processes that control life in bacteria, from understanding the transfer of chemical energy to movement in the bacterial flagellar motor to unveiling new insights into the mechanisms behind bacterial immunity. Here we show our studies of the Zorya phage defence system[11] and the flagellar motor stator unit, which share structural similarities. We use designed proteins as us mimics of active conformers and test different hypotheses for the mechanisms of action and signalling pathways in tandem with functional studies to describe the mechanisms of immunity by Zorya and energy transfer in the flagellar motor complex.

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MAASTY: A (dis)ordered copolymer for structural determination of human membrane proteins in native lipid nanodiscs

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Integral membrane proteins represent an important class of therapeutic targets, necessitating comprehensive understanding of their functional properties and structural attributes to facilitate rational drug development[1]. The development of amphiphilic copolymers capable of extracting membrane proteins directly from lipid bilayers into "native nanodiscs" offers a simplified approach for preparing membrane protein samples for structural and functional studies. Polymer amphiphilicity, length, and composition influence the performance of copolymers, in addition to the protein itself and the purification conditions used[2,3]. In this study, we introduce a copolymer composed of methacrylic acid and styrene, which we term MAASTY. We leverage the inherent reactivity ratios of the individual monomers to create an anionic copolymer with a statistically determined distribution of monomers. MAASTY copolymers effectively solubilize a broad range of lipid species and a wide array of tested eukaryotic membrane proteins from mammalian cells. We show that MAASTY can be used for high-resolution structural determination of a human membrane protein with single particle cryogenic electron microscopy (cryo-EM), preserving endogenous lipids. The MAASTY copolymers are promising as effective solubilizers of membrane proteins and offer a new chemical platform for their structural and functional characterization in a detergent-free system.

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Mechanistic insights into the auto-inhibition and filament formation of caspase-9

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Apoptosis, referred to as programmed cell death, comprises an intricate biochemical signaling cascade and aids in the regulation of cellular differentiation and homeostasis by eliminating damaged cells. Dysregulation in apoptotic signaling pathways plays a central role in unregulated inflammatory responses, neurodegenerative diseases, tumor progression, and cancer development. Caspase-9 (C9), a cysteineaspartic protease, plays a key role as an initiator of the intrinsic apoptotic pathway and regulates physiological and pathological cell death processes by activating downstream effector caspases. Under basal conditions, C9 is present as an inactive zymogen and requires activation by the apoptosome, a heptameric complex comprising Apaf-1 and cytochrome C. Failure to activate C9 aborts caspase-mediated apoptosis and enables evasion of this mode of programmed cell death. At the molecular level, C9 is comprised of a protease domain and a caspase activation and recruitment domain (CARD) that are connected by a 40-residue linker. In certain cancers, ERK1/2-mediated phosphorylation of the C9 linker prevents its activation and thus shuts down the apoptotic cascade. However, the molecular mechanism by which phosphorylation of the linker inhibits the enzymatic activity remains unclear, especially because the phospho-sites in the linker are located nearly 50 Å from the protease domain. Here, we investigated how the C9 linker might allosterically regulate the activation of this apoptotic caspase. With a combination of nuclear magnetic resonance (NMR) spectroscopy and biophysical methods, we characterized the structure and dynamics of the C9 CARD domain with and without the 40-residue linker.

Our data indicate that the linker docks onto the CARD domain at a specific site. We further identified that certain Arg residues in the CARD domain bind to phosphate anions in solution, suggesting a mechanism by which phosphorylation of the linker may additionally regulate binding to the CARD domain. Thus, our data lead us to hypothesize that the C9 linker may occupy a similar binding surface as Apaf-1, and thereby regulate activation by the apoptosome. We will perform a detailed structural and functional analysis of C9 to determine how the phosphorylation of its linker inactivates its catalytic activity in the full-length protein. Finally, we have identified with negative-stain electron microscopy that, like other death domains, the C9 CARD can assemble into filamentous structures. We have characterized the solution conditions that drive filament formation, and our ability to precisely control the conformational landscape of the C9 CARD will provide a model system to characterize the structural and kinetic mechanisms of filament formation. We aim to capture the early or on-pathway intermediate states that are involved in filament formation via NMR spectroscopy. Our study will provide molecular insights into the auto-inhibitory mechanism of C9 and the formation and oligomerization of filamentous death domains.

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N-terminal toxin signal peptides efficiently load therapeutics into a natural nano-injection system

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Targeted delivery of therapeutics to specific cells is a major bottleneck towards personalized medicine. Extracellular injection systems (eCIS) offer promising potential for drug delivery purposes [*1,2*]. However, the precise mechanism of action, toxin location, and eCIS loading remain unclear. Here, we reveal a minimal N-terminal signal peptide (NtSP) of an eCIS toxin, that plays a key role in toxin packing. By engineering fusion proteins, we demonstrate that the NtSP can shuttle effectors for CIS loading. We packed non-eCIS effectors, including CRISPR-Cas protein from Biggiephage, Casϕ-2, and a human antimicrobial peptide, LL37, into an eCIS. Additionally, NtSPs from eCIS effectors of other species facilitate loading of Casϕ-2. We observed cargo being packed inside the eCIS tail tube through cryo-EM analysis. *In vivo*, experiments on *Galleria mellonella* larvae resulted in increased killing when cells were challenged with bacterial cells expressing modified eCIS vs. cells expressing non-modified eCIS. The presented results enhance our understanding of toxin packing and contribute to eCIS development as targeted delivery systems.

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Structural basis and mechanism of membrane translocation of fully folded Rieske protein by the unusual AAA-ATPase BCS1

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One of the central roles of mitochondria is that of being the powerhouse of the cell. To fulfil this purpose, mitochondria rely on a robust ensemble of protein complexes which transport electrons and build up an electrochemical gradient across the mitochondrial inner membrane. The assembly of the complexes is assisted by dedicated chaperones, which take part in crucial steps and whose disruption can lead to cell death (1). One of the more unusual biogenesis pathways is that of bc1, the cytochrome c oxidoreductase complex, because it involves the translocation of a fully folded protein, the iron-sulphur cluster containing protein Rieske (ISP), across the inner mitochondrial membrane (2). This transport activity is carried out by the translocase and chaperone Bcs1, an unusual heptameric AAA-ATPase that, in contrast to other members of the AAA-ATPase family, couples the energy stored in ATP not to unfolding of its cargo but to its translocation in a folded conformation (3, 4). How a membrane protein such as Bcs1 can transport another folded protein across a lipid bilayer without compromising the permeability barrier of the membrane is an intriguing question from a structural and biochemical perspective. The main aim of this work is unveiling the mechanistic details of this translocation mechanism. We used an *in vitro* reconstitution reaction of Bcs1 with a transport competent Rip1 substrate and aimed to capture different stages of their interaction by stabilizing distinct nucleotide states of the transporter. After obtaining structural snapshots of the process by cryo-EM, we expect to analyse the 3D-reconstructions from single-particle analysis, to uncover the main interaction sites that line the pathway of Rip1 translocation across the inner mitochondrial membrane. Understanding the structural basis of these interactions during translocation and their relation to the conformational transitions of Bcs1 during ATP binding and hydrolysis will elucidate the underlying mechanism of translocation

and could potentially help to explain why specific point mutations in Bcs1 are major causes of human diseases such as the Björnstad and GRACILE syndromes (5).

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Exploring the mechanisms of Pif1 helicase function during DNA replication

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Faithful DNA replication is essential for maintaining genome stability and, consequently, for healthy development and ageing. Eukaryotic genomes are replicated by complex molecular machines called 'replisomes'. In every cell cycle, replisomes must duplicate the entire length of every chromosome once and only once, whilst minimizing errors that can cause pathogenic genome instability[1]. Key to this process is the activity of helicases – proteins that can unwind the DNA duplex. The hexameric MCM helicase forms the motor at the core of the replisome and is the primary replicative helicase; however, the MCM helicase alone is insufficient for faithful genome replication. Additional 'accessory' helicases, including the Pif1 helicases, also play a crucial role, particularly in assisting the replisome to overcome 'hard-to-replicate' regions of the genome. Whilst increasing evidence suggests that replication-associated accessory helicases play an important role in maintaining genome stability and human health[2], mechanistically, it remains unclear how any accessory helicase functions with the replisome to facilitate faithful DNA replication field[3]. To address this question, we employ an integrative approach that combines protein interaction screening, biochemistry, and electron microscopy, to determine how the budding yeast Pif1 helicases are recruited to, and function at replication forks. Preliminary results reveal direct interactions between Pif1 helicases and core components of the replisome, shedding light on how these helicases may be recruited to stalled replication forks to facilitate faithful replication past obstacles to DNA replication.

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Serial Lift-Out: Towards Volume Cryo-Electron Tomography

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Cryo-focused ion beam (cryo-FIB) milling has become a commonly used method for the preparation of frozen hydrated biological samples destined for cryo-electron tomography (cryo-ET). This technique uses ions to ablate away material above and below a region of interest, yielding electron transparent lamellae which can then be used for tomographic data acquisition. Until recently, samples amenable to this preparation method were limited to single cells plunge frozen on grids. Recently, thicker samples frozen under high pressure have been made accessible for FIB-milling, however, the milling process of samples prepared this way is tedious and time-consuming. Tedious because a volume extraction process called cryo-lift-out is required, and time-consuming because milling times are extended due to the large sample thicknesses and relatively low ablation rates with gallium ions. Besides, a substantial amount of material and contextual information is lost in such a lift-out lamella preparation. For single cells, only around 1% make it into the final tomographic volume, and for thick samples this figure is even lower.

Here we introduce a new method called ‘serial lift-out’. This method, reminiscent of serial sectioning of embedded material at room temperature, modifies the lift-out process to expose the biology of frozen hydrated material by creating a series of lamellae. While these lamellae are not perfectly sequential, as is the case with serial mechanical sections, it increases throughput when milling HPF samples and maintains a greater level of contextual information. The latter can be fine-tuned by varying the section thickness, with thinner sections (down to 1 μm) allowing for the tracking of sub-cellular morphological features between lamellae. While the sections obtained are coarser than with other volumetric electron microscopy techniques, the higher resolution offered by cryo-ET is critical to gaining new biological insights.

Much like in other volume EM methods, fluorescence can be incorporated into the workflow in order to target specific regions of interest. Fluorescence is initially used to guide trench milling around region of interest within the HPF sample. Then, after serial

lift-out, the individual sections can be screened for fluorescence, allowing the biological feature of interest to be tracked throughout the extracted volume. This correlation also permits site specific tilt-series acquisition.

As a proof-of-concept, serial lift-out has been applied to *C. elegans* L1 larvae. Using METEOR, a FIB-integrated light microscope, mKate-tagged INA-1 signal was used to target a single organism. A 180x40x25 um block was extracted from a waffle HPF sample, sectioned in 4 μm increments and milled down to ~200 nm lamellae. Prior to fine milling, the sections were screen for INA-1 fluorescence in order to target this structure for data acquisition. The resulting lamellae were used for tilt-series acquisition, allowing tomographic reconstructions of the organism from cross-sections along the anterior-posterior axis, with focus on acquiring regions correlating to the INA-1 fluorescence signal.

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Correlative Light and Electron Microscopy workflows in cells and tissues using an integrated fluorescence light microscope

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Cryo-electron tomography is a powerful tool for visualizing proteins in their native state and understanding their molecular interactions within the cellular context. The addition of an integrated fluorescent light microscope to the existing cryo-FIB/SEM instrument enables cryogenic correlative light and electron microscopy (cryo-CLEM) to be performed within the same microscope.

Finding target sites for tomography in high-pressure frozen (HPF) samples is a particular challenge. The samples and areas of interest are often buried in the bulk ice of the sample carrier and hardly detectable by SEM imaging. However, if the samples are fluorescently labelled, for example by genetically encoded fluorescent tags, then an integrated fluorescence light microscope (iFLM) can be used to localize the target sites in such bulk samples. Here we demonstrate how the iFLM is guiding the targeted preparation of cryo-lamellae from fluorescently labelled HPF samples, and we introduce a new method for needle-sample attachment which makes the cryo-lift-out process more applicable.

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Advancing Structural Biology of Infectious Diseases through CryoEM: Insights from the CSSB Multi-User CryoEM Facility

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In recent years, Cryo-Electron Microscopy (CryoEM) has emerged as a transformative technique for unraveling the intricate details of biomolecular structures. The cryo-EM Multi-User-Facility at the Center for Structural Systems Biology (CSSB) Hamburg serves as a forefront hub for cryo-electron microscopy in infection biology research, driving innovation in both technology and methodology. This presentation offers a comprehensive overview of the CSSB CryoEM Facility and highlights the cutting-edge methods applied and developed on campus.

This talk will delve into the facility's state-of-the-art infrastructure, including advanced cryoEM instruments and data analysis pipelines, which empower researchers to visualize the mechanisms of different host-pathogen interactions with unprecedented precision. The focus encompasses studies of purified protein complexes through single particle analysis, alongside investigations of pathogens and infected cells through cryo-tomography. Attendees will gain insight into how the CSSB CryoEM Facility trains scientists in the technologies and shares knowledge through workshops, and training programs.

The presentation will also spotlight some key results obtained by research groups at the CSSB, that have been instrumental in deciphering molecular processes underlying infection. Ongoing methodological developments which expand the facility’s

capabilities in sample preparation, electron diffraction, and correlative imaging will be shown - made possible by the collaborative environment on Campus Bahrenfeld, fostering the exchange of ideas and expertise between research groups and facilities.

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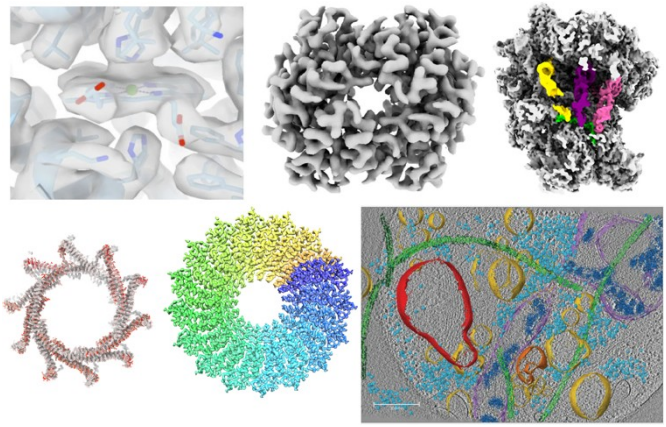
The Ernst Ruska Centre, located at Forschungszentrum in Julich, Germany, is a leading national user facility that provides access to advanced cryo-EM infrastructure for the global scientific community. Our state-of-the-art equipment includes the Talos L120C, Talos Arctica 200kV, TitanKrios 300 kV, and Aquilos 2 FIB-SEM, which are all capable of supporting standard structural analysis techniques such as Negative Stain, Cryo-EM Single Particle Analysis, Tomography, andCryo-CLEM. Our Titan Krios 300 kV, equipped with Falcon 4 and GIF-K3, has achieved impressive resolutionranging from 1.7 Å to 2 Å for benchmark Apoferritin proteins. Additionally, the Titan Krios 300 kV is equipped with the Panther detector system, which includes 4 segments and annular dark- field detectors that can be used for iDPC and HAADF-STEM imaging.



Our Aquilos 2 FIB-SEM platform has been upgraded with the METEOR system and CERES ice shield (Delmic), allowingin-situ fluorescence microscopy to be performed directly on samples inside the system, reducing transfer steps and associated ice contamination. This upgrade also improves the efficiency of the cryo-CLEM pipeline. In addition to our impressive imaging equipment, our facility is equipped with arange of sample preparation equipment.

Our Vitrobot Mark IV is the standard, butwe have also upgraded our facility with VitroJet and Leica EM GP2 equipment. At the ER-C cryo-EM user facility, we provide access to the entire pipeline from sample preparation to image acquisition. Experienced users can operate the equipment with minimal supervision, and we are committed to teaching and training new users to be independent.

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In situ ribosome clustering and ultrastructure reorganisation of Polar tube proteins in germinated microsporidia polar tube

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During host cell invasion, microsporidia translocate their entire cytoplasmic contents through a thin, hollow superstructure known as the polar tube. To achieve this, the polar tube transitions from a compact spiral-like state inside the environmental spore to an long needle-like tube capable of long-range sporoplasm delivery. The molecular and structural factors enabling this ultrafast firing process and the state of cargo during delivery remain a mystery. Likely, the unique mechanical properties of the tube enable an explosive transition from compact to extended state and facilitate previously undescribed morphological changes during the rapid cargo translocation process. Here, we use light microscopy and in situ cryo-electron tomography to visualise multiple ultrastructural states of the polar tube, allowing us to characterise its structural transitions and evaluate the kinetics of its germination. We describe a cargofilled state with a unique ordered arrangement of microsporidian ribosomes, which cluster along the thin tube wall, and an empty post-translocation state with a reduced diameter but a thicker wall. Together with compositional information from an affinitypurified polar tube, our work provides comprehensive novel data on the infection mechanism in microsporidia and demonstrates that ribosomes are efficiently transported through polar tubes in a spiral-like parallel arrangement.

M-Ionic: Prediction of metal ion binding sites from sequence using residue embeddings

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Motivation

Understanding metal-protein interaction can provide structural and functional insights into cellular processes. As the number of protein sequences increases, developing fast yet precise computational approaches to predict and annotate metal binding sites becomes imperative. Quick and resource-efficient pre-trained protein language model (PLM) embeddings have successfully predicted binding sites from protein sequences despite not using structural or evolutionary features (multiple sequence alignments). Using residue-level embeddings from the PLMs, we have developed a sequence-based method (M-Ionic) to identify metal-binding proteins and predict residues involved in metal-binding.

Results

On independent validation of recent proteins, M-Ionic reports an area under the curve (AUROC) of 0.83 (recall=84.6%) in distinguishing metal-binding from non-binding proteins compared to AUROC of 0.74 (recall =61.8%) of the next best method. In addition to comparable performance to the state-of-the-art method for identifying metal-binding residues (Ca2+, Mg2+, Mn2+, Zn2+), M-Ionic provides binding probabilities for six additional ions (i.e., Cu2+, Po43-, So42-, Fe2+, Fe3+, Co2+). We show that the PLM embedding of a single residue contains sufficient information about its neighbours to predict its binding properties.

Availability and Implementation

M-Ionic can be used on your protein of interest using a Google Colab Notebook (https://bit.ly/40FrRbK). GitHub repository (https://github.com/ TeamSundar/m-ionic) contains all code and data.

cryoEM of NOA1-bound human mitoribosome small subunit suggests alternative assembly pathway

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Assembly of the mitochondrial ribosome (mitoribosome) is a critical step in organellar protein synthesis providing the organism with subunits of oxidative phosphorylation machinery. Despite the recent progress in the structural and functional characterization of mitochondrial translation machinery, many details of the process remain an enigma. One example is the function of NOA1, GTPase involved in the assembly of the small subunit (mtSSU) of the human mitochondrial ribosome. Recently reported structure of NOA1 bound to the body of the immature small subunit found it to be responsible for the maturation of h44 of 12S rRNA at the stage before head region of mtSSU becoming mature [1]. However, it is still not clear how the factor couples its GTPase activity with the ribosome maturation; additionally, there may be other not reported mtSSU assembly states where NOA1 is present.

In our work, we aimed at elucidating the role of NOA1 further by structurally characterizing mtSSU complexes having FLAG-tagged NOA1 as a bait using cryoEM. The data we obtained suggest at least two sets of assembly intermediates deviating from those reported structurally in the literature [1, 2]. In the first one, mtSSUs having distorted h44 are still capable of being assembled further having fully mature head region; mtIF2 may be involved in the assembly process as well. In the second one, NOA1 is still bound to mtSSU while the head is mature.

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A nucleotide-sensing oligomerization mechanism that controls NrdR-dependent transcription of ribonucleotide reductases

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Ribonucleotide reductase (RNR) is an essential enzyme that catalyzes the synthesis of DNA building blocks in virtually all living cells. NrdR, an RNR-specific repressor, controls the transcription of RNR genes and, often, its own, in most bacteria and some archaea. NrdR senses the concentration of nucleotides through its ATP-cone, an evolutionarily mobile domain that also regulates the enzymatic activity of many RNRs, while a Zn-ribbon domain mediates binding to NrdR boxes upstream of and overlapping the transcription start site of RNR genes. Here, we combine biochemical and cryo-EM studies of NrdR from Streptomyces coelicolor to show, at atomic resolution, how NrdR binds to DNA. The suggested mechanism involves initially a dodecamer loaded with two ATP molecules that cannot bind to DNA. When dATP concentrations increase, an octamer forms, loaded with one molecule each of dATP and ATP per monomer. A tetramer derived from this octamer then binds to DNA and represses transcription of RNR. In many bacteria, including well-known pathogens such as Mycobacterium tuberculosis, NrdR simultaneously controls multiple RNRs and hence DNA synthesis, making it an excellent target for novel antibiotics development.

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pCF10-encoded PrgK is a regulated cell wall remodelling enzyme required for conjugation in *E. faecalis*

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Enterococcus faecalis is one of the causes of nosocomial infection not only carrying multiple resistance, but also transmit those resistance genes. The dissemination is facilitated via conjugative Type 4 Secretion System (T4SS). T4SS, encoded by plasmids or mobile gene elements, is a multi-protein complex embedded in the membrane, with a transport channel in the center. However, unlike Gram-negative ones, we have limited structural knowledge of any Gram-positive T4SSs, which is surely key knowledge to fight the spread of antibiotic resistance. PrgK, an essential protein for pCF10-encoded Gram-positive T4SS, possessed three domains homologous to cell wall hydrolase domains: LytM, SLT, and CHAP. They are all capable of binding cell wall substrate, but only SLT showed significant cell wall digestion activity in *E. coli*. We hypothesize that PrgK is regulated to only create a localized lesion in the cell wall for conjugation, but it is unclear about the domains' activities in *E. faecalis*, and how the regulation takes place.

To tap into the function and regulation of PrgK, we expressed and purified the domains with FPLC for crystallographic structures and enzymatic activity analysis. Full-length PrgK was also over-expressed in *E. faecalis* to dissect its detrimental effect in the absence of potential regulators in pCF10.

Here, we demonstrate that over-expressing PrgK in *E. faecalis* without endogenous regulation would result in lower viable cell number and abnormal cell morphology, but no lethal consequences. Furthermore, *E. faecalis* cell wall substrates digested by different PrgK domains, and then analyzed by mass spectrometry indicates that the CHAP domain is not active, and that the SLT domain on its own has muramidase

activity. However, when the SLT domain digests in presence of the CHAP and LytM domains, its activity drops dramatically. This implies that the CHAP and LytM domains may play a regulatory role in PrgK, and thus in conjugation. Additionally, we also present the crystallographic structure of LytM domain, which shares structural homology with M23 peptidase family but lacks active site. Alpha-fold modelling provides structural information for the other two domains. The potential dimerization of PrgK and interaction with another T4SS channel protein PrgL were also predicted and later proved by SEC-MALS and single particle cryoEM.

Here we reported the activity of PrgK in *E. faecalis* relies on SLT domain majorly, and the potential regulation from the other two domains, whereas the structural information of PrgK suggests clues for further understanding of intermolecular regulation to PrgK and T4SS function.

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Molecular Mechanisms of CRISPR-Cas-Associated Transposons

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Our knowledge of traditional CRISPR-Cas systems has been greatly improved by structural biology. However, non-canonical systems remain poorly understood. Some of these non-canonical systems, referred to as CRISPR-Cas associated transposons (CAST), have had their interference effector recruited by mobile genetic elements to carry out target recognition and direct DNA integration [1-3]. This process has led to the evolution of nucleoprotein complexes with unknown structures and mechanisms. Given that CAST combines the site selection precision of CRISPR-Cas with the DNA integration properties of transposons, they would be better suited for genome editing than traditional CRISPR-Cas systems. Here, we aim to biochemically characterize and obtain the structures of the CAST complexes using cryo-EM. So far, the available subcomplex structures of subtype V-K and I-F CASTs [4-7] reveal snapshots of the target recognition mechanism, shedding light on the first steps of transposon integration. However, the optimization of these systems for biotechnological applications will require further structural information on the Cas-Tn protein interactions as well as data on other CAST subtypes.

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Totally tubular?

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Bin/Amphiphysin/Rvs (BAR) domain containing proteins are cytosolic, peripheral membrane proteins that regulate the curvature of membranes in eukaryotic cells. [1–4] The proteins typically form dimers in solution that have a characteristic cresent shape, where the concave side binds to membranes. Endophilin B1 (EnB1) is a is a BAR domain containing protein with an amphipathic N-terminal helix (H0) and an additional amphipathic insert in helix 1 (H1i). [5–10] Studies show that EnB1 plays a key role in multiple fundamental processes that are important for cell homeostasis. [11–14] The protein has a high affinity for membranes containing cardiolipin, a key signalling molecule that is also involved in the regulation of cell death, and the knockdown of endophilinB1 in HEK293 cells appears to inhibit the intrinsic Bax-mediated apoptotic pathway.[6,15–17] It has been shown previously that EnB1 can bind to and tubulate LUVs in vitro by oligomerizing at the membrane surface and forming helical scaffolds,[7] and that this tubulation activity is dependant upon H0, whose function is likely modulated by the C-terminal SH3 domain.[10] So far structures of BAR proteins have been limited to either static crystal structures, not bound to a membrane (e.g. PDB IDs 4avm, 1x03, 3ok8 and 3qe6), [18–21] solution NMR structures of small individual domains (e.g. PDB IDs 1x43 and 2rnd), [22,23] and low resolution electron microscopy (EM) maps of helical scaffolds bound to tubulated vesicles (e.g. EMDB IDs EM-1471 and EM-20835) [10,24] . The mechanisms that enable EnB1s diverse functions in vivo remain unclear. Here we present two models of EnB1 bound to nanodiscs, the first atomic models of a BAR protein bound to a membrane. The final EM maps show clear density for both H0 and the H1i in two distinct conformations. Data processing with CryoSPARC and cryoDRGN independently confirmed the presence of multiple complexes with different stoichiometries present in the sample. [25–29] CryoDRGN analysis of specific particle species also display continuous conformational changes which indicate that individual EnB1 dimers are capable of remodeling their local membrane environment. This could

potentially be an intrinsic feature of EnB1 (and other BAR domain containing proteins) and gives a vital clue to how the protein can serve in such different roles in the cell.

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Complete structure of the *Perkinsus* mitochondrial respiratory supercomplex II2-III2-IV2 with 114 lipids

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The electron transport chain involves four protein complexes (CI-IV) that reside in the inner mitochondrial membrane, using the energy from redox reactions to establish an electrochemical proton gradient used for ATP production. Recent biological studies, especially using single-particle cryo-EM analysis, have revealed several different types of supramolecular organizations of these enzymes. In our research, we found two new types of respiratory supercomplexes, CIICIII2CIV(1/2), in Perkinsus marinus,in which complex II(CII) is for the first time seen to be involved . Furthermore, we observed two previously unknown extra proteins (named ISPR1/2) binding to the cytochrome c binding site at complex III (CIII) and interacting with Rieske ironsulfur protein (ISP) during the c1-state of ISP, indicating a unique mechanism of electron transfer. Our structure provides strong evidence for the existence of CII in a supercomplex and reveals frameshifting in all three protein-coding genes in the mitochondria. The unique arrangement of CII, CIII, and CIV in the supercomplex reveals a specific adaptation in the electron transfer chain of P. marinus and the discovered interaction between CIII and ISPR1/2 suggests a novel regulatory mechanism in oxidative phosphorylation.

Autophagy preferentially degrades non-fibrillar polyQ aggregates

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Aggregation of proteins containing expanded polyglutamine (polyQ) repeats is the cytopathologic hallmark of a group of dominantly inherited neurodegenerative diseases, including Huntington's disease (HD). Huntingtin (Htt), the disease protein of HD, forms amyloid-like fibrils by liquid-to-solid phase transition. Macroautophagy has been proposed to clear polyQ aggregates, but the efficiency of aggrephagy is limited. Here, we used cryo-electron tomography to visualize the interactions of autophagosomes with polyQ aggregates in cultured cells *in situ*. We found that an amorphous aggregate phase exists next to the radially organized polyQ fibrils. Autophagosomes preferentially engulfed this amorphous material, mediated by interactions between the autophagy receptor p62/SQSTM1 and the non-fibrillar aggregate surface. In contrast, amyloid fibrils excluded p62 and evaded clearance, resulting in trapping of autophagic structures. These results suggest that the limited efficiency of autophagy in clearing polyQ aggregates is due to the inability of autophagosomes to interact productively with the non-deformable, fibrillar disease aggregates.

Programming maturation time of an RNA kinetic trap

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RNA origami is a method for designing RNA nanostructures that can self-assemble through co-transcriptional folding. However, to advance the method further, an improved understanding of RNA structural properties and folding principles is required. We use cryogenic electron microscopy (cryo-EM) to determine the structure of RNA origamis down to 4-Å resolution and derive structural parameters of RNA motifs, such as kissingloops and crossovers. Interestingly, we discovered a kinetic folding trap that causes an RNA origami bundle to initially form an open conformation that after 10 hours matures into a more compact conformation [1]. To study the mechanism of this transition we used cryo-EM single particle analysis and individual particle cryo-electron tomography (IPET). It was found that the trap was caused by the formation of a kissing loop in the open structure, that had to be broken and reformed, before the compact conformation could occur [1]. We are further exploring the trap in relation to kinetics by modulating the energy of the kissing loops and its function in vitro and in vivo with the aim of constructing a molecular timer for use in nanomedicine and synthetic biology.

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In silico purification of Membrane Proteins from E.coli Cell Lysates

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Cryo electron microscopy (cryo-EM) accelerated the generation of high resolution structures. Yet this is not the most impressive feature of this technique. CryoEM allowed for the imaging of complex samples at interfaces that are challenging for other structure biology techniques. In particular, the structural investigation of proteins at the membrane interface has been aided by high-resolution cryo-EM. Further, cryoEM samples have very few restrictions which is especially important for proteins and complexes isolated from their native environment. This has given rise of protein structures with native composition.

This project is a focuses on reconstituted native membrane proteins. There are different strategies to obtain native proteins. Several labs have fractionated cell lysates and reconstructed the most abundant protein species. This approach holds a lot of promise, because EM data collection is fast enough (10,000 images/movies per day) to generate data for structure determination of endogenous proteins. By using this approach, this project reconstructed three membrane proteins from E.coli cell lysates. This technique also holds a lot of promise for the investigation of potential drug targets because artificial expression systems can lead to protein complexes that are assembled in a non-native stoichiometry, leading to wrong target structure.