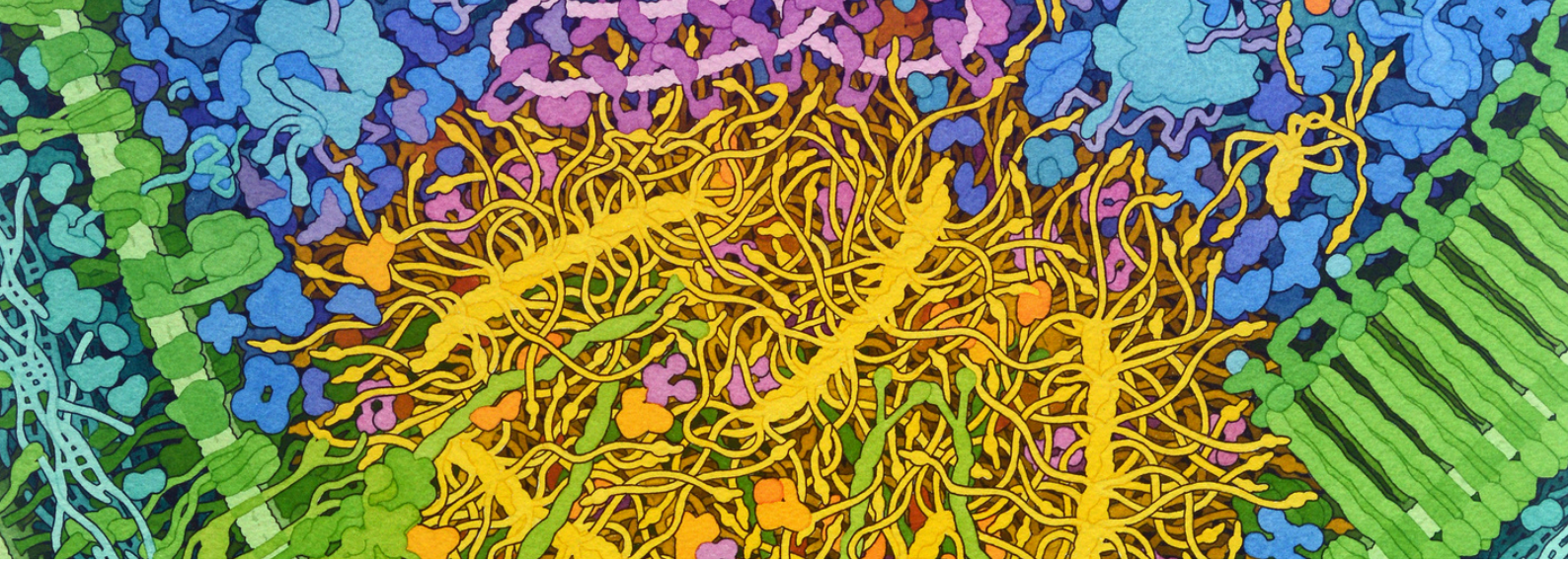


13TH SYMPOSIUM ON STRUCTURAL PROTEOMICS

6-8 OCTOBER 2025
HUMAN TECHNOPOLE, MILAN (IT)

PROGRAMME

MONDAY OCTOBER 6TH

8:30-9:30	Arrival, registration, welcome coffee (Reception Auditorium)
9:30-9:45	**Welcome Remarks** (Auditorium)
9:45-12:10	**Crosslinking MS for structural and systems biology** (Auditorium)
9:45-10:10	David Schriemer - "Redesigning the crosslinking reaction for high-fidelity cellular interactomics"
10:10-10:25	Selected talk - Asat Baischew
10:25-10:50	Petr Novak - "Singlet Oxygen as a Covalent Labeling Probe for Structural Proteomics"
10:50-11:05	Selected talk - Yi He
11:05-11:30	Stephen Fried - "A structural proteomic perspective on the basis for age-related cognitive decline"
11:30-11:45	Selected talk - Lutz Fischer
11:45-12:10	Florian Stengel - "Studying proteome organization and cellular compartmentalization: from proteins to functional compartments".
12:10-13:10	Lunch and sponsor exhibition (Restaurant area, 4 floor)
13:10-13:40	**Sponsored Talk by Thermofisher: Weijing Liu - "Expanding Structural Insights with Advanced Mass Spectrometry"** (Auditorium)
13:40-14:10	**Data standards in structural proteomics (Juri Rappsilber/Juan Antonio Vizcanio) + Round table** (Auditorium)
14:10-16:00	**Poster session** with coffee and sponsor exhibition (Restaurant area, 4 floor)
16:00-18:05	**Chemical proteomics and hybrid approaches** (Auditorium)
16:00-16:25	Alessandro Vannini - "The RNA polymerase III transcription machinery: more than a housekeeping complex at the interface of transcription, chromatin organization and native immunity "
16:25-16:40	Selected talk - Dmitry Loginov
16:40-16:55	Selected talk – Michael Karpisiek
16:55-17:20	Francesca Coscia - "Unlocking Thyroid protein complexes with Cryo-EM and Cross-linking MS"
17:20-17:35	Selected talk – Edward Marcotte
17:35-17:50	Selected talk – Noelle Potier
17:50-18:05	Selected talk – Evgeniya Biryukova
18:10-19:10	**Keynote Lecture by Paola Picotti – "Decoding the protein dance"** (Auditorium)

TUESDAY OCTOBER 7TH

9:00-10:35	**Hydrogen-Deuterium Exchange** (Auditorium)
9:00-9:25	Argyris Politis - "Probing the structural dynamics of native membrane proteins"
9:25-9:40	Selected talk - Jonathan Zöller
9:40-10:05	Malvina Papanastasiou – "An ultrasensitive nanoHDX-MS platform for probing protein dynamics and interactions"
10:05-10:20	Selected talk – Sarah Mundingl
10:20-10:35	Selected talk – Tereza Nešporová
10:35-11:15	Coffee break and sponsor exhibition (Restaurant area, 4 floor)
11:15-12:50	**Native MS & Top Down, conformational changes** (Auditorium)
11:15-11:40	Michal Sharon - "Circulating 20S Proteasomes: New Insights from Native and Top-Down Mass Spectrometry"
11:40-11:55	Selected talk – Julia Bieber
11:55-12:20	Kevin Pagel - "Infrared Spectroscopy in a Mass Spectrometer - Molecular Fingerprints for Omics Research"
12:20-12:35	Selected talk – Konstantinos Thalassinos
12:35-12:50	Selected talk - Idlir Liko
12:50-13:50	Lunch and sponsor exhibition (Restaurant area, 4 floor)
13:50-14:20	**Sponsored talk by Bruker: Christian Albers - "Revolution - timsOmni as Swiss-Army Knife for Biologics Characterization"** (Auditorium)
14:20-16:05	**Computational modeling and structure prediction** (Auditorium)
14:20-14:45	Tiziana Bonaldi - "Breaking Boundaries in MS-based Epigenetic Profiling: a Tailored Search Strategy for Unrestricted identification of novel epigenetic marks"
14:45-15:00	Selected talk – Konstantin Gilep
15:00-15:25	Riccardo Pellarin - "Macromolecular modeling using cross-linking data: how crosslinks are formed?"
15:25-15:50	Agnes Toth-Petroczy - "Beyond structure: disordered regions and biomolecular condensates"
15:50-16:05	Selected talk – Dina Schneidman
16:05-18:00	**Poster session** with coffee and sponsor exhibition (Restaurant area, 4 floor)
18:00-19:00	**Keynote lecture by Andrew Emili - "One Ring to Rule them All: Atomic Resolution Spatial Proteomics" - REMOTE** (Auditorium)

Bus pickup and transfer to the social dinner:
Villa Campari (Via Davide Campari, 23, 20099 Sesto San Giovanni MI)

WEDNESDAY OCTOBER 8TH

9:30-10:30	**Keynote lecture by Fan Liu - "Revealing cellular structural interactomes by cross-linking mass spectrometry"** (Auditorium)
10:30-11:15	**Proteomics and interactomics at scale** (Auditorium)
10:30-10:45	Lucia Santorelli
10:45-11:00	Selected talk – Dollie Lajoie
11:00-11:15	Selected talk - Manuel Matzinger
11:15-11:40	Coffee break and sponsor exhibition (Restaurant area, 4 floor)
11:40-12:10	**Proteomics and interactomics at scale** (Auditorium)
11:40-11:55	Selected talk – Caitlyn McAfferty
11:55-12:10	Selected talk – Suparat Scheu
12:10-12:30	**Poster prize + final remarks** (Auditorium)

SUPPORTED BY

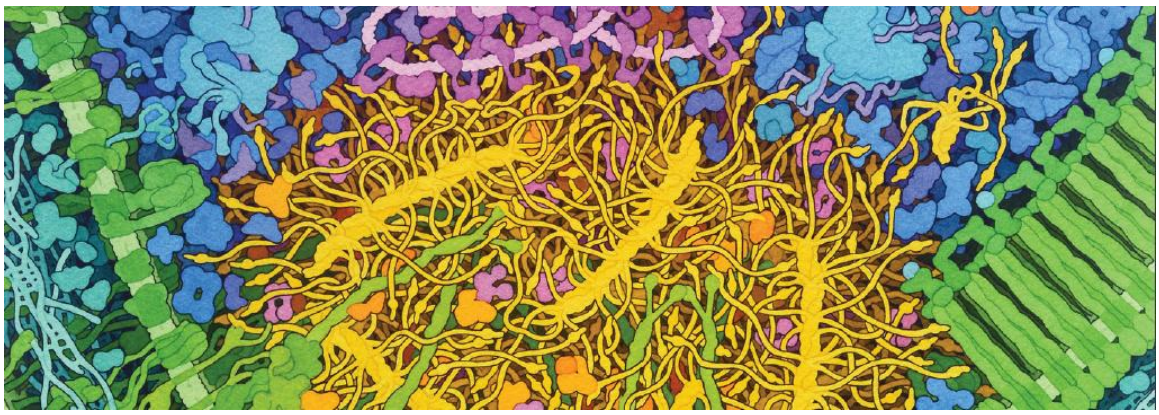
BRUKER

MSVision

HUMAN TECHNOPOLE

affipro

ThermoFisher SCIENTIFIC



13th Symposium on Structural Proteomics

6-8 October 2025 Human Technopole, Milan (IT)

ABSTRACT BOOK

From Structure to Biological Function: Unveiling the Role of the N-Terminus of Neuropeptide Y2 Receptor by Cross-linking Mass Spectrometry

DAY 1 - SELECTED TALK

Asat Baischew[1], Juan C. Rojas Echeverri[1], Claudio Iacobucci[1], Maik Pankonin[2], Davide Sala[2], Dirk Tänzler[1], Christian Ihling[1], Ronny Müller[2], Annette G. Beck-Sickinger[2], Peter Schmidt[2], Jens Meiler[2], Peter W. Hildebrand[2], Andrea Sinz[1]

[1] Martin Luther University Halle, [2] Leipzig University

The neuropeptide Y (NPY) receptors comprise a family of rhodopsin-like G-protein coupled receptors (GPCRs) that are involved in controlling food intake, memory retention, and circadian rhythm. The multireceptor/multiligand nature of NPY receptors, such as the neuropeptide Y2 receptor (Y2R), requires a detailed understanding of their interactions with their ligands. However, traditional structural biology approaches, such as X-ray crystallography or cryo-electron microscopy, have not been able to capture the ensemble of dynamic conformers. To overcome this challenge, cross-linking mass spectrometry (XL-MS)1 was employed to capture ligand-receptor contacts including transient binding states.

A photo-reactive NPY analogon allowed mapping the interaction between NPY and the intrinsically disordered N-terminus of Y2R resulting in 40 cross-links2. Cross-link were analyzed by a novel workflow3, which uses Parallel Accumulation-Serial Fragmentation (PASEF) on a timsTOF Pro mass spectrometer (Bruker Daltonik). The cross-links provided distance constraints for deriving structural models of the interaction. Molecular dynamics simulations highlight the structural flexibility and rapid interconversion of ligand-receptor contacts. Mutagenesis of Y2R and functional characterization suggest that the cross-linking hotspots electrostatically control its conformational ensemble. Moreover, the established workflow allowed in-cell XL-MS studies of the interaction in a native environment. This will serve as a basis for developing novel targeted and sensitive MS-based strategies to delineate the molecular mechanisms underlying Y2R-NPY interactions.

1. Piersimoni, L. et al. Chemical Reviews 122.8 (2021)

2. Kaiser et al. in revision

3. Rojas Echeverrri, J. C. et al. Anal Chem 96 (2024)

Singlet Oxygen as a Covalent Labeling and Cross-linking Probe for Structural Proteomics and Clinical Applications

DAY 1 – TALK

Jan Rasl[1], Jana Fialová[1], Marek Polák[1], Jan Fiala[1], Marta Cruz[1], Tereza Kadavá[1], Zuzana Kalaninová[1], Dmitry Loginov[1], Zdeněk Kukačka[1], Karel Holada[2], Petr Man[1], Michael Volný[1], **Petr Novák**[1]

[1] Institute of Microbiology, The Czech Academy of Sciences, Czechia, [2] First Faculty of Medicine, Charles University, Czechia

Introduction: Singlet oxygen ($^1O_2$) is a highly reactive oxygen species capable of covalently modifying a wide range of biomolecules, including proteins. This reactivity makes $^1O_2$ a promising probe for mass spectrometry (MS)-based structural proteomics. In this study, we employed a photodynamic approach to generate singlet oxygen under mild conditions using phthalocyanine as a photosensitizer and red laser irradiation. The generated $^1O_2$ selectively targets solvent-accessible residues such as methionine, cysteine, histidine, tryptophan, tyrosine, and phenylalanine.

Methods: Singlet oxygen was produced in a quench-flow apparatus via photodynamic activation of phthalocyanine with a red laser. Sodium azide was used to quench the reaction at defined time points. Short peptides and model proteins were labeled under varying pH conditions and in the presence or absence of ammonium ions to assess the modification spectrum. Samples were analyzed using LC-MS/MS on a Vanquish Neo UHPLC system (Thermo Scientific) coupled to a timsTOF SCP mass spectrometer (Bruker Daltonics). Data processing and interpretation were performed using DataAnalysis (Bruker), MASCOT (MatrixScience), and custom R scripts. Preliminary

Results: We applied this labeling strategy to study structural differences between monomeric and fibrillar forms of α-synuclein both in vitro and in serum-derived ex vivo samples. Our data show that singlet oxygen labeling sensitively detects conformational differences, supporting its applicability in structural proteomics. Notably, the method reliably discriminates between monomeric and oligomeric α-synuclein in human serum, highlighting its potential utility in clinical diagnostics of neurodegenerative diseases.

Novel Aspect: We present singlet oxygen labeling as a promising clinical tool for the early detection of neurodegenerative diseases through structural proteomics analysis.

Innovative data-independent acquisition (DIA) workflows for protein–nucleic acid interactions in living cells

DAY 1 – SELECTED TALK

Yi He[1], Sergei Moshkovskii[2,3] Olexandr Dybkov[3], Arslan Siraj[4], Timo Sachsenberg[4], Oliver Kohlbacher[4], Robert Justin Grams[5], Ku-Lung Hsu[5], Henning Urlaub [2,3], Rosa Viner[1]

[1] Thermo Fisher Scientific, San Jose, USA, [2] Bioanalytical Mass Spectrometry Group, Max Planck Institute for Multidisciplinary Sciences, Göttingen, Germany, [3] Bioanalytics Group, Institute of Clinical Chemistry, University Medical Center Göttingen, Göttingen, Germany, [4] Institute of Bioinformatics and Medical Informatics, University of Tübingen, Germany, [5] Department of Chemistry, University of Texas at Austin, Austin, TX, USA

Introduction Mass spectrometry can effectively resolve protein-RNA crosslinking induced by UV light and chemical methods at both the peptide and amino acid levels. UV light crosslinks various amino acids primarily to uracil, while chemical methods mainly connect amino acids to guanosine and adenosine. However, analyzing these crosslinks remains challenging for complex systems, particularly when assessing changes under different cellular conditions or states. Previous studies on protein-RNA crosslinking have mostly used data-dependent acquisition (DDA) approach. In this work, we developed innovative Data-Independent Acquisition (DIA) workflows to identify and quantify protein-RNA interactions. We applied the new workflows to ribonucleoprotein (RNP) complexes extracted from living cells to investigate protein-RNA binding domains and binding activity within the human proteome. Methods E. coli ribosome or ribonucleoprotein (RNP) complexes from living cells were modified using UV- or chemically-induced protein-RNA crosslinking. After crosslinking reactions and enrichment, samples were separated using a Thermo Scientific™ Vanquish™ Neo LC system with a 60 min gradient using an IonOpticks Aurora Ultimate™ (75 um x 25 cm) column. Crosslinked peptide-RNA oligonucleotides were detected using a Thermo Scientific™ Orbitrap™ Astral™ mass spectrometer in data-dependent mode or data-independent mode with isolation windows of 2 Th and 4 Th. Identification and quantification of crosslinking data were accomplished using the NuXL node in Thermo Scientific™ Proteome Discoverer™ 3.0 and DIA-NN 1.9.1. Results Crosslinking mass spectrometry (XL-MS) provides a straightforward approach for identifying proteins and protein domains that interact with RNA and/or DNA at the molecular level. However, analyzing complex samples, such as those from entire cellular systems, remains challenging. In this study, we developed data-independent acquisition (DIA) workflows using UV or chemically crosslinked E. coli ribosomes. The E. coli ribosome is easily obtainable and reproducible, making it an excellent model for quantitative crosslinking studies, as it serves as a well-defined standard for protein-RNA interactions. We performed DIA mass spectrometry analyses using 2 Th or 4 Th isolation windows. The spectral library for each experiment was generated from data-dependent acquisition (DDA) runs, adhering to the following criteria: both crosslinks (mono- and dinucleotides) and linear peptides included, precursor charge 2-3, CSM > 1, NuXL localization score > 0 (for crosslinks). For the sample crosslinked with nitrogen mustard (NM), we identified 1,099 and 1,058 crosslinks for the 2 Th and 4 Th isolation windows,

respectively, with an 86% overlap in NuXL identifications. Additionally, the DIA quantitation results, based on precursor ion intensity, showed a correlation of 0.97 between the two isolation windows. Similar findings were observed with the UV-crosslinked E. coli ribosomes. Importantly, we applied the DIA workflow to ribonucleoprotein (RNP) complexes derived from living cells using a photo-activatable-competition and chemoproteomic enrichment (PACCE) method. We quantified RNA binding activity of proteins including the specific residue(s) involved in samples with and without UV irradiation. In conclusion, we have successfully developed novel DIA workflows that enhance the investigation of protein-nucleic acid interactions in complex systems.

How Accurate Are FDR Estimates in Crosslinking MS?

DAY 1 – SELECTED TALK

Lutz Fischer, Juri Rappsilber

TU Berlin

Crosslinking mass spectrometry (MS) is a powerful approach for investigating protein structures and interactions. However, reliable error estimation remains a major challenge, and misleading error rates can undermine confidence in the method. Today, multiple software pipelines are available for crosslink identification, ranging from applications on individual proteins to proteome-wide studies. As sample complexity increases, accurate error control becomes critical for ensuring reliable interpretation. In a community effort, we benchmarked several widely used crosslinking MS tools in close collaboration with their developers. Rather than focusing on the number of reported crosslinks, our aim was to assess how well each tool controls false discovery rates (FDR) and reports protein–protein interactions at stated confidence levels. We found that while about half of the tools produced robust error estimates, others underestimated error, reporting up to 31-fold more false interactions than suggested by their own FDR values. Importantly, our collaborative approach also enabled improvements to one of the initially underperforming tools. Our study highlights both the strengths and limitations of current crosslinking MS analysis software. It underscores the need for an accurate error estimation to ensure that crosslinking MS delivers reliable insights into protein interactions and complex biological systems.

Towards unified reporting standards for protein footprinting: leveraging ProForma 2.1

DAY 1 – SELECTED TALK

Douwe Schulte[1], Juan Antonio Vizcaíno[2], Eric W. Deutsch[3], Joshua A. Klein[4], Petr Novák[5], **Dimitry Loginov[5]**

[1] Utrecht University, [2] EMBL-EBI, [3] Institute for Systems Biology Ralf Gabriels, Ghent University, [4] Boston University, [5] Institute of Microbiology, CAS

Structural proteomics methods, such as protein footprinting, have become pivotal in studying protein folding, protein-protein and protein-ligand interactions, and more. The increasing popularity of footprinting methods is driven by recent advancements, including the development of cost-effective radical probes [1] and easy to use instrumentation [2]. Consequently, the volume of available datasets is rapidly expanding, posing challenges in standardizing data reporting and its re-usability. Unlike cross-linking and HDX methods, there are currently no guidelines for reporting the protein footprinting data within the structural mass spectrometry community. To cover this gap, we propose a solution involving the standardization protocol to report all possible outcomes of the footprinting experiments utilizing ProForma 2.1. ProForma, initiated by the Proteomics Standards Initiative (PSI) of the Human Proteome Organization, focuses on describing proteoforms/peptidoforms [3]. These are amino acid sequences represented in one-letter code, supplemented with information on modifications (either known or unidentified mass shifts) enclosed in brackets following specific amino acids. ProForma 2.1 supports two widely used controlled vocabularies for protein modifications, namely Unimod and PSI-MOD, and also allows for the use of custom reference systems through the 'Custom' tag, accommodating implementation-specific needs. This flexibility makes ProForma 2.1 fully compatible with the diverse requirements for footprinting experiments. Such a format provides comprehensive rules for describing peptide modifications, whether they are precisely localized or ambiguously assigned. In cases where the exact site of modification is unknown but limited to a specific set of amino acids, ProForma 2.1 offers clear syntax to represent such ambiguity. Additionally, it includes specific guidelines for reporting isobaric peptides—an essential feature for accurate data representation in any radical footprinting studies, where multiple modification sites can be occupied by identical mass increments. Through these features, ProForma 2.1 enables standardized and unambiguous reporting of complex peptide data sets and its re-usability for structural proteomics. This work was financially supported by the Technology Agency of the Czech Republic (ODEEP-EU TH86010001).

[1] Fojtík, L. et al. Fast Fluoroalkylation of Proteins Uncovers the Structure and Dynamics of Biological Macromolecules. J Am Chem Soc 143, 20670–20679 (2021).

[2] Sharp, J. S. et al. Flash Oxidation (FOX) System: A Novel Laser-Free Fast Photochemical Oxidation Protein Footprinting Platform. J Am Soc Mass Spectrom (2021)

[3] Leduc, R. D. et al. Proteomics Standards Initiative's ProForma 2.0: Unifying the Encoding of Proteoforms and Peptidoforms. J Proteome Res 21, 1189–1195 (2022).

Expanding the FFAP protocol for structural characterization of membrane proteins

DAY 1 – SELECTED TALK

Michael Karpisek[1,2], Zdenek Kukacka[2], Jasmina Maria Portasikova[1,2], Petr Man[1,2], Petr Novak[1,2]

[1] Faculty of Science, Charles University, Prague, [2] Institute of Microbiology, The Czech Academy of Sciences, Prague

Structural mass spectrometry (MS) offers multiple methods (e.g. protein footprinting, chemical cross-linking, hydrogen/deuterium exchange) that provide valuable structural information even for samples that are difficult to study using standard high resolution techniques of structural biology (e.g. X-ray crystallography, NMR, cryo-EM). Although the resolution of these MS-based techniques is lower, they have several advantages over conventional techniques of structural biology such as low sample consumption, ability to analyze protein in native-like conditions, no size limitation etc. Protein footprinting is one of such MS-based methods that provides information about solvent accessible residues through covalent modification. Because the extent of modification depends on the environment surrounding the modifiable residues (i.e. whether they are exposed or buried), this method can be used to monitor protein-protein or protein-ligand interactions, structural comparison of wild type and mutant proteins or assess influence of buffer composition on the protein structure. In this work, we applied the Fast FluoroAlkylation of Proteins (FFAP) protocol developed in our laboratory (JACS, 143: 20670-20679), to study membrane Cl-/H+ transporter (ClC-ec1). Specifically, we focused on the comparison of the wild type and the QQQ mutant, that was designed to mimic the outward facing state of the channel. The FFAP workflow involves mixing the studied protein with a Togni reagent II (Chem. Eur. J., 12: 2579-2586) that releases a CF3 radical upon activation by ascorbic acid. The released radicals can modify side chains of aromatic residues and cysteine. The studied protein was analysed by mass spectrometry (timsTOF Pro, Bruker Daltonics) using bottom-up approach. The results show that we were able to modify the ClC-ec1 membrane protein using the FFAP protocol. Overall, twelve residues were modified in the wild-type and eleven in the QQQ mutant. Comparison of the calculated extent of modification of labeled residues between the wild type and QQQ mutant suggest minimal difference between these two proteoforms. This finding is consistent with results of hydrogen-deuterium exchange experiment. Funding of the Charles University Grant Agency (grant no. 448425) and of the Czech Science Foundation (grant number 25-18181S) is gratefully acknowledged.

Proteomics across deep evolutionary time to decode human genetics

DAY 1 – SELECTED TALK

Rachael M. Cox[1], Ophelia Papoulas[1], Shirlee Shril[1], Chanjae Lee[2], Tynan Gardner[2], Anna M. Battenhouse[2], Muyoung Lee[2], Kevin Drew[2], Claire D. McWhite[3], David Yang[4], Janelle C. Leggere[4], Dannie Durand[4], Friedhelm Hildebrandt[1], John B. Wallingford[5], **Edward M. Marcotte[5]**

[1] Division of Nephrology, Department of Pediatrics, Boston Children's Hospital, Harvard Medical School, Boston, MA 02215, USA, [2] Department of Biological Sciences, University of Illinois at Chicago, Chicago, IL 60607, USA, [3] Lewis-Sigler Institute for Integrative Genomics, Princeton University, Princeton, NJ 08544, USA, [4] Department of Biological Sciences, Carnegie Mellon University, 4400 5th Avenue, Pittsburgh, PA 15213, USA, [5] Department of Molecular Biosciences, The University of Texas at Austin, Austin, TX 78712, USA

All eukaryotes share a common ancestor from roughly 1.5 – 1.8 billion years ago, a single-celled, swimming microbe known as LECA, the Last Eukaryotic Common Ancestor. Nearly half of the genes in modern eukaryotes were present in LECA, and many current genetic diseases and traits stem from these ancient molecular systems. To better understand these systems, we compared genes across modern organisms and identified a core set of 10,092 shared protein-coding gene families likely present in LECA, a quarter of which are uncharacterized. We then integrated >26,000 mass spectrometry proteomics analyses from 31 species to infer how these proteins interact in higher-order complexes. The resulting interactome describes the biochemical organization of LECA, revealing both known and new assemblies. We analyzed these ancient protein interactions to find new human gene-disease relationships for bone density and congenital birth defects, demonstrating the value of ancestral protein interactions for guiding functional genetics today.

Probing antimicrobial peptide structures with cross-linking mass spectrometry: example of PGLa and magainin2

DAY 1 – SELECTED TALK

Noelle Potier[1], Emilie Hirschler[2], Emmanuelle Leize[2], Elise Glattard[2], Burkhard Bechinger[2]

[1] Laboratoire de Spectrométrie de Masse des Interactions et des Systèmes, UMR 7140, Strasbourg, France, [2] Laboratoire de Biophysique des Membranes et RMN, UMR 7177, Strasbourg, France

Introduction : This work focuses on the structural characterization of two antimicrobial peptides (PGLa and magainin2) in a membrane-mimetic environment. Both peptides are amphipathic peptides exhibiting antimicrobial properties on their own but they also show synergism of one order of magnitude when added in 1/1 cocktail. While synergistic mechanism of action is not well understood, we proposed to use cross-linking mass spectrometry (XLMS) to help deciphering this behavior in a context of major public health threats, as antimicrobial peptides are thought to be less prone to antimicrobial resistance. Methods : XL experiments were carried out in HEPES buffer in presence of DDM detergent micelles or lipid bilayers (POPE:POPG 3:1 and DMPE:DMPG 3:1). Dissucinimidyl suberate (DSS) or disuccinimidyl glutarate (DSG) were used as cross-linkers. The XL reaction was monitored by MALDI-MS and cross-linked peptides were analysed before- or after- enzymatic digestion with trypsin and pepsin using nano liquid chromatography-MS/MS (easy-nLC1000-QExactivePlus orbitrap). Linkage sites identification was achieved using pLink2. Results : XLMS performed in a DDM micelle environment showed the formation of intramolecular cross-linked monomers but also provided direct evidence of a specific PGLa/magainin2 heterodimer while no other oligomeric states were detected. Interestingly such heterodimer was only observed in a membrane mimetic environment when proper folding occurred (verified by circular dichroism). Monitoring the reaction using MALDI-MS facilitated a rapid optimization of conditions to achieve the best balance between stabilizing complex formation and avoiding unspecific aggregation or multi-linkages. It also revealed some biases likely to be introduced insidiously during XLMS experiments, such as significant loss of the cross-linked heterodimer during C18 spin column desalting, inefficient quenching the XL reaction with Tris buffer, or targeted resistance of the cross-linked heterodimer towards trypsin digestion. To avoid misinterpretation of cross-linked peptides with consecutive sequences, the search for intra- and inter-molecular cross-links was performed separately: undigested samples were used to unambiguously identify intramolecular links, whereas digested samples were used for the characterization of heterodimeric links. Performing XL in liposomes appeared much more difficult in terms of XL efficiency, even at high cross-linker ratio, although the heterodimer was still the only multimeric species detected. Hydrolyzed monomeric species were mainly identified on each monomer, indicating that one head of the cross-linker reacted with the peptide, while the other was not able to do so. Thus, the low abundance of cross-linked species in liposome medium is more of a XL efficiency issue related to a restricted access of the XL reagent or to unreactive lysines. It is important to note that helix formation alone is not sufficient to explain the antimicrobial activity of these peptides

as various parameters related to the lipidic environment can affect the molecular recognition. Changing the lipid composition from unsaturated POPE:POPG to satured DMPE:DMPG bilayers revealed different behaviors by MALDI, suggesting that interaction between the peptides might occur differently in both membrane-mimicking media, and that XLMS can also reflect the topological differences observed by NMR. Conclusion : Optimizing the workflow for a simple purified system as antimicrobial peptides may lead to different choices at several steps when compared with large-scale mapping of protein-protein interactions at the proteome level. MALDI-MS proved to be a key asset in our study, in order to avoid technical biases arising from the rapid nature of the XL reaction. The optimized conditions could be used in more complex peptidomic studies realized with intact bacteria or applied to the characterisation of membrane protein complexes.

Development of Software for LC-MS Data Processing of Oligonucleotides

DAY 1 – SELECTED TALK

Evgeniya Biryukova[1,2], Marek Polák[1], Petr Novák[1]

[1] Institute of Microbiology of the CAS, Prague 4, Czech Republic, [2] Faculty of Science, Charles University, Prague 2, Czech Republic

We present a Python-based software tool designed for the efficient analysis and interpretation of oligonucleotide fragmentation patterns. Initially developed for the study of oxidative cleavage induced by FPOP (Fast Photochemical Oxidation of Proteins), the software tool generates theoretical libraries of monoisotopic fragment masses across multiple charge states and matches them to experimentally detected peaks within a defined mass error tolerance. The newly expanded functionality goes beyond radical-induced fragmentation. The tool now supports simulation of enzymatic cleavage, including products generated by RNases T1 and U2 acting on RNA, and performs automatic annotation of the corresponding fragments. Additional features include graphical output and fragment quantification, enabling a flexible and efficient workflow. In future development phases, the software could be applied to miRNA analysis from body fluids to support early diagnostics of inflammatory or cancer-related diseases. The tool supports both qualitative and quantitative analysis and, thanks to its intuitive graphical user interface, is accessible even to users with minimal programming experience.

Structural dynamics of the Hc/groove interaction in MLKL investigated by HDX-MS

DAY 2 – SELECTED TALK

Jonathan Zöller[1], Uris Ros[1,2], Veronica Martinez-Osorio[2], Raed Shalaby[1,2], Julian D. Langer[1], Ana J. García-Sáez[1,2]

[1] Max Planck Institute of Biophysics, Frankfurt am Main, Germany, [2] Institute of Genetics and Cologne Excellence Cluster on Cellular Stress Responses in Aging-Associated Diseases (CECAD), University of Cologne, Cologne, Germany

Necroptosis is an inflammatory form of regulated cell death with many implications in human pathologies like neurodegeneration or cardiovascular injury. During its execution, the cell releases danger-associated molecular patterns (DAMPS) following plasma membrane permeabilization and triggers an inflammation guided immune response. The most downstream executer of this pathway is the pseudokinase mixed lineage kinase domain-like (MLKL) which translocates to the plasma membrane, inducing cell death via yet unknown mechanisms. Previous studies established phosphorylation by RIPK3 as a trigger for conformational changes of MLKL and its activation. However, we identified a splicing-dependent isoform of MLKL, which has an insertion of a short amino acid sequence in the c-terminal helix (Hc) and functions as a negative regulator for the active, necroptosis-triggering MLKL isoform. Here, we employed an integrative strategy that combines several biochemical and biophysical approaches to dissect the functional role of MLKL and its different isoforms. Functional consequences of the isoforms were assessed using cell-based necroptosis assays that monitored phosphorylation, oligomerization, and membrane translocation in response to necroptosis induction. To investigate the structural role of the Hc in the reaction mechanism, we utilized hydrogen/deuterium exchange mass spectrometry (HDX-MS) complemented by molecular dynamics (MD) simulations and mutagenesis experiments. Those experiments guided the design of small allosteric inhibitors for mouse and human MLKL, which proved to inhibit Necroptosis in vivo. In this study, we identified alternative splicing-dependent isoform (mMLKL1 in mouse and hMLKL0 in human) with a short sequence insertion in the C-terminal α-helix (Hc) of MLKL as a critical determinant of necroptosis sensitivity. This isoform abolished killing capacity and functions as an antagonist for the active MLKL variant (mMLKL2 in mouse and hMLKL1 in human) in both mice and humans. Experiments of the expression confirmed that this inactive isoform reduced MLKL phosphorylation, oligomerization, and membrane translocation, thereby acting as a negative regulator. In vivo experiments in mouse models showed that the relative expression of the two isoforms regulate necroptosis sensitivity. To assess the structural basis for the functional discrepancies of the two MLKL isoforms we used MD simulations and HDX-MS experiments. First, MD simulations showed that the Hc of the inactive MLKL isoform is partially unfolded, which is not the case in the active isoform. The structured Hc of mMLKL2 and hMLKL1 is accommodated in a previously unrecognized groove formed by residues of the 4 helix bundle (4HB), the brace (Br), and pseudokinase (psK) domain. Mutations disrupting the Hc/groove interaction drastically altering the functionality of MLKL and demonstrated the importance of the Hc for the activation of MLKL. Next, we performed

HDX-MS in the absence (control) and presence (binding) of small inhibitors with mMLKL2 and hMLKL1. We reasoned that binding of those inhibitors pushes the Hc out of the groove, resulting in increased deuterium uptake of the Hc and the groove. Binding of a well-known hMLKL inhibitor, NSA, resulted in a significant deuterium increase in the Hc and groove of hMLKL1. Interestingly, we observed that NSA binding induced increased deuterium uptake in segments involved in RIPK3 binding of the psK domain. Similar experiments with a small inhibitor and the mouse mMLKL2 protein corroborated the observation, as they showed similar effects in the psK domain. Taken together, those results highlight the importance of the Hc/groove interaction for the reaction mechanism of MLKL during necroptosis. Our findings establish a two-way regulatory system for MLKL: first, alternative splicing generating antagonistic isoforms that tune necroptosis sensitivity; second, intramolecular Hc/groove interaction acting as a structural switch for activation. HDX-MS provided detailed information on the Hc/groove interaction and showed that small inhibitor binding resulted in allosteric re-arrangements in the psK domain. The altered effects on deuterium uptake may indicate that the inhibitor not only inhibits by interfering with Hc /groove interactions but also with RIPK3 binding, potentially preventing necrosome formation. Future experiments aim to investigate whether the identified inhibitors fully prevent the association of RIPK3 with MLKL. Additionally, we will characterize another small molecule that might act as an activator of MLKL in both mouse and human models and identify its binding site and structural mode of action. Finding answers to these points will provide a more complete picture of the cellular mechanisms of MLKL and potentially guide the development of therapeutic modulators.

From Broad to Precise: Mutual Dependence of HDX-MS and Modelling in Epitope Identification

DAY 2 – SELECTED TALK

Sarah Mundigl[1], Catherine Wong[2], Cornelia Wagner[1], Maximiliane König[1];

[1] Mass Spectrometry, Large Molecule Research, Roche Innovation Center Munich, Roche Pharma Research and Early Development, [2] Computational Engineering & Data Science, Large Molecule Research, Roche Innovation Center Munich, Roche Pharma Research and Early Development

Hydrogen Deuterium Exchange coupled to Mass spectrometry (HDX-MS) was used to map the epitope of a therapeutic antibody on its antigen, revealing a broad interaction region potentially encompassing more than just the direct epitope. As HDX-MS alone cannot differentiate between epitope and allosteric effects, we integrated computational modelling. Guided by the HDX-MS data, modelling refined the epitope to a smaller, more precise region within the initial broad area. The remaining HDX-affected regions were identified as likely allosteric changes. This study underscores the synergistic relationship between HDX-MS, providing initial localization, and computational modelling, enabling precise epitope definition and mechanistic insights, which would not be achievable by one method alone.

Allosteric Disruption of the SARS-CoV-2 nsp10–nsp14 Exonuclease Complex Revealed by HDX-MS

DAY 2 – SELECTED TALK

Tereza Nešporová[1], Matěj Danda[2], Michaela Rumlová[2], František Filandr[1]

[1] Institute of Organic Chemistry and Biochemistry of Czech Academy of Science, Prague, Czech Republic, [2] Department of Biochemistry and Microbiology, University of Chemistry and Technology, Prague, Czech Republic

The accuracy of coronavirus genome replication is safeguarded by a specialized proofreading system centered on the non-structural proteins nsp14 and nsp10. Nsp14 is a bifunctional enzyme containing an N-terminal 3′–5′ exoribonuclease domain essential for error correction and a C-terminal N7-methyltransferase domain required for mRNA capping. Nsp14 exonuclease activity is markedly stimulated by nsp10, a small cofactor that binds and stabilizes nsp14, thereby enabling high-fidelity genome replication. Structural studies have shown that nsp10 does more than stabilize: it induces conformational changes in nsp14 consistent with allosteric activation. This complex therefore represents a key therapeutic target, and disrupting the nsp10–nsp14 interaction has the potential to undermine the virus's ability to replicate accurately. In this work, we investigated the mechanism of action of a small-molecule inhibitor against the nsp10–nsp14 complex by combining biochemical and structural proteomics approaches. Microscale thermophoresis, crosslinking analysis, SDS PAGE and other biochemical approaches provided an initial assessment of binding unspecificity. The inhibitor bound weakly to isolated nsp14 or nsp10. Strikingly, however, in nsp14-nsp10 complex, significant inhibitory effects emerged. These findings suggested that the compound does not act by direct competition at nsp14's catalytic site but instead by altering conformational states stabilized by nsp10. Such behavior is consistent with non-competitive, allosteric inhibition. To resolve this mechanism in molecular detail, we employed hydrogen–deuterium exchange mass spectrometry (HDX-MS), which provides peptide-level insights into protein dynamics and interfaces. In the absence of inhibitor, HDX-MS confirmed stable formation of the nsp10–nsp14 complex. Distinct protection from deuterium exchange was observed at two specific regions of nsp10 and nsp14, precisely mapping the protein–protein interface. These data are fully consistent with crystallographic structures, which highlight these regions as central to cofactor binding and stabilization of the exonuclease active site. Beyond the interface, additional protection was detected in nsp14's RNA-binding region, reflecting the allosteric effect of nsp10 binding and its role in modulating RNA interaction surfaces. Addition of the inhibitor profoundly altered this landscape. HDX-MS measurements revealed a complete loss of protection at the nsp10–nsp14 interface, with deuterium uptake reverting to levels typical of the unbound proteins. This effect was reproducible whether nsp10 or nsp14 was preincubated with the inhibitor, underscoring that complex assembly could not occur in its presence. Importantly, the inhibitor's impact was not limited to disrupting the interface. The strongest destabilization occurred in the RNA-binding region of nsp14, where peptides lost protection and became highly dynamic. These results point to an allosteric inhibitory mechanism in which the

compound perturbs nsp14's RNA-binding domain, preventing it from adopting the conformation necessary for stable interaction with nsp10. For nsp10, the loss of protection in the specific region further confirmed that its normal stabilization by nsp14 was abolished. Taken together, biochemical approaches and HDX-MS converge on a coherent mechanistic model: the small-molecule inhibitor destabilizes nsp14's RNA-binding region, disrupts its conformational response to nsp10 binding, and thereby blocks productive assembly of the exonuclease complex. This mechanism explains why inhibitory activity depends on the presence of nsp10, even though the inhibitor binds only weakly to nsp14 alone. Rather than acting as a classical active-site competitor, the compound interferes with conformational communication between RNA-binding elements and the protein–protein interface. These findings have broad implications for antiviral strategy. The data demonstrate that protein–protein interactions within essential viral complexes can be effectively disrupted by allosteric inhibitors, even when direct active-site competition is weak. By abolishing the assembly of the nsp10–nsp14 complex, the inhibitor undermines SARS-CoV-2 proofreading capacity and opens the possibility of inducing mutational overload or impaired replication. In conclusion, this study highlights the value of HDX-MS for elucidating inhibitor mechanisms at peptide-level resolution. The approach not only confirmed the interaction interface of the nsp10–nsp14 complex but also revealed how a small molecule specifically destabilizes nsp14's RNA-binding region to disrupt allosteric regulation and cofactor engagement. Such studies is critical for validating this mechanism and optimizing small molecules that target the exonuclease complex as antiviral candidates.

The combination of native mass spectrometry and chemical cross-linking provides insights into the regulation of SNARE complex assembly by Complexin-1 and Synaptotagmin-1

DAY 2 – SELECTED TALK

Julia Bieber and Carla Schmidt

Johannes Gutenberg University Mainz, Department of Chemistry - Biochemistry, Germany

Signal transmission between neurons is mediated by the SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) complex, which is responsible for fusion of synaptic vesicles with the presynaptic membrane thereby releasing neurotransmitters into the synaptic cleft. The ternary SNARE complex assembles from vesicular Synaptobrevin-2 (Syb2) as well as SNAP25 and Syntaxin-1A (Stx1A), which are both anchored to the presynaptic membrane, forming a stable four-helix bundle. SNARE assembly is highly regulated by several proteins; for instance, the vesicular calcium sensor Synaptotagmin-1 (Syt1), which contains two cytosolic membrane binding domains termed C2A and C2B. Together with the cytosolic protein Complexin-1 (Cpx1), Syt1 was shown to be important for calcium-triggered membrane fusion. However, the underlying regulatory mechanism is largely unknown and contradictory models were proposed. In the so-called 'clamping model', Syt1 binds to the SNARE complex upon calcium influx leading to the disassembly of previously assembled Cpx1 from the SNARE complex. In the 'cooperativity model', on the other hand, Syt1 binds to the SNARE complex causing dissociation of an inhibitory domain of Cpx1 without displacing the protein from the complex. Using native mass spectrometry, we studied interactions between Cpx1 and the cytosolic domains of Syt1 as well as binding of the proteins to the fully assembled SNARE complex. We first investigated interactions between Cpx1 and the intact soluble variant of Syt1 (Syt1C2AB) and then pre-assembled the SNARE complex to provide an interaction surface for the two proteins. For this, SNAP25, Syb2 and Stx1A were mixed, and Cpx1 or Syt1 were added. Complex formation was then followed by native mass spectrometry. Interestingly, in the absence of SNARE proteins, formation of a stable complex between Syt1 and Cpx1 was not observed. However, specific interactions between the two regulators and the SNARE complex were observed, when the SNARE complex was present. While Cpx1 immediately associates with the SNARE complex as previously described (Hesselbarth, Schmidt (2023) Commun Biol 6(1):198), addition of Syt1C2AB did not result in formation of a stable complex. When employing the individual C2 domains of Syt1, binding to the SNARE complex was only observed for Syt1C2A and a complex involving Syt1C2B was not observed. We therefore investigated sequential binding of the two regulators to the SNARE complex and pre-assembled the SNARE:Cpx1 complex. Interestingly, initial binding of Cpx1 enabled binding of Syt1C2AB, Syt1C2A and Syt1C2B to the SNARE complex resulting in stable complexes of equimolar stoichiometry. Notably, Cpx1:Syt1C2AB as well as Cpx1:Syt1C2A complexes of 1:1 stoichiometry were also observed when the SNARE complex was present. We conclude that Cpx1 and Syt1 do not compete for their binding site on the SNARE complex. Instead, Cpx1 mediates binding of Syt1 and further allows dissociation of a complex involving both proteins. In

summary, our results support a combination of the proposed “clamping” and “cooperativity” models. To address the structural (re-) arrangements induced by Syt1 binding, we will explore interactions within the SNARE:Cpx1:Syt1C2AB assembly by chemical cross-linking in future studies.

Top-down ion mobility mass spectrometry reveals a disease associated conformational ensemble of alpha-1-antitrypsin

DAY 2 – SELECTED TALK

Sarah Vickers[1], Ibrahim Aldobiyan[2], Sarah M. Lowen[2], James A. Irving[2], David A. Lomas[2], **Konstantinos Thalassinos**[1]

[1] Institute of Structural and Molecular Biology, Division of Biosciences, University College London, London, U.K., [2] Centre for Respiratory Biology, Division of Medicine, University College London, London, U.K.

Modern protein prediction methods using deep neural networks can now achieve extremely accurate results when predicting protein structures from the protein's sequence. Despite this impressive achievement these approaches still struggle to predict the structure of flexible proteins, and those where a single folded state does not represent the majority conformation. By contrast, structural mass spectrometry approaches, and in particular ion mobility and crosslinking mass spectrometry, can probe the dynamic conformational landscape of proteins and proteins in complex with other molecules. We have used ion mobility mass spectrometry and electron-capture dissociation to directly observe and characterise novel alpha-1-antitrypsin intermediates formed during polymerisation. Our data are congruent with an ensemble of conformations that are monomeric but maintained in a partially misfolded metastable state in which ~12% of the molecule at the C-terminus is displaced. The application of these techniques to Z alpha-1-antitrypsin polymers isolated from human liver revealed a molecular species most consistent with a polymer mediated by an intermolecular C-terminal domain insertion. These findings establish a previously unobserved progression of pathogenic structural changes and thereby extend the mechanism of alpha-1-antitrypsin polymerisation. They additionally demonstrate the strengths of native top-down ion mobility mass spectrometry in characterising misfolding intermediates and proteins isolated from human tissue.

High-Throughput Native MS and Proteoform-Resolved Analysis of Membrane Protein–Ligand Interactions in Native Lipid Environments

DAY 2 – SELECTED TALK

Idlir Liko

EIT Oxford

Understanding membrane protein–ligand interactions within native lipid bilayers remains a major challenge in drug discovery. Conventional cell-based assays often fail to capture the critical influence of post-translational modifications (PTMs) and membrane context on protein function and ligand specificity. To overcome these limitations, we developed two complementary native mass spectrometry (MS)–based approaches: a high-throughput screening platform combining direct injection with ultra-high mass resolution (UHMR), enabling label-free screening of up to 50,000 compounds per day against membrane protein targets; and a laser-induced injection method that selectively releases intact membrane protein complexes directly from native lipid environments, allowing detailed structural and interaction analysis. We applied this integrated platform to study rhodopsin, a prototypical G protein-coupled receptor (GPCR), in its native lipid bilayer. Using infrared laser-induced ejection, intact rhodopsin-effector complexes were released directly from retinal rod disc membranes. Subsequent gas-phase isolation and infrared multiphoton dissociation (IRMPD) enabled proteoform-level sequencing and PTM mapping. This native membrane proteomics approach uncovered distinct lipidated Gβγ subunits and identified a previously uncharacterized Gβγ proteoform that abolishes membrane association. Additionally, we localized labile palmitoylations on rhodopsin that influence receptor structure and ligand binding, and observed lipid modifications on G proteins that modulate their assembly and interactions. To explore the pharmacological relevance of these findings, we employed the high-throughput MS screening workflow to assess off-target interactions of the phosphodiesterase 5 (PDE5) inhibitors sildenafil and vardenafil with native retina membrane proteins. Both compounds showed differential binding to proteoforms of phosphodiesterase 6 (PDE6), with a preference for lipidated G protein–associated complexes. These results provide mechanistic insight into the visual side effects observed with PDE5 inhibitors and highlight the importance of proteoform-specific targeting in drug development. The combination of UHMR-native MS, laser-based membrane protein release, and automated data analysis offers a powerful, unified platform for characterizing protein–ligand interactions within native membrane assemblies. This workflow overcomes limitations associated with detergent solubilization or reconstituted systems and enables in-depth exploration of proteoform diversity, including lipidation, phosphorylation, and other modifications that govern biological function. Together, these advances demonstrate how native proteomics can enable both the large-scale discovery of bioactive compounds and the mechanistic resolution of membrane protein interactions, ultimately facilitating the identification of novel therapeutic targets and drugs with improved specificity and reduced off-target effects.

AF3x: Improving AlphaFold 3 structural modeling by incorporating explicit crosslinks

DAY 2 – SELECTED TALK

Konstantin Gilep[1], Agnieszka Obarska-Kosinska[2], Jan Kosinski[1]

[1] EMBL Hamburg, Centre for Structural Systems Biology (CSSB), [2] Max Planck Institute of Biophysics

Recent advances in neural network–based structural modeling have substantially transformed structural biology. Among these, AlphaFold 3 (AF3) extends modeling capabilities beyond protein chains to include nucleic acids, post-translational modifications, and small molecules, including covalently bound ones. Nevertheless, accurate prediction of protein-protein interactions and alternative conformations remains a major challenge. Previous studies have shown that such difficult tasks can be facilitated by incorporating experimental residue distance restraint data derived from methods such as crosslinking mass spectrometry (XL-MS). Here, we present AF3x, a framework for integrating crosslinking data directly into the AF3 prediction pipeline by explicitly introducing covalently bound crosslinking molecules as structural components. Unlike standard residue distance restraints, AF3x embeds atomistic crosslinkers directly into the model, allowing their geometry and the spatial volume between crosslinked atoms to define the accessible surface for crosslink formation and to impose more realistic physical constraints on conformational sampling. AF3x supports the inclusion of variable crosslinking molecules, including custom-designed ones. Our results demonstrate that this strategy improves the accuracy of standard AF3 predictions and achieves performance comparable to other approaches that employ residue distance restraints.

Integrative modeling of large and dynamic protein assemblies

DAY 2 – SELECTED TALK

Dina Schneidman

Recent progress in protein structure prediction has significantly enhanced integrative structure modeling of large macromolecular assemblies by providing improved structural coverage for individual proteins and protein-protein interactions. However, direct prediction of large protein assemblies remains a challenge due to their size, dynamics, and disordered regions. I will introduce CombFold, a hierarchical and combinatorial assembly algorithm designed to predict structures of large protein complexes utilizing pairwise interactions between subunits predicted by AlphaFold. We test the method on a benchmark of large heteromeric assemblies (up to 30 chains and 18,000 amino acids) and obtain a success rate of ~70%. Distance restraints derived from cross-linking mass spectrometry can be directly integrated into the assembly algorithm, thereby further improving accuracy. We further develop CombFold to model protein interactions with disordered regions and account for compositional and conformational heterogeneity based on cross-linking datasets. Shor B, Schneidman-Duhovny D. CombFold: Predicting structures of large protein assemblies using combinatorial assembly algorithm and AlphaFold2. Nat Methods, 21(3):477-487, 2024

Characterizing novel interactors of the membrane remodeling protein CHMP7 using photo-crosslinking MS

DAY 3 – SELECTED TALK

Dollie LaJoie, Swantje Lenz, Letitia Fernandez, Andrea Knaust, Andrej Shevchenko, Alexander von Appen

MPI-CBG, Dresden

The endosomal sorting complex required for transport (ESCRT) pathway mediates diverse membrane remodeling events within cells. Given how ubiquitous this machinery is, it must be tightly regulated in space and time through specific interactions. CHMP7 is a structurally unique constituent of the ESCRT family of proteins, for which very few interactors have been identified, let alone characterized biologically. Using protein motif annotation, a priori information from the literature, and structural modelling, we sought to identify novel interactors of CHMP7. Candidate interactions were confirmed with photo-crosslinking mass spectrometry and further characterized biochemically. Additionally, we use approaches in cell biology and light microscopy to study the function of these interactions in cells.

Streamlined In Vivo Crosslinking and Dual Enrichment Workflow Enables High-Throughput Mapping of the Nuclear Interactome

DAY 3 – SELECTED TALK

Philipp Bräuer[1], Laszlo Tirian[2], Fränze Müller[1], Karl Mechtler[123], **Manuel Matzinger**[1]

[1] Research Institute of Molecular Pathology (IMP), Vienna BioCenter (VBC), Vienna, Austria, [2] Institute of Molecular Biotechnology (IMBA), Austrian Academy of Sciences, Vienna BioCenter (VBC), Vienna, Austria, [3] Gregor Mendel Institute of Molecular Plant Biology (GMI), Austrian Academy of Sciences, Vienna BioCenter (VBC), Vienna, Austria

Protein–protein interactions (PPIs) are central to virtually all cellular processes, yet their comprehensive mapping in native cellular environments remains a major challenge. Traditional approaches such as co-immunoprecipitation or proximity labeling often suffer from limitations in capturing transient or weak interactions and typically require cell lysis, disrupting native complexes. Cross-linking mass spectrometry (XL-MS) has emerged as a powerful alternative, offering structural insights into PPIs in situ. However, proteome-wide XL-MS studies are hindered by low crosslinking efficiencies, complex sample matrices, and the computational burden of data analysis. To address these challenges, we developed a robust and scalable in vivo crosslinking workflow based on the MS-cleavable, enrichable crosslinker azide-a-disuccinimidyl bis-sulfoxide (DSBSO). Our approach integrates two orthogonal enrichment strategies—affinity enrichment via strain-promoted azide-alkyne cycloaddition (SPAAC) and size exclusion chromatography (SEC)—to selectively isolate DSBSO-crosslinked peptides while minimizing background from linear and mono-linked species. We applied this workflow to K562 cells and achieved the identification of over 5,000 unique crosslinks, representing one of the most comprehensive in vivo interactome datasets to date. Notably, analysis of a single SEC fraction was sufficient to recover approximately 90% of all crosslinks, significantly reducing instrument time and enhancing throughput. Within the nuclear sub-proteome, we identified 393 PPIs, including 56 previously unreported interactions, underscoring the method's sensitivity and discovery potential. To further explore the method's utility in detecting low-abundance interactions, we applied DSBSO crosslinking to nuclear extracts. This enabled deeper coverage of interactions involving less abundant proteins, exemplified by the DEAD-box RNA helicase DDX39B. Structural mapping of crosslinks revealed that DDX39B exists in both monomeric and dimeric forms and interacts with its paralog DDX39A, suggesting a dynamic interplay within nuclear RNA processing complexes. In summary, our optimized DSBSO-based workflow enables efficient, high-throughput, and proteome-wide mapping of PPIs in their native context. The combination of orthogonal enrichment steps significantly improves crosslink yield and data quality for in vivo crosslinking studies, making this approach also highly suitable for large-scale interactome studies. Our findings not only expand the known nuclear interactome but also demonstrate the feasibility of capturing dynamic and low-abundance interactions, paving the way for future applications in systems biology and disease research. References: Bräuer P. et al.

In vivo crosslinking and effective 2D enrichment for proteome wide interactome studies. Commun Chem 8, 245 (2025). https://doi.org/10.1038/s42004-025-01644-6

In situ cross-linking mass spectrometry contextualizes cryo-ET to study the mammalian ciliary base

DAY 3 – SELECTED TALK

Caitlyn L McCafferty[1], Hugo van den Hoek[1], Marine Brunet[2], Tim Stearns[3], Paul Guichard[2], Virginie Hamel[2], Benjamin D. Engel[1]

[1] University of Basel, Biozentrum, Basel, Switzerland, [2] University of Geneva, Geneva, Switzerland, [3] Rockefeller University, New York, USA

In recent years, cross-linking mass spectrometry (XL/MS) has emerged as a powerful tool for in situ structural biology, allowing both temporal and spatially controlled mapping of protein–protein interactions. When combined with cryo-electron tomography (cryo-ET), XL/MS bridges the molecular identification gap that often comes with label-free cellular imaging by providing candidate proteins for unassigned densities. These candidates can then be further validated by ultrastructure expansion microscopy (U-ExM confirming their precise localization within cells. We apply this integrated approach to mammalian multiciliated cells, generating the first XL/MS-based interactome of the ciliary base, comprising >10,500 unique cross-links across 1,661 proteins and 1,171 protein–protein interactions. Guided by cross-links, we were able to assign identities to microtubule inner proteins (MIPs) and microtubule-associated proteins (MAPs), including the unexpected discovery of MLF1 and DNAJB6 as novel tubulin-binding proteins. U-ExM confirmed their distinct localizations, revealing DNAJB6 enrichment at the transition zone where cryo-ET showed a previously undescribed inner helical density. Together, these results highlight XL/MS as a powerful complement to in situ imaging—providing molecular identification where cryo-ET alone cannot—and establish a roadmap for using proteomics to contextualize cellular cryo-ET data.

Porous Graphitic Carbon (PGC)-HPLC Fractionation Enhances Detection of Cross-Linked Peptides for Robust XL-MS Workflows

DAY 3 – SELECTED TALK

Suparat Scheu[1], Tara Bartolec[2], Mandy Rettel[1], Clement Potel[2], Nassos Typas[2], Mikhail Savitski[1,2]

[1] Proteomics Core Facility, EMBL, Germany, [2] Molecular Systems Biology, EMBL, Germany

Cross-linking mass spectrometry (XL-MS) enables mapping of protein–protein interactions (PPIs) and extracting structural information from proteins in their native context within complex mixtures. XL-MS employs cross-linkers to covalently link proximal amino acids, generating cross-linked peptides upon enzymatic digestion that are subsequently identified by LC–MS/MS. Despite many recent outstanding studies employing XL-MS approaches, several limitations remain to be overcome to establish XL-MS as an effective and robust workflow suitable for service infrastructures. Detecting and identifying cross-linked peptides is particularly challenging due to the broad dynamic range and high complexity of proteomes, and is especially problematic for low-abundance cross-links, which often represent protein-protein interactions. Therefore, peptide separation approaches are necessary to enhance the sensitivity and depth of cross-link identification. Here, we establish porous graphitic carbon high-performance liquid chromatography (PGC-HPLC) as an alternative offline fractionation method for XL-MS samples. Using Escherichia coli (E. coli) cell lysate cross-linked with PhoX, which is an enrichable crosslinker, we benchmarked PGC-HPLC against conventional workflows including size exclusion chromatography (SEC) and high-pH reversed-phase (High-pH RP) fractionation, and optimized pooling and concatenation schemes to maximize recovery of cross-linked peptides. PGC-HPLC outperformed both SEC and High-pH RP; from 24 fractions analyzed in a single day of LC-MS/MS acquisition, it facilitated the identification of approximately 5,400 unique cross-linked peptides and ~900 PPIs, corresponding to roughly a two-fold increase in detected inter-protein cross-links relative to High-pH RP and SEC. PGC-HPLC was further applied to PhoX cross-linking of purified E. coli membrane proteins, yielding ~10,000 unique cross-linked peptides and ~1,200 PPIs from 24 fractions. These results establish PGC-HPLC as a scalable offline fractionation strategy that substantially deepens XL-MS coverage.

Investigating low crosslink abundance in whole cell XL-MS

DAY 1 – POSTER 1

Bruno C. Amaral, Nicholas I. Brodie, Andrew R.M. Michael, D. Alex Crowder, Pauline Douglas, Morgan F. Khan, David C. Schriemer.

Department of Biochemistry & Molecular Biology, University of Calgary, Calgary, Alberta, Canada

Crosslinking mass spectrometry (XL-MS) is often positioned as the bridge between systems and structural biology, yet in whole-cell settings its apparent signal remains stubbornly low and vulnerable to experimental artifacts. We asked a simple question with a difficult-to-achieve answer: are the disappointing crosslink numbers a chemistry problem where they are not produced in enough quantity, or are we losing them in downstream steps like sample handling, acquisition, and identification? We will present these investigations combining our formaldehyde-based cell-preserving workflow (FIX-MS) with our newly developed Click-Linking. Single in situ experiments using Click-Linking resulted in threefold increases in detected PPIs even when compared to enrichment-capable linkers like PhoX. Combining these two strategies, crosslinks can account for up to 30% of the entire proteome signal, illustrating that chemical yield is not the main issue. We will illustrate possible points for crosslink losses during sample preparation and MS analysis that could account for the unfortunate reality in which we only identify a small fraction of what is formed in cells, opening venues to explore and regain some of that signal back in order to make XL-MS a viable method for whole-cell interactome mapping.

QproMS: a web application for label-free proteomic data analysis

DAY 2 – POSTER 1

Linda Andreoli[1], Giorgia Cucina [2], Fabio Bedin[1], Andrea Graziadei[3], Alessandro Cuomo[1]

[1] Department of Molecular Oncology, European Institute of Oncology IRCCS, Milan, Italy, [2] University of Milan, Milan, Italy, [3] Fondazione Human Technopole, Milan, Italy

The significance of mass spectrometry-based proteomics in biological and clinical research has steadily increased, becoming one of the most powerful tools. Therefore, modern proteomics has experienced a rapid evolution of algorithms and data analysis approaches aimed at extracting robust statistical insights and identifying potentially intriguing biological outliers. We present here Quantitative Proteomics Made Simple (QProMS), a user-friendly, data analysis and visualisation pipeline aimed at guiding the scientist through all steps from preprocessing and statistical analysis to visualization in a graphical interface. QProMS is capable of importing and processing proteomics data generated from various software platforms including MaxQuant, FragPipe and Spectronaut. Statistical tests rely on established R functions and are compatible with all types of label-free quantification experiments. The pipeline recapitulates features from different available software packages and introduces mixed imputation, an improved framework for handling missing values. QProMS can also perform interaction analyses based on gene ontology, or by querying protein-protein interaction databases. All figures in QProMS are interactive, allowing for the investigation of individual proteins of interest before exporting. The analysis steps and parameters can be saved within a standalone report, ensuring reproducibility for future analyses. In summary, QProMS is built to offer a comprehensive and robust platform for both beginner and experienced researchers, enabling state-of-the-art data analysis of a wide variety of label-free proteomic workflows ranging from global proteome profiling to targeted methods such as proximity labelling.

Mass spectrometry of palmitoylated peptides: challenges and prospects

DAY 1 – POSTER 2

E. Badin, C. Schmidt

Johannes Gutenberg University Mainz

Protein lipidation such as cysteine palmitoylation, consisting of a of a thioester-linked palmitate on the cysteine residues, is an important, reversible post-translational modification of proteins determining not only their stability and folding but also their interactions with other proteins or membranes. However, in contrast to other post-translational modifications, lipidation is less explored and lipidated proteins are underrepresented in large-scale studies. We, therefore, set out to advance the analysis of S-palmitoylation by mass spectrometry. For this, we chose a model peptide of the synaptosome associated protein 25 (SNAP25), a SNARE protein involved in membrane fusion during neurotransmitter release. SNAP25 is naturally anchored to the synaptic plasma membrane through four proximate S-palmitoylated cysteine residues in its linker region. By selectively introducing S-palmitoylation in the four potential modification sites, we generated a set of multiply modified tryptic peptides differing in the sites as well as the degree of modification. Importantly, we noticed that solubility of the peptides decreased tremendously with increasing degree of modification requiring the use of alternative solvents. Nonetheless, using direct-infusion mass spectrometry, we characterised the ionisation and fragmentation behaviour of the differently modified peptides. We show that lipidation is stable during tandem mass spectrometry and that the sites of modification can be unambiguously identified. The use of dimethyl sulfoxide during electrospray ionisation further improved signal intensity of multiply modified peptides. In summary, we found that the identification of S-palmitoylation even in multiply modified peptides is possible; however, further improvements are required for large-scale analyses.

Enzyme Activity Regulates Substrate Diffusion by Modulating Viscosity in Crowded Milieu

DAY 2 – POSTER 2

Alessandro Bevilacqua[1], Mauricio Rios Maciel, Mark V. Sullivan, Stefano Pascarelli, Mirco Dindo, Amy Q. Shen, Paola Laurino

[1]Okinawa Institute of Science and Technology

Enzymatic activity and its tight regulation are fundamental to cellular metabolism and life. While classical models of enzyme kinetics explain the behavior of enzymes in dilute buffer solutions, there are elusive properties that emerge from enzymes in their native, crowded environments. In this study, we harness liquid-liquid phase separation (LLPS) to create controlled in vitro droplets that mimic cytosolic protein crowding, offering a unique system to understand enzyme kinetics in complex microenvironments. We uncover a mechanism in which enzyme-induced changes in shear viscosity arise from dynamic interactions among the substrate, product, and the protein crowder. Using fluorescence microscopy, bulk shear rheometry and microrheology, we show that enzymatic activity modifies the apparent viscosity of both protein-rich droplets and the surrounding PEG-rich phase, enhancing substrate mobility and improving substrate access to catalytic sites. Our findings suggest that this enzymatic-viscosity coupling affects substrate availability and influences the organization and dynamics of macromolecular crowding within droplets. These results provide new insights into how enzymes impact both their physical environment and metabolic processes in the cell.

The PRIDE Crosslinking Resource: An Archive for Crosslinking MS Data and Results

DAY 1 – POSTER 3

Colin W. Combe[1], Suresh Hewapathirana[2], Arthur Zalevsky[3], Chakradhar Bandla[2], Lutz Fischer 1, Jingwen Bai[2], Deepti J. Kundu [2], Kolja Stahl[1], Andrej Sali[3], Stephen K. Burley[3,4], Sameer Velankar [2], Brinda Vallat[4], Juan Antonio Vizcaíno [2], Juri Rappsilber[1]

[1] Technische Universität (TU) Berlin, [2] European Molecular Biology Laboratory, European Bioinformatics Institute (EMBL-EBI), Cambridge, [3] Research Collaboratory for Structural Bioinformatics Protein Data Bank, Department of Bioengineering and Therapeutic Sciences, Quantitative Biosciences Institute (QBI), and Department of Pharmaceutical Chemistry, University of California, San Francisco, USA, [4] Research Collaboratory for Structural Bioinformatics Protein Data Bank and the Institute for Quantitative Biomedicine, Rutgers, The State University of New Jersey, Piscataway, USA; Rutgers Cancer Institute, Rutgers, The State University of New Jersey, New Brunswick, USA

Crosslinking Mass Spectrometry (MS) provides distance restraints for modelling protein complexes and mapping protein–protein interactions. PRIDE, the leading ProteomeXchange repository, now includes a dedicated resource for crosslinking MS data and results. As part of PRIDE, this resource has neutrality and long-term support that an archive supported by an individual laboratory cannot. It is based on mzIdentML as the file format for submitting crosslinking MS experimental results. The goal of the archive is to facilitate the validation and reuse of crosslinking MS results. The PRIDE Crosslinking Resource provides an API for programmatic access to the archive and web pages to browse the information. The web pages include summary pages for projects with onward links to other related resources, and visualisation in xiVIEW. The Protein Data Bank has been closely involved in the work, with the aim of using mzIdentML and this resource to validate the crosslinking component of integrated hybrid modelling outputs. With these advances, mzIdentML can now be used as the basis for tracking the provenance of crosslinking data from spectra to 3D structure.

Resolving the dimerisation core of human amylin (hIAPP) through native top-down ion mobility and electron-capture dissociation

DAY 2 – POSTER 3

Zijie Dai

University College London, Institute of Structural and Molecular Biology

Human islet amyloid polypeptide (hIAPP), a 37-residue pancreatic hormone co-secreted with insulin, is essential in regulating glucose metabolism. However, hIAPP is prone to misfolding and aggregation. Its pathological assembly into oligomers and insoluble amyloid fibrils underlies pancreatic β-cell dysfunction and is a hallmark of Type-2 Diabetes (T2D). Mounting evidence indicates that early, soluble oligomeric intermediates of hIAPP, rather than mature fibrils, are the principal toxic species. However, the structural basis of the toxic oligomeric intermediates remains elusive due to their transient, heterogeneous, and intrinsically disordered nature. Defining the nucleation interfaces and conformational dynamics that govern early oligomerisation is therefore central to understanding disease-relevant aggregation mechanisms. Here, we present the first conformer-specific and residue-specific structural interrogation of the oligomerisation process of intrinsically disordered hIAPP, using state-of-the-art native top-down cyclic ion-mobility mass spectrometry (cIM-MS) integrated with electron-capture dissociation (ECD) and collision activation (CA). This workflow allows the isolation and differentiation of discrete hIAPP oligomeric conformers under native-like conditions, followed by conformer-targeted structural activation and fragmentation, yielding residue-level mapping of dimerisation interfaces. By combining native top-down cIM-MS with ECD fragmentation, we mitigate the long-standing challenge of conformational averaging in native MS, providing conformer- and residue-specific evidence of structural protection and conformational change within transient assemblies. Our study focused on wild-type hIAPP and the clinically relevant S20G variant, a natural mutation that accelerates aggregation and exacerbates T2D pathogenesis. We were able to separate co-existing dimeric conformers by cIM-MS and track the conformational changes along their unfolding trajectories under progressive collision activation. This strategy revealed the conformer-dependent differences in their structural stability and dynamics, providing evidence for sequential structural rearrangements preceding aggregation. Top-down ECD fragmentation further identified consistent protection near the C-terminal region as the nucleation core at a residue-specific level, while exposing a flexible N-terminal segment. Notably, we found that the S20G variant exhibits higher local stability around the proposed nucleation core compared to wild-type during activation, correlating with its accelerated aggregation propensity. Both hIAPP sequences exhibited conserved sequence-specific protection motifs, indicating the presence of the shared structural core that is critical for early oligomerisation. Collectively, these findings provide the first conformer- and residue-resolved insights into the structural cores that nucleate hIAPP oligomerisation. More broadly, they establish a generalisable framework for probing conformational changes in intrinsically disordered proteins. This work demonstrates the capability of native top-down cIM-MS with ECD to dissect transient, heterogeneous protein assemblies with

unprecedented structural detail at conformer-specific and residue-specific levels, advancing mechanistic understanding of amyloid formation and its role in T2D pathogenesis. Importantly, by pinpointing conserved nucleation motifs and variant-specific conformational changes, our study provides a molecular foundation that may aid the design of targeted therapeutic strategies to modulate or disrupt early oligomerisation events in hIAPP.

Increasing the Analytical Depth of Large-Scale Cross-Linking Studies with the Astral Mass Analyzer

DAY 1 – POSTER 4

Philipp Deist[1,2], Shakir Shakir[3], Zhengrui Zhang[2], Guillaume Dumenil[2], Julia Chamot-Rooke[1]

[1] Institut Pasteur, Université Paris-Cité, CNRS UAR 2024, Mass Spectrometry for Biology, Paris, France, [2] Institut Pasteur, Université Paris Cité, INSERM UMR1225, Pathogenesis of Vascular Infections, Paris, France, [3] Thermo Fisher Scientific, Villebon-sur-Yvette, France

Cross-linking mass spectrometry (XL–MS) is a well-established technique to study protein–protein interactions of multiprotein complexes. The transition from multiprotein to large-scale interactome studies, however, remains challenging. Currently, high sample complexity of large-scale studies outreaches state-of-the-art analytical capacities, creating an analytical bottleneck. The bottleneck unfolds as only a limited number of cross-links of the most abundant proteins are confidently identified, limiting the depth and biological relevance of large-scale interactome analyses. In this study, we introduce the Orbitrap Astral mass analyzer to mitigate this constraint. We cross-linked a whole cell lysate of the gram-negative pathogen Neisseria meningitidis with our enrichable cross-linker NNP9. Enriched, cross-linked peptides were separated over a 2 h chromatographic gradient, gas-phase separated in the FAIMS interface, and analyzed with the Orbitrap Astral or the Orbitrap Eclipse, the current XL–MS gold standard. Raw data was searched with xiSearch v1.8 for NNP9-generated cross-links against the whole Neisseria meningitidis proteome (2,000 entries). Five times more MS2 spectra (total: 210,000) were recorded on the Orbitrap Astral. For a cross-link triplicate, this translates to a two-fold increase of cross-link spectrum matches (total: 2,000 CSMs/replicate), a 1.8-fold increase of unique peptide pairs, and a 1.3-fold increase of cross-linked protein groups, compared to the Orbitrap Eclipse. For the most abundant proteins, the number of cross-links was doubled, and new ones were also discovered in previously unattained proteins. At most, on the Orbitrap Astral platform, we identified up to 2,850 CSMs for a single run. The numbers highlight that migrating large-scale XL–MS studies to the Orbitrap Astral platform yields deeper insight into the cellular interactome, mitigating the analytical bottleneck. Next, we will take advantage of the increased analytical depth to structurally better resolve the multiprotein complex of interest in Neisseria meningitidis.

Structural analysis of the full-length Bcl2/Beclin 1 complex reveals alternative Bcl-2 binding sites on Beclin 1

DAY 2 – POSTER 4

Miray Turk, Umut Cagan Ucar, **Gizem Dinler Doganay**

Istanbul Technical University Molecular Biology and Genetics & Biotechnology Research Center, Istanbul, Türkiye

Autophagy and apoptosis balance maintains homeostasis and survival. Any dysregulation in this interplay functions as a driver factor for a wide range of diseases, e.g. neurodegenerative diseases and cancer. Beclin 1 is the major macroautophagy regulator due to its crucial role in the preautophagosomal complex formation, which is essential for autophagy initiation. Its interaction with Bcl-2 negatively regulates Beclin 1 activity by preventing its interaction with other complex members. For this reason, Bcl-2/Beclin 1 complex creates a pivotal juncture for cell homeostasis. Although both Bcl-2 and Beclin 1, as well as their complex have been considered as drug target for therapy and some interaction points have also been identified so far, drugs have not yet achieved the desired success, primarily due to lack of resolved full-length structure and protein-protein interaction surface. Therefore, comprehensive studies are still required to fully elucidate their interaction surface(s). We investigated their interaction interface(s), which may constitute alternative drug target site(s). Determining this surface could identify druggable sites for potential anticancer therapies. To achieve this, full-length Bcl-2 and Beclin 1 proteins were produced and affinity purified (> 80%). The activity of these proteins was confirmed by in vitro interaction assays. The interaction regions of Bcl-2 on Beclin 1 were determined based on deuterium incorporation levels of Beclin 1 in the presence and absence of Bcl-2 using HDX-MS. Given the lack of full-length structures for Bcl-2, Beclin 1, and their complex, molecular dynamics simulations were conducted to visualize the HDX-MS data. Our findings reveal that although the interaction between Bcl-2 and Beclin 1 traditionally involves the BH3 domain of Beclin 1 and the cavity of Bcl-2, additional interaction regions of Bcl-2 on Beclin 1 were identified. These surfaces can potentially be used to target and inhibit Bcl-2 for therapy.

Aptamer-Based GANAB Enzyme Blockade Guided by Artificial Intelligence for Therapeutic Intervention in Pancreatic Cancer

DAY 1 – POSTER 5

Anna Drabik[1], Przemyslaw Mielczarek[1], Juseong Kim[2]

[1] Department of Analytical Chemistry and Biochemistry, AGH University of Science and Technology, Krakow, Poland, [2] Division of Artificial Intelligence, Pusan National University, Busan, Republic of Korea

Background: Pancreatic cancer remains one of the most aggressive and lethal malignancies, characterized by rapid disease progression, early metastasis, and poor prognosis with a five-year survival rate often below 10%. A major contributor to pancreatic cancer pathophysiology is aberrant glycosylation—a post-translational modification wherein sugar moieties are enzymatically attached to proteins, profoundly affecting their structure and function. Glycosylation governs key cellular processes, including protein folding, stability, cell-cell adhesion, immune recognition, and signaling pathway modulation. In pancreatic tumors, dysregulated glycosylation mediated by enzymes such as GANAB (Glucosidase II alpha subunit), a critical player in the N-linked glycosylation pathway, results in the accumulation of extensively glycosylated protein forms that promote oncogenic behaviors, including immune evasion, enhanced cell adhesion, and invasiveness. Targeting the enzymatic activity of GANAB presents a novel therapeutic avenue by disrupting these pathological glycosylation profiles, potentially impeding tumor progression and metastasis. Method: In this study, we explored an innovative therapeutic strategy to inhibit GANAB enzyme activity in pancreatic cancer cells utilizing aptamers—short, single-stranded oligonucleotides capable of high-affinity, specific binding to target proteins. Aptamers against GANAB were generated through an iterative in vitro selection procedure, further refined using a sophisticated artificial intelligence (AI) algorithm framework, AptaTrans. This AI-driven platform accelerated aptamer design by optimizing sequence prediction, enhancing binding affinities, and improving molecular specificity for the GANAB enzyme, surpassing conventional SELEX methods in both speed and efficacy. We conducted comprehensive biochemical assays to determine the extent of GANAB inhibition, specifically by measuring N-linked glycosylation levels on key glycoproteins following aptamer treatment. Lectin-based proteomic profiling was employed for detailed quantitation of changes in glycosylated protein forms, complemented by mass spectrometry analyses to confirm glycosylation pattern alterations. To assess functional consequences of GANAB inhibition, we performed in vitro assays evaluating pancreatic cancer cell proliferation rates, adhesion capabilities, and invasive potential, all hallmark traits linked to glycosylation-mediated tumor aggressiveness. Parallel toxicity assays ensured aptamer specificity, confirming minimal off-target effects on normal pancreatic tissue cells. Results: Our results demonstrate that application of the AI-designed aptamer remarkably reduced GANAB enzymatic activity, effectively disrupting N-glycan processing in pancreatic cancer cells. This blockade led to a significant decrease in the abundance of extensively glycosylated oncogenic proteins—critical mediators of tumor cell adhesion and metastatic dissemination—as verified through lectin-binding assays and high-

resolution glycoproteomic mass spectrometry profiling. Functionally, GANAB inhibition by the aptamer corresponded with a notable reduction in pancreatic cancer cell adhesion and invasiveness, indicating effective disruption of glycan-dependent cellular mechanisms that facilitate metastasis. Importantly, this targeted inhibition did not produce significant cytotoxic effects on normal pancreatic tissue cells, underscoring the therapeutic specificity and safety potential of the aptamer approach. These findings align with growing evidence that aberrant glycosylation enhances malignant phenotypes in pancreatic cancer by altering cell surface glycoproteins such as integrins and cadherins which mediate extracellular matrix interactions, and tumor-associated antigens which modulate immune evasion. By blocking GANAB's upstream role in glycosylation, the modified aptamers essentially "normalize" glycoprotein profiles, impairing the tumor's ability to metastasize and survive in hostile tissue microenvironments. Conclusion: This study validates the feasibility and therapeutic promise of using AI-guided aptamer technology to inhibit GANAB enzymatic activity in pancreatic cancer. By targeting GANAB-mediated glycosylation, this approach strategically interrupts the formation of aberrant glycoprotein forms that are foundational for tumor progression, adhesion, immune escape, and metastasis. The integration of artificial intelligence in aptamer development offers a rapid, precise, and scalable modality to generate highly specific inhibitors for challenging molecular targets such as glycosylation enzymes. The resulting aptamer-based GANAB blockade shows high efficacy and specificity with negligible toxicity in normal pancreatic cells, highlighting its potential for clinical translation. Future research will focus on in vivo validation, pharmacokinetic characterization, and optimization of delivery methods for aptamer therapeutics targeting glycosylation pathways. Moreover, coupling GANAB inhibition with existing chemotherapies may enhance treatment response and overcome resistance by modulating the tumor glycoproteome. Collectively, this work presents a pioneering molecular intervention against pancreatic cancer's glycosylation-dependent oncogenic machinery, opening pathways for precision oncology applications that harness AI-powered molecular therapeutics to combat this formidable disease.

Defining the Molecular Basis for Altered DNA-Binding of p53 Isoforms by Structural Mass Spectrometry

DAY 2 – POSTER 5

J. Fehr[2], A. Sinz[1,2] C. Arlt[1,2]

[1] Martin Luther University Halle Wittenberg, Department of Pharmaceutical Chemistry and Bioanalytics, Halle, Germany, [2] Martin Luther University Halle Wittenberg, Center for Structural Mass Spectrometry, Halle, Germany

P53 acts as a major tumor suppressor in human cells that is often referred to as the "guardian of the genome" and plays a pivotal role in cancer prevention. Upon detection of cellular stress, such as DNA damage, p53 is activated and orchestrates a series of protective responses. These include regulating cell cycle, initiating DNA repair, promoting cellular senescence (growth arrest), and triggering apoptosis. Through these mechanisms, p53 ensures that damaged or abnormal cells do not survive and divide, thus maintaining genetic stability and preventing tumorigenesis. The importance of p53 is underscored by the fact that the TP53 gene is the most frequently mutated gene in human cancers. When p53 is lost or impaired in function, cells lose a critical line-of-defense against malignant transformations. The TP53 gene ultimately results in a network of at least 12 protein isoforms through mechanisms, such as alternative promoter usage, alternative splicing, and alternative translation initiation. Besides the p53 wild-type, the main N-terminally truncated isoforms are Δ40p53, Δ133p53, Δ160p53. C-terminally altered isoforms (p53β, p53γ) diversify the structure and functional repertoire of p53 in cells (Mehta et al., 2021). Aforementioned isoforms modulate both the canonical and non-canonical functions of the p53 pathway, providing a additional layer of regulatory control over cell fate and stress responses. Aside of fine-tuning gene expression (Guo et al. 2024), some isoforms, like p53β/γ and Δ40p53, function independently of full-length p53 to direct unique gene expression programs or to carry out transcriptional regulation that does not overlap with p53. Others, such as Δ133p53 and Δ160p53, impact the outcome by forming mixed oligomers with p53α, fine-tuning responses to cellular stress. For example, Δ40p53α in hetero-oligomers can stabilize p53α by protecting it from MDM2-mediated degradation and shift its transcriptional activity from cell cycle arrest genes, like CDKN1A, to pro-apoptotic genes (gain-of-function). In contrast, Δ133p53 acts as dominant-negative regulator by inhibiting p53α's ability to induce apoptosis and senescence, while promoting the expression of DNA repair genes (Guo et al. 2024). In a disease context, p53 isoforms are often dysregulated resulting in tumor progression or suppression, depending on the isoform. Our work aims at gaining insights into the DNA-binding behavior, aggregation tendencies, and hetero-tetramerization capabilities with full-length p53 of various isoforms. The recombinantly expressed and purified p53 isoforms are currently being analyzed by native MS to investigate hetero-oligomerization and qualitative DNA binding. DNA-binding affinities of p53-DNA complexes will be determined by surface plasmon resonance (SPR) and compared to wild-type p53. Selected p53-DNA complexes will be in -depth characterized by a combination of cross-linking MS (XL-MS) for structural characterization and native ion mobility mass spectrometry (IM-MS) to determine

tetrameric conformation and collision-induced unfolding (CIU) experiments. Our results will help clarifying the molecular basis for altered DNA-binding of p53 isoforms and help identifying their specific roles in different cancer types.

Integrative Structural Modeling of Intrinsically Disordered Regions in a Human HDAC2 Chromatin Remodeling Complex

DAY 1 – POSTER 6

Jules Nde[1], Cassandra G. Kempf[2], Rosalyn C. Zimmermann[1], Joseph Cesare[1,2] , Ying Zhang[2], Jerry L. Workman[2], **Laurence Florens**[2], Michael P. Washburn[1]

[1] Department of Cancer Biology, University of Kansas Medical Center, Kansas City, USA
[2] Stowers Institute for Medical Research, Kansas City, USA

Intrinsically disordered regions (IDRs) and intrinsically disordered proteins (IDPs) play pivotal roles in cellular signaling, molecular recognition, and the regulation of various biological processes. These flexible and conformationally dynamic protein segments are difficult to study using structural analysis methods and computational approaches including AlphaFold. A critical challenge arises when attempting to understand the structural basis of protein-protein interactions involving IDRs. IDRs often govern the formation of transient and flexible complexes and are difficult to study using conventional structural biology techniques. Here we demonstrate that the poorly characterized C16orf87 protein, which we rename MHAP1, forms a stable novel complex with HDAC2 and MIER1. These three proteins all contain IDRs whose structure is unknown. We implemented an integrative approach combining experimental crosslinking mass spectrometry data with computational modeling techniques (I-TASSER, HADDOCK, AlphaFold) to probe the IDR-driven assembly of the HDAC1:MIER2:MHAP1 complex and build an integrative structural model of the heterotrimer. The C-terminal domain of HDAC2, a poorly characterized IDR, promotes interactions between the ELM2 domain of MIER1 as well as the N- and C-termini of MHAP1. These results contrast with most current literature, including the results from AlphaFold alone that are missing structural information on HDAC C-domain. Our pipeline can be generalized to study other complexes, emphasizing the need for such integrative approaches in determining the 3D structures of IDR/IDP-driven complexes.

Monitoring Conformational Changes in the Tumor Suppressor Protein p53 Induced by DNA-Binding

DAY 2 – POSTER 6

Jacopo Giorgi, Christian Arlt, Christian Ihling, Marc Kipping, Andrea Sinz

Martin-Luther-University Halle-Wittenberg, Department of Pharmaceutical Chemistry and Bioanalytics, Institute of Pharmacy, Halle (Saale), Germany; Martin-Luther-University Halle-Wittenberg, Center for Structural Mass Spectrometry, Halle (Saale), Germany)

The tumor suppressor protein p53 is one of the most important regulators of cellular response to stress, including DNA repair, senescence, and apoptosis, making it highly relevant for studies in cancer biology. Mutation of the TP53 gene is a common event in tumorigenesis with missense mutations accounting for ~50% of human tumors. These missense mutations result in single amino acid substitutions in the sequence of the p53 protein, often disrupting its tumor-suppressive function and, in many cases, conferring gain-of-function properties that promote malignancy. In this study, 11 constructs, combining p53's DNA-binding domain (DBD) and tetramerization (TET) domain (amino acids 93-356), were expressed in E. coli and purified. The p53 constructs comprise the wild-type protein as well as 10 DBD single-point mutants. six mutants were selected from literature and database searches regarding their roles in cancer development, while four mutations were introduced based on a previous study from our lab [1]. These latter mutants are located in two conserved segments of p53's DBD (aa 138–145 and 229–236) and were selected to further investigate their influence on DNA binding. Both conserved segments have been shown to enhance stability in p53's DNA-free form, suggesting a pivotal role in the regulation of p53-DNA interaction. Following expression and purification of p53 wild-type and mutant constructs, DNA-binding will be evaluated using a library of p53 consensus DNA-response elements. To capture conformational changes in p53 upon DNA-binding in the different mutants, various methods of structural MS will be employed, such as HDX-MS, XL-MS, native MS, covalent labeling, and ion mobility MS.

[1] Di Ianni, A., Tüting, C., Kipping, M., Ihling, C. H., Köppen, J., Iacobucci, C., Arlt, C., Kastritis, P. L. & Sinz, A. (2023) Structural assessment of the full-length wild-type tumor suppressor protein p53 by mass spectrometry-guided computational modeling. Scientific reports, 13(1), 8497. https://doi.org/10.1038/s41598-023-35437-5

Evidence of TTR tetramer stabilization by RBP by limited proteolysis-mass spectrometry

DAY 1 – POSTER 7

Francesco Greco[1], Veronica Musetti[2], Lidia Ciccone[3], Maria Franzini[3], Liam A. McDonnell[4], Michele Emdin[1], Giuseppe Vergaro[1]

[1] Fondazione Toscana Gabriele Monasterio, Pisa, Italy, [2] Scuola Superiore Sant'Anna, Pisa, Italy, [3] Department of Pharmacy, University of Pisa, Pisa, Italy, [4] Fondazione Pisana per la Scienza ONLUS, San Giuliano Terme, Pisa, Italy

Background: Transthyretin (TTR) is a circulating protein, transporter of retinol-binding protein (RBP) and thyroid hormone T4. In its physiological status, TTR is an homotetramer bound to one or two RBP. TTR can dissociate into dimers and monomers; monomers unfold, and aggregates into beta-sheet fibrils whose deposition is responsible for TTR amyloidosis (ATTR). Currently ATTR is diagnosed only in the symptomatic stage of the disease. TTR stabilizing drug (such as tafamidis and acoramidis) slow down the TTR tetramer dissociation but cannot remove the already deposited fibrils. For this reason, early diagnosis through monitoring TTR destabilization and fibril formation directly in bloodstream would be crucial to prevent fibril deposition. Limited proteolysis-mass spectrometry (LiP-MS) is a powerful technique to investigate protein structure in the presence of a complex background. LiP-MS has never been applied to the investigation of TTR, but it could be a promising tool for early detection of TTR dissociation and aggregation in plasma of ATTR patients. We applied LiP-MS to the preliminary investigation of the interaction of TTR with RBP. Methods: TTR-RBP complex was produced by reaction of native recombinant TTR and RBP. Complex formation was verified by native gel electrophoresis. Native tetrameric TTR and native RBP were compared to TTR-RBP complex by LiP-MS in the presence of a complex yeast lysate. A first proteolytic reaction was carried on using proteinase K (PK), followed by enzyme denaturation at 100 C°. A second enzymatic reaction was performed with trypsin in denaturing conditions. Enzymes were added with an automated liquid handler robot to ensure reproducibility. Purified structural peptides were separated by liquid chromatography and analysed by tandem mass spectrometry (data independent analysis). Results: LiP-MS allowed to distinguish both TTR and RBP from TTR+RBP complex based only the intensity of structural peptide markers in the presence of a complex yeast lysate. RBP peptide LLNNWDVCADMVGTFTDTEDPAK and TTR peptide TSESGELHGLTTEEEFVEGIYK were protected from the PK digestion by the complex formation. These peptides are close to the TTR-RBP interaction surface. Interestingly, TTR peptide YTIAALLSPYSYSTTAVVTNPK, involved in monomer-monomer interaction in TTR dimers, is also protected upon the formation of TTR-RBP complex. This result support the hypothesis that TTR-RBP interaction slows down tetrameric TTR dissociation, possibly having a role in ATTR progression. Conclusions: LiP-MS is a powerful tool to probe protein structure in a complex environment. We performed a preliminary investigation on the potential of this technique in gaining information about TTR and RBP isoforms. Since both TTR and RBP are abundant proteins, the presented approach could

be extended to directly probe the native distribution of TTR and RBP isoforms in ATTR patient plasma.

Toward Residue-Resolved Protection Factors from HDX-MS data

DAY 2 – POSTER 7

Antonio Grimaldi and Emanuele Paci

University of Bologna

Hydrogen/deuterium exchange mass spectrometry (HDX-MS) is rapidly establishing itself as a pivotal technique for probing protein conformational dynamics, folding pathways, and molecular interactions. At the core of HDX interpretation lies the Linderstrøm-Lang (LL) model, in which each backbone amide hydrogen exists in either solvent-inaccessible ("closed") or solvent-accessible ("open") states, with exchange occurring only in the latter. Exchange rates therefore encode information on protein structure and dynamics, and are conventionally reported in terms of protection factors (PFs). Residue-level PFs are the thermodynamic quantities providing the link between raw uptake data and structural modelling. Yet, extracting PFs from measurements is notoriously difficult. HDX-MS records uptake across peptides that overlap in sequence domain, and such signals must be deconvolve to recover kinetics at the residue level, hence PFs. Current strategies often rely on two assumptions: (i) maximally deuterated controls can correct for back exchange, and (ii) residue-level signals can be recovered by subtractive analysis of overlapping peptides. Both assumptions are problematic. Fully deuterated controls implicitly assume uniform back exchange across residues, an assumption known to be false. Likewise, peptide subtraction are prone to artefacts: in experimental datasets, we find cases where a longer peptide exhibits a smaller corrected uptake than its nested shorter counterpart, or where the kinetics for the same residue inferred from different peptide pairs yield apparent rates that do not match. These inconsistencies propagate directly into estimation of PFs, ultimately undermining structural inferences (manuscript in preparation). To address these issues, we argue that more robust modelling frameworks are needed — ones that explicitly account for back exchange, during both the labeling reaction and subsequent processing steps. As a step in this direction, we developed a generalised Linderstrøm-Lang (GLL) model that incorporates back exchange effects in H2O/D2O mixtures. In collaboration with Prof. Theodoros K. Karamanos (UCL), we validated the GLL model leveraging HDX-NMR data, demonstrating that protection factors can be correctly retrieved by experiments performed in mixtures (manuscript in preparation). This represents, to our knowledge, the first successful extension of the LL model that explicitly integrates back exchange that naturally occurs in mixtures into PFs inference. The implications for HDX-MS are significant. Incorporating physical models of back exchange directly into data analysis will not only improve the accuracy of PFs inference, but also reduce reliance on error-prone correction schemes. This shifts opens the door to make HDX-MS a more quantitative and predictive tool for structural biology, at the same time allowing for more quantitative comparisons. I will present (i) the limitations of current HDX-MS analysis strategies, (ii) the theoretical framework of our generalised LL model, (iii) validation against HDX-NMR data, and (iv) perspectives for extending this methodology to routine HDX-MS experiments. These results represent a first step toward realistic, physically grounded modelling of HDX-MS, paving the way for more reliable extraction of

residue-level protection factors and, ultimately, more powerful structural insights from exchange data.

Native MS of urea-unfolded proteins

DAY 1 – POSTER 8

Nicklas Österlund, Jacob S. Jordan, Eleonora Renzi, **Michael Götze** and Kevin Pagel

Freie Universität Berlin and Fritz Haber Institute of the Max Planck Society

Protein stability and folding mechanisms are commonly probed using chemical denaturants. Urea is widely employed due to its ability to disrupt noncovalent interactions by competing for hydrogen bonds and destabilizing hydrophobic packing. At concentrations up to 8 M, urea effectively unfolds most proteins, providing a tunable system to explore structural transitions. Denaturation is typically monitored by optical spectroscopy, which yields averaged spectra of conformational ensembles. Resolving unfolding intermediates or distinct oligomeric assemblies can therefore be challenging in such experiments. Native mass spectrometry (MS) is a powerful tool for characterizing proteins under these conditions. By preserving noncovalent interactions to varying extents, native MS allows direct measurement of oligomeric states, ligand binding, and charge state distributions as sensitive readouts of folding states. This circumvents reliance on indirect spectroscopic probes such as circular dichroism or fluorescence, enabling a universal approach: any protein amenable to electrospray ionization can be interrogated. Our approach leverages nanoelectrospray ionization (nanoESI) with precisely defined, small-diameter emitters, allowing stable ionization of solutions containing up to 8 M urea. This technical advance enables monitoring of protein folding behavior under highly denaturing conditions previously deemed incompatible with MS. By coupling denaturation with mass spectrometric analysis, we establish a workflow to probe folding intermediates, oligomer stoichiometries, and the extent of unfolding without the need for protein-specific labeling strategies. We analyzed urea-dependent folding states of single- and multi-domain proteins, multimeric complexes, and protein–ligand interactions. Our results agree with traditional spectroscopy-based techniques, while extending their scope by providing non-averaged snapshots of coexisting folding states and oligomeric assemblies. The combination of urea-based solution-phase denaturation and native MS offers a simple yet powerful alternative to traditional biophysical methods for assessing protein folding.

Leveraging cross-linking mass spectrometry-guided integrative modeling for modeling antibody-antigen complexes

DAY 2 – POSTER 8

Andrea Di Ianni[1,2], Alessio Di Ianni[3], Kyra Cowan[4], Luca M. Barbero[1], Federico Riccardi Sirtori[1]

[1] NBE-DMPK Innovative BioAnalytics, Merck Serono RBM S.p.A., an affiliate of Merck KGaA, Italy, [2] Molecular Biotechnology Center, Department of Molecular Biotechnology and Health Sciences, University of Turin, Italy, [3] Martin Luther University Halle-Wittenberg, Department of Pharmaceutical Chemistry and Bioanalytics, Center for Structural Mass Spectrometry, Institute of Pharmacy, Halle/Saale, [4] New Biological Entities, Drug Metabolism and Pharmacokinetics (NBE-DMPK), Research and Development, Merck KGaA, Darmstadt, Germany

Elucidating antibody-antigen complexes at the atomic level is of utmost interest for understanding immune responses and designing better therapies. Cross-linking mass spectrometry (XL-MS) has emerged as a powerful tool for mapping protein-protein interactions, suggesting valuable structural insights. However, the use of XL-MS studies to enable epitope/paratope mapping of antibody-antigen complexes is still limited up to now. XL-MS data can be used to drive integrative modeling of antibody-antigen complexes, where cross-links information serves as distance restraints for the precise determination of binding interfaces. In this approach, XL-MS data are employed to identify connections between binding interfaces of the antibody and the antigen, thus informing molecular modeling. Current literature provides minimal input about the impact of XL-MS data on the integrative modeling of antibody-antigen complexes. In this work, we applied XL-MS to retrieve information about binding interfaces of three antibody-antigen complexes. We leveraged XL-MS data to perform integrative modeling using HADDOCK (active-passive residues and distance restraints strategies) and AlphaLink2. We then compared these three approaches with initial predictions of investigated antibody-antigen complexes by AlphaFold Multimer. This work emphasizes the importance of cross-linking data in resolving conformational dynamics of antibody-antigen complexes, ultimately enhancing the design of better protein therapeutics and vaccines.

In-Cell Cross-linking Mass Spectrometry with DSBU and tBu-PhoX and Orbitrap Astral Data Acquisition

DAY 1 – POSTER 9

Christian Ihling[1,2], Dirk Tänzler[1,2], Marcell Lederer[3], Jana Richter[4], Tabiwang N. Arrey[4], Rosa Viner[5], Andrea Sinz[1,2]

[1] Martin Luther University Halle Wittenberg, Department of Pharmaceutical Chemistry and Bioanalytics, Halle, Germany, [2] Martin Luther University Halle Wittenberg, Center for Structural Mass Spectrometry, Halle, Germany, [3] Martin Luther University Halle-Wittenberg, Institute of Molecular Medicine, Halle, Germany, [4] Thermo Fisher Scientific, Bremen, Germany, [5] Thermo Fisher Scientific, San Jose, CA, USA

Cross-linking mass spectrometry (XL-MS) has become an essential technique for investigating protein 3D-structures and protein-protein interactions in their native environment. To date, the majority of studies have been performed in cell lysates rather than in intact cells, largely as experimental conditions are more easily controlled in disrupted cells. Furthermore, intact membranes restrict the diffusion of most cross-linkers and even membrane-permeable reagents frequently undergo non-specific reactions with membrane proteins, lipids, or sugars. A further challenge in proteome-wide XL-MS lies in the inherent sample complexity. Therefore, strategies using affinity-enrichable cross-linkers or MS-cleavable reagents are employed. Especially MS-cleavable cross-linkers allow a more reliable identification of cross-links in complex mixtures by generating characteristic fragmentation patterns in MS/MS analysis. In this study, we assessed the performance of the membrane-permeable, affinity-enrichable cross-linker tBu-PhoX [1] and the MS-cleavable reagent DSBU [2] for XL-MS in intact HEK293T cells. For cross-linking with tBu-PhoX, ~1*106 HEK293T cells were washed and incubated with 2.25 mM tBu-PhoX for 1 h at room temperature. The reaction was quenched with ammonium bicarbonate. For experiments with DSBU, 1 mM reagent was added three times at 15 min intervals and quenched after 1 h at room temperature. For comparison, experiments with cell lysates were performed under identical conditions, adding 1 mM DSBU only once. Cross-linked intact cells were lysed, reduced with DTT, alkylated with iodoacetamide, and digested with LysC and trypsin. tBu-PhoX samples were additionally dephosphorylated using shrimp alkaline phosphatase and cross-linked products enriched with Fe-NTA spin columns. DSBU samples were fractionated by size-exclusion chromatography (SEC), and the first five fractions were collected for nano-HPLC/nano-ESI-MS/MS analysis. LC/MS analysis was carried out on a Vanquish Neo nano-HPLC system coupled to an Orbitrap Astral or Astral Zoom mass spectrometers equipped with FAIMS. For each run, 200 ng of sample was loaded onto an EASY-Spray™ PepMap™ C18 column (75 µm x 250 mm, 2 µm particle size) column and separated using a 60-min gradient. Each sample was analyzed in duplicate under two different FAIMS settings. Cross-linking products are identified using different software tools available for XL-MS data analysis, namely pLink3, MS Annika, MeroX and XlinkX. A comparison between XL-MS results of intact cells versus cell lysates as well as between tb-PhoX and DSBU will be made.

[1] P.-L. Jiang, C. Wang, A. Diehl, R. Viner, C. Etienne, P. Nandhikonda, L. Foster, R. D. Bomgarden, F. Liu, Angew. Chem. Int. Ed. 2022, 61, e202113937; Angew. Chem. 2022, 134, e202113937.

[2] M. Q. Mueller, F. Dreiocker, C. H. Ihling, M. Schaefer, A. Sinz, Analytical Chemistry 2010, 82, 6958-6968.

RNA Polymerase III and Herpesvirus Interaction: A Mass Spectrometry-based Approach to study Dynamic Complexes

DAY 2 – POSTER 9

Fabiola Iommazzo, Fabio Pessina, Mariavittoria Pizzinga, Alessandro Vannini

Structural Biology Research Centre, Human Technopole, Milan, Italy

RNA Polymerase III (Pol III) is a multi-subunit complex responsible for transcribing small non-coding RNAs. While usually associated with nuclear activity, recent studies highlight a cytoplasmic activity connected to the innate immune response. Pol III recognizes viral DNA sequences and transcribes them in 5'-ppp RNAs, triggering RIG-I activation and, in turn, the interferon response. While mutations in Pol III subunits are linked to herpesvirus susceptibility, the precise mechanisms of Pol III-mediated viral DNA recognition remain unclear. This project aims at uncovering how Pol III senses herpesvirus infection, including: i) the involved viral and host factors; ii) the cellular context of its anti-viral activity; iii) how this affects the innate immune response. HSV-1 infection (6 hours) causes Pol III to relocalize to a perinuclear region, forming cytoplasmic structures that co-localize with components of the virus itself, Golgi, and trans-Golgi markers. To better assess Pol III dynamics during HSV-1 infection (0-3-6-12-24 hours), I apply co-fractionation mass spectrometry (CF-MS) on cytoplasmic and nuclear fractions. Preliminary CF-MS analysis of the cytoplasm shows that at early infection stages (3 and 6 hours), Pol III forms higher molecular weight peaks than the uninfected condition, suggesting complex formation with additional factors and/or viral DNA. These peaks diminish significantly by 12 and 24 hours. Viral proteins profiles partially overlap with Pol III at 3 and 6 hours, supporting potential interaction. >From CF-MS profiles, suggestive localization information about Pol III emerges: at 6 hours, Pol III subunits show co-elution with a set of Golgi marker proteins, consistent with the imaging observation of perinuclear Pol III structures that overlap Golgi/TGN markers and viral particles. At later infection stages (12 and 24 hours) Pol III subunits co-elute with vesicles and autophagosome markers. Importantly, because CF-MS was performed on bulky cytoplasm rather than physical compartments, these data should be interpreted as co-association with Golgi/vesicle-enriched biochemical environments rather than a proof of cellular localization or trafficking. To strengthen this model, we are orthogonally validating some markers with confocal/live imaging. This project integrates proteomics and virology to investigate Pol III role in antiviral defense. CF-MS reveals dynamic changes in Pol III assembly, interaction with viral components and indicative cellular localization. Ongoing CF-MS analyses aim to further explore the precise stage and context of Pol III-virus interaction and identify new host factors involved.

The power of integrative structural proteomics in retroviral–host interplay

DAY 1 – POSTER 10

Karolína Burešová[1], Jan Prchal[1], Markéta Častorálová[1], Tereza Nešporova[2], František Filandr[2], Tomáš Ruml[1] and **Petra Junnková**[2]

[1] Department of Biochemistry and Microbiology, University of Chemistry and Technology, Prague, Czech Republic, [2] Institute of Organic Chemistry and Biochemistry of Czech Academy of Science, Prague, Czech Republic

Viruses exploit host cellular calcium signaling pathways to support essential stages of their life cycle, including entry, replication, assembly, and release. They manipulate calcium signaling both by employing Ca^{2+}-regulated proteins and pathways, and by directly binding Ca^{2+} to subvert host cell functions and compromise cellular integrity. One of the most studied Ca^{2+}-regulated proteins is calmodulin (CaM), a ubiquitous calcium sensor that undergoes a conformational change from a closed to an open state upon Ca^{2+} binding. This transition enables CaM to interact with more than 300 cellular targets—including kinases, phosphatases, ion channels, and transcription factors—thereby orchestrating diverse cellular processes. Importantly, CaM expression and activity are modulated upon infection by phylogenetically diverse viruses, such as rotavirus, Ebola virus, geminivirus, human papillomavirus, and retroviruses. To elucidate how retroviruses exploit CaM, we investigated the interaction between human CaM and the matrix protein (MA) of Mason-Pfizer monkey virus (M-PMV). Using microscale thermophoresis, protein cross-linking, and native mass spectrometry, we confirmed a direct, Ca^{2+}-dependent interaction between CaM and MA in vitro, forming predominantly a heterodimeric complex. Co-immunoprecipitation further validated this interaction in vivo. Biochemical analyses revealed that binding to CaM plays a crucial role in the maturation of M-PMV structural proteins. Additionally, we obtained evidence that CaM binding alters MA conformation to enable a myristoyl switch, thereby facilitating membrane interaction prior to viral budding. To gain structural insight into the complex, we applied cross-linking mass spectrometry (XL-MS), NMR spectroscopy, and hydrogen–deuterium exchange mass spectrometry (HDX-MS). The resulting data were integrated using HADDOCK, with NMR chemical shifts and XL-MS restraints as input parameters. The generated models were validated against results from all three structural proteomics approaches. Our model suggests that the loop between helices I and II and helix II of MA are oriented towards the N-terminal domain of CaM in the complex. It needs to be mentioned that this orientation resembles that of truncated (8-43)MA of HIV-1 in complex with CaM, as reported previously [1]. Our study exemplifies how the synergy of XL-MS, NMR, and HDX-MS, supported by computational modeling, can reveal structural principles of viral protein regulation by host factors, thereby advancing the field of virus–host interaction research.

[1] Vlach J, Samal AB, Saad JS. Solution structure of calmodulin bound to the binding domain of the HIV 1 matrix protein. J Biol Chem. 2014;289:8697–8705. doi:10.1074/jbc.M113.543694.

Structural Dynamics of the CyaA Acylated Segment Drive Membrane Invasion: Insights from HDX-MS

DAY 2 – POSTER 10

Zuzana Kalaninova[1,2] , Petr Man[1], Adriana Osickova[1], Jiri Masin[1], Peter Sebo[1]

[1] Institute of Microbiology of the CAS, Prague, Czech Republic, [2] Faculty of Science, Charles University, Prague, Czech Republic,

The adenylate cyclase toxin (CyaA) from Bordetella pertussis is a bifunctional virulence factor essential for early host colonization, exhibiting both adenylate cyclase (cytotoxic) and pore-forming (hemolytic) activities. Proper folding of its acylated segment (AS) is crucial for membrane interaction and functional activity. In this study, we developed a robust hydrogen-deuterium exchange mass spectrometry (HDX-MS) workflow to investigate structural dynamics of full-length CyaA and assess the impact of specific mutations and differential acylation on AS folding. Wild-type CyaA, mutants (W876F and D880L), and variants with distinct acylation patterns were expressed, purified, and refolded in vitro under calcium-dependent conditions. Optimized proteolytic digestion offered excellent coverage of the acylated region across a range of urea concentrations required for protein solubility. HDX-MS analysis revealed that Trp876 plays a key role in stabilizing acylated loops critical for ß2-integrin-independent cell entry. Its substitution disrupted AS structure and impaired activity on CR3-negative cells. In contrast, the D880L mutation, which alters a newly described calcium-binding site, enhanced structural stability and increased cell binding, translocation efficiency, and hemolytic activity. Comparative analysis of protoxin (non-acylated), non-canonical acylation (C14), and WT-like C16 acylation variants highlighted the importance of acyl chain composition in modulating AS folding and function. The HDX-MS platform established here enables high-resolution mapping of conformational changes in complex, multidomain bacterial toxins and provides mechanistic insight into virulence regulation, informing future efforts in therapeutic targeting.

Optimization of DDA PASEF Acquisition Modes for the Detection Extremely Low Abundant Crosslinking Products on a timsTOF Ultra 2 Mass Spectrometer

DAY 1 – POSTER 11

Christian Ihling[1,2], **Marc Kipping**[1,2], Andrea Sinz[1,2]

[1] Department of Pharmaceutical Chemistry and Bioanalytics, Martin Luther University Halle-Wittenberg, [2] Center for Structural Mass Spectrometry, Martin Luther University Halle-Wittenberg

Crosslinking Mass Spectrometry (XL-MS) is a powerful method for detecting conformational states of proteins and protein interactions. It can help to gain information, when classical methods of structural biology, such as X-ray crystallography or cryo-electron microscopy, can't be successfully applied for reasons such as dynamic nature of the protein or low protein amount. MS signals of crosslinking products in proteolytic mixtures have abundancies in the lowest detectable order of magnitude. For combinatoric reasons, a targeted analysis of these products after prediction of possible crosslinks is impossible. Untargeted DIA analysis without information about the existence of a specific crosslinking product is not possible as well. This leaves the traditional DDA experiment as the method for de-novo detection of crosslinks. DDA methods are typically optimized to detect signals in the order from highest to lowest intensity (TOP N), which is suitable for Proteomics experiments for example. In the case of crosslinking products, such algorithms are sub-optimal. MS signal in the first and second order of magnitude (starting from the most abundant signal) can't be crosslinking products, because the nature of the crosslinking reaction and protein dynamics do not allow products with high yield. On the other hand, the detection of the low abundant crosslinking products has a very high value for the structural information, which can be gained from a XL-MS experiment. After implementation of the ion mobility dimension and some related optimizations, the analysis with first timsTOF instruments in PASEF mode opened up a new level of quality and depth in the XL-MS field [1][2]. Here we want to discuss our experiments to implement capabilities of the newest timsTOF Ultra2 (like highest sensitivity, dynamic range controls and PASEF timing controls) to improve XL-MS results.

[1] Steigenberger et al. (2020) MCP 19, 1677–1687.

[2] Ihling et al. (2021) Anal. Chem. 93, 11442–11450.

Characterizing the N-Terminus of Neuropeptide Y1 Receptor by Cross-Linking Mass Spectrometry

DAY 2 - POSTER 11

Joshua Kraft[1], Asat Baischew[1], Karl Leitner[2], Anette Kaiser[2], Andrea Sinz[1]

[1] Center for Structural Mass Spectrometry, Department of Pharmaceutical Chemistry and Bioanalytics, Martin Luther University Halle-Wittenberg, [2] Department of Anesthesiology and Intensive Care, University of Leipzig Medical Center

Neuropeptide Y receptors, such as the Y1R and Y2R, are membrane proteins that bind to their endogenous ligand Neuropeptide Y (NPY) and belong to the rhodopsin-like family of G-protein-coupled receptors (GPCR). GPCRs are an integral part of signal transduction in cells and constitute highly important drug targets, with ca. 30% of drugs targeting GPCRs. The N-termini (NT) of rhodopsin-like GPCRs are still structurally and functionally elusive. It has been suggested that the NT are intrinsically disordered and exist as conformational ensembles to fulfil their distinct functions, influencing both ligand binding and receptor signaling. The Y1R mediates food intake and stress response, and is overexpressed in several cancer types, making it a promising drug target. Our recent work suggests that the NT of Y2R plays a cucial role in ligand binding and arrestin recruitment [1]. Cross-linking mass spectrometry (XL-MS) allows us to investigate the ligand binding behavior of Y1R. XL-MS gives in-cell snapshots of the highly dynamic interactions between NPY and Y1R, providing detailed molecular insights into the ligand binding behavior of the receptor. The dynamic and transient interactions between NPY and Y1R require the use of a fast reaction chemistry, such as photo-reactive diazirines, that react in the µs to ms time range [2]. NPY and Y1R were cross-linked using a photo-leucine modified NPY analog, carrying a diazirine group. For photo-XL-MS, three Y1R systems were employed: i) a 36-aa peptide representing the NT of Y1R, (ii) Y1R reconstituted in bicelles, iii) Y1R overexpressed in HEK293 cells. The cross-links were identified using a workflow for data processing that relies on Parallel Accumulation-Serial Fragmentation (PASEF) using a timsTOF Pro mass spectrometer (Bruker Daltonik) [3]. This workflow is the foundation for integrating data-independent-acquisition (DIA) in XL-MS, enabling unbiased sampling and increasing sensitivity as well as reproducibility. It allowed analyzing XL-MS data from low-abundant membrane proteins, such as the Y1R, directly in their cellular environment, supported by an in-vitro generated spectral library. Distance constraints imposed by the cross-links will be used as basis for computational modeling studies and molecular dynamic simulations. To complement 3D-structural data mutagenesis studies of Y1R and nano-bioluminescence resonance energy transfer (nanoBRET) assays are being conducted. Integrating XL-MS with computational modeling and functional assays will delineate the role of the intrinsically disordered NT of the Y1R. Combined with our previous findings from Y2R [1], our insights will contribute to a deeper understanding of the NT of rhodopsin-like GPCRs.

[1] Kaiser, A. et al. Nat. Commun, in press (2025)
[2] Iacobucci, C. et al. Analytical Chemistry, 90(4), 2805–2809 (2018)

[3] Rojas Echeverri, J. C. et al. Analytical Chemistry, 96(19), 7373–7379 (2024)

Development of a fluoroalkyl radical chemistry for mapping the surface accessible area of membrane proteins

DAY 1 – POSTER 12

Zdenek Kukacka[1], Michael Karpisek[1,2], Giacomo Ambrosino[3], Jasmina Portasikova[1,2], Petr Man[1,2] and Petr Novak[1,2]

[1] Institute of Microbiology of the Czech Academy of Sciences, Prague, Czech Republic, [2] Faculty of Science, Charles University, Prague, Czech Republic, [3] Faculty of Pharmaceutical and Pharmacological Sciences, University of Padova, Padova, Italy

Covalent labeling combined with mass spectrometry is a robust method for studying protein structure, interactions, and dynamics. Recently, a new technique called Fast FluoroAlkylation of Proteins (FFAP) has expanded the toolbox of covalent labeling techniques. FFAP is a novel protein labeling method that uses fluoroalkyl radicals generated from Togni reagents. These radicals target aromatic amino acids and cysteine and have been proven being beneficial for structural characterization of protein complexes and therapeutical antibodies as well as epitope/paratope mapping. All Togni reagents are based on hypervalent iodine core and can be decomposed by ascorbic acid to form fluoroalkyl radicals, which then label the solvent-accessible aromatic amino acid side chains of studied protein or protein complex. The structure of the released fluoroalkyl radical can vary. However, only Togni reagents bearing the CF3 radical or the CF2CF2-imidazole radical have been introduced so far. Therefore, in this study we expand the scope of Togni chemistry by testing a broader pallet of Togni reagents that release different kind of fluoroalkyl radicals. In particular, we focused on selecting Togni reagents beneficial for studying topology and dynamics of membrane and membrane associated proteins in its native environment. We synthetized several Togni reagents bearing a CF2-CF2-R group where R represents azido, benzimidazole, pyrazoline, phenoxy, phenylthiol or trifluoromethylfenoxy group, and tested them on model proteins. For pilot labeling experiments we used apomyoglobin, which has been well characterized by FFAP and other methods of structural biology. For experiments on membrane proteins Cl-/H+ antiporter from E. coli (CLC-ec1) was selected. The labeling conditions were optimized, and modified proteins were analyzed by top-down (solariX XR 15T, Bruker Daltonics) or bottom-up mass spectrometry approach (timsTOF Pro, Bruker Daltonics). Obtained data show that all tested Togni reagents were able to modify model proteins using the FFAP protocol. However, the extent of modification and modified residues varied across reagents. These results suggest that the combination of different hydrophobicity of fluoroalkyl radical and the leaving group play an important role in the selectivity against hydrophilic and hydrophobic surfaces. This work was mainly supported by the Czech Science Foundation (25-18181S), by grant provided by the Ministry of Education, Youth and Sports of the Czech Republic grant Talking microbes - understanding microbial interactions within One Health framework (CZ.02.01.01/00/22_008/0004597).

Systematic identification of motifs in the human intrinsically disordered proteome

DAY 2 – POSTER 12

Swantje Lenz[1], Katelyn C. Cook[1], Chi Fung Chow [1,2], Barbara Szewczyk[1], Dollie LaJoie[1], Doris Richter[1], Maxim Scheremetjew[1], Andrej Shevchenko[1], Alexander von Appen[1], Agnes Toth-Petroczy[1,2,3]

[1] MPI-CBG, Dresden, [2] Center for Systems Biology, Dresden, [3] Cluster of Excellence Physics of Life, Technische Universität Dresden

Human proteins contain a substantial fraction of intrinsically disordered regions (IDRs), estimated to constitute about one third of the proteome. Despite this, the majority of these regions are not functionally characterized, largely due to experimental limitations as low binding affinities and high structural flexibility. One approach to annotate function of IDRs is to identify short linear motifs, however, rapid evolution of IDRs limits the effectiveness of alignment-based tools for conservational analyses. To address this, we previously developed SHARK-capture, an alignment-free motif detection tool which outperforms existing methods in our benchmarking analyses. Here, we apply SHARK-capture to human IDRs in order to systematically identify conserved motifs. By integrating motif predictions with complementary metadata, we will be able to uncover novel instances of known motifs as well as previously uncharacterized motifs shared across different proteins.

High-Throughput Screening of Molecular Glues Using Native Mass Spectrometry Followed by Cryo-EM Structure Determination

DAY 1 – POSTER 13

Weijing Liu [1], Kheewoong Baek[2], Eric Fischer[2], Wenfei Song[3], Christopher Mullen[1], Yuan Xiong[2], Ken Durbin[4], Rosa Viner[1], Thomas Moehring[5]

[1] Thermo Fisher Scientific, San Jose, CA, USA, [2] Dana-Farber Cancer Institute, Boston, MA, USA; Harvard Medical School, Boston, MA, USA, [3] Thermo Fisher Scientific, Eindhoven, Netherlands, [4] Proteinaceous, Evanston, IL, USA, [5] Thermo Fisher Scientific, Bremen, Germany

Introduction Targeted protein degradation targeting conventionally undruggable proteins is a transformative approach in drug discovery. Molecular glues (MGs) achieve this by enhancing weak intrinsic interactions between targets and E3 ligase, enabling ubiquitin-proteasome-mediated degradation. While online affinity selection mass spectrometry (MS) struggles to identify weak binders due to on-column dissociation and lack of ternary complex interrogation, native MS facilitates the direct identification of E3-MG-target complexes. However, manual sample preparation and direct infusion limit its throughput. This study demonstrates high-throughput MG screening using nMS for WEE1 binding to CRBN-DDB1. It enables multiplex screening and analysis of over 2,500 compounds per day. Gas-phase ligand release and fragmentation support identification of unknown binders, and cryo-EM analysis further characterizes ligand-bound complexes, advancing MG discovery and validation. Methods Compound library, CRBN-DDB1 and WEE1 were provided by Dr. Eric Fischer. Thermo Fisher ScientificÔ VanquishÔ Flex LC equipped with a UV detector, fraction collector, dual-injection autosampler, and dual Flex pumps was used. Proteins were online buffer exchanged into ammonium acetate (AmAc) using a 3-cm prototype SEC column and collected into a 96-well plate at the rate of 0.7 min/run. Each well of the plate was preloaded with ≥4 ligands. Protein-ligand mixtures were incubated and flow-injected into Thermo Fisher Orbitrap Ascend using Thermo ScientificÔ EASY-SprayÔ source for MS analysis. MS1 identified ternary complexes, MS2 determined bound ligands, and MS3 elucidated unknown ligands. Data processing was done using High-Throughput Screening feature in Prosight Native (Proteinaceous). Cryo-EM data was acquired on Krios 5 Cryo-TEM. Results We initially compressed 96 compounds into 24 mixtures (4 compounds/well) and employed a 3-cm prototype SEC column for rapid online buffer exchange. LC-MS screening of all 96 compounds via native MS (nMS) took less than an hour, enabling throughput exceeding 2,500 compounds per day. Strong ternary complex formation between CRBN-DDB1 and WEE1 was observed in 4 of 24 mixtures, with 2 additional samples showing moderate binding. However, identifying individual binders within mixtures was challenged by compound multiplexing, native adduct interference, and non-specific interactions. To resolve this ambiguity, ternary complexes were isolated in the quadrupole and subjected to collision-induced dissociation. Released binders, potentially uncharged in the gas phase, were detected via polarity switching. MS2 analysis of low m/z ions enabled accurate mass determination and comparison with the compound library. Ligands with unmatched masses were classified as "Unknown" and further analyzed by MS3 fragmentation to assist structural elucidation.

This workflow leverages MS1 to detect intact ternary complexes, MS2 to identify bound ligands, and MS3 to characterize unknowns, thereby increasing both throughput and specificity in MG screening. To eliminate compound competition and ensure sufficient molar excess of each ligand, we also screened compounds individually. This approach confirmed 16 of 96 compounds as potential molecular glues, exhibiting varying degrees of binding strengths. This nMS-based workflow enhances screening throughput, resolves compound ambiguity, and facilitates discovery of novel MGs. Selected hits were further characterized using cryo-electron microscopy (cryo-EM), yielding high-resolution structures of WEE1-MG-CRBN-DDB1 ternary complexes reflecting open and closed conformations. These structures reveal how MGs mediate and stabilize protein–protein interactions, offering critical mechanistic insights to guide drug design and optimization.

Studying PI4P 5-kinases in Plants by Cross-linking Mass Spectrometry, Hydrogen-Deuterium Exchange MS and Native MS

DAY 2 – POSTER 13

Philip Lude[1,2], Dirk Tänzler[1,2], Wolfgang Hoehenwarter[1,2], Alexandra Schutkowski[3], Johanna Uhlenberg[3], Ingo Heilmann[3], Andrea Sinz[1,2]

[1] Department of Pharmaceutical Chemistry and Bioanalytics, Martin Luther University Halle-Wittenberg, Halle (Saale), Germany; [2] Center for Structural Mass Spectrometry, Martin Luther University Halle-Wittenberg, Halle (Saale), Germany, [3] Department of Plant Biochemistry, Martin Luther University Halle-Wittenberg, Halle (Saale), Germany.

The plasma membrane of plant cells is a dynamic interface that mediates cellular responses to environmental stimuli. One of its central regulators is the phospholipid phosphatidylinositol 4,5-bisphosphate (PIP2), which is involved in different physiological processes at the plasma membrane like endocytosis, exocytosis, and cytoskeleton dynamics1. By recruiting specific proteins to distinct membrane subdomains, PIP2 plays a pivotal role in shaping the specialized function of these membrane regions. Cellular levels of PIP2 change dynamically when exposed to abiotic stresses, such as salt and osmotic stress, or exposure to elevated temperatures2,3. Its synthesis is driven by phosphatidylinositol 4-phosphate 5-kinases (PIP5Ks), which are potential targets for phosphorylation by upstream protein kinases, linking their activity to environmental triggers. The natural genetic variation in Arabidopsis thaliana encompasses numerous single nucleotide polymorphisms (SNPs) within PIP5K genes, including non-synonymous SNPs4. These introduce amino acid exchanges that potentially impact PIP5K structure and function, enabling plants to adapt to their specific ecological niche. Understanding how such structural variation in PIP5K proteoforms translates into altered function can shed a light on molecular principles of plant adaptation. This project aims to dissect PIP5K 3D-structure and conformational dynamics that might be influenced by the presence of SNPs. For this, various structural MS techniques are employed, namely cross-linking MS (XL-MS), hydrogen-deuterium exchange MS (HDX-MS), and native MS in combination with computational modelling. Initial in vitro studies will focus on MBP- and HLT-tagged fusion proteins with particular emphasis on the N-terminal intrinsically disordered regions (IDRs) and the membrane occupation and recognition nexus (MORN) domain, as these are unique to plant PIP5Ks. Given the high flexibility of IDRs, they might serve as interaction hubs mediating contacts between proteins. XL-MS studies with known protein interactors will be conducted to map potential contact sites of PIP5K/protein complexes. Finally, in-situ XL-MS will give a comprehensive picture of the PIP5K conformational dynamics and protein-protein interactions in planta.

[1] Heilmann, M., & Heilmann, I. (2022). Regulators regulated: Different layers of control for plasma membrane phosphoinositides in plants. Current opinion in plant biology, 67, 102218.

[2] Zarza, X., Van Wijk, R., Shabala, L., Hunkeler, A., Lefebvre, M., Rodriguez-Villalón, A., ... & Munnik, T. (2020). Lipid kinases PIP5K7 and PIP5K9 are required for polyamine-triggered K+ efflux in Arabidopsis roots. The Plant Journal, 104(2), 416-432.

[3] Kuroda, R., Kato, M., Tsuge, T., & Aoyama, T. (2021). Arabidopsis phosphatidylinositol 4-phosphate 5-kinase genes PIP5K7, PIP5K8, and PIP5K9 are redundantly involved in root growth adaptation to osmotic stress. The Plant Journal, 106(4), 913-927.

[4] Alonso-Blanco, C., Andrade, J., Becker, C., Bemm, F., Bergelson, J., Borgwardt, K. M., ... & Zhou, X. (2016). 1,135 genomes reveal the global pattern of polymorphism in Arabidopsis thaliana. Cell, 166(2), 481-491.

SpectraXceed: Extracting MS Metadata from raw Files to Enable FAIR Data Practices, QC Optimization, and Troubleshooting

DAY 1 – POSTER 14

Ahmad Lutfi, Juri Rappsilber

Technische Universität Berlin, Rappsilber Lab

Efficient mass spectrometry (MS) workflows rely on fast access to detailed metadata for quality control (QC), method optimization, and troubleshooting. SpectraXceed is a lightweight, versatile tool that bridges the gap between Thermo Fisher raw files and actionable insights. With both a graphical user interface and command-line functionality, it offers flexibility for manual tasks and integration into automated pipelines. Using Thermo Fisher's RawFileReader, SpectraXceed extracts essential details about the sample, MS and LC methods, and scan-specific metrics, such as retention time, total ion current, and ion injection time at the MS1/MS2 level. The software processes multiple raw files in one session and exports data in tabular formats for downstream analysis. SpectraXceed aligns with FAIR data principles by structuring MS metadata, improving findability, accessibility, interoperability, and reusability. This metadata enhances data deposition in public repositories, making mass spectrometry data more discoverable and usable for secondary analysis. By integrating detailed acquisition parameters, SpectraXceed improves metadata logging, allowing researchers to track key experimental details and ensure reproducibility in large-scale proteomics studies. SpectraXceed also generates basic visualizations of MS1 and MS2 scans, helping users identify issues with instrument performance, sample preparation, or method settings. Additionally, it provides direct access to complete acquisition measurements from public repositories without requiring specialized data viewers, facilitating the replication and optimization of published methods. It supports QC workflows, method refinement, and large-scale computational mass spectrometry applications, including machine learning and deep learning model training and reinforcing data quality across the proteomics community.

MS-cleavable Cross-linking analysis on Orbitrap Astral Zoom mass spectrometer

DAY 2 – POSTER 14

Yi He[1], Tabiwang Arrey[2], Eugen Damon[2], Erum Raja[3], Leigh Foster[3], **Luce Maldini[4],** Ryan Bomgarden[3], Rosa Viner[1], Thomas Moehring[2]

[1] Thermo Fisher Scientific, San Jose, CA, USA, [2] Thermo Fisher Scientific (Bremen) GmbH, Bremen, Germany, [3] Thermo Fisher Scientific, Rockford, Illinois, USA, 4 Thermo Fisher Scientific, Milan, Italy

Introduction Cross-linking mass spectrometry (XL-MS) has rapidly become a powerful tool for elucidating the higher-order structures of proteins and mapping protein-protein interaction (PPI) networks on a proteome-wide scale. Cleavable crosslinkers are commonly used in PPI analysis, especially in complex systems like intra-cellular crosslinking, due to their unique fragmentation patterns observed in MS/MS. However, these applications can also present challenges regarding the sensitivity and speed of mass spectrometry instruments. In this study, we optimized XL-MS workflows for MS-cleavable crosslinkers using Thermo Scientific™ Orbitrap™ Astral™ Zoom mass spectrometer, resulting in significant improvements in XL identifications and spectrum quality. Methods Two cleavable crosslinkers: DSSO (disuccinimidyl sulfoxide) and DSBSO (disuccinimidyl bis-sulfoxide) were used to crosslink standard proteins, E.coli ribosome, and 64 human protein mix. XL samples were digested and DSBSO crosslinked samples were further enriched using dibenzocyclooctyne (DBCO) coupled magnetic beads. Digest of crosslinked samples was also spiked into Hela digest in various ratios for the sensitivity test. Separation was achieved using Thermo Scientific™ Vanquish™ Neo LC system with a 60 min gradient using Thermo Scientific™ EASY-Spray™ PepMap™ Neo column. Following separation, the peptides were detected on Orbitrap Astral Zoom mass spectrometer, and data were analyzed using XlinkX node in Thermo Scientific™ Proteome Discoverer™ v3.2. Identified XLs were visualized using xiVIEW. Results XL-MS has emerged as a universal tool for studying protein structures and protein-protein interaction networks. However, the challenges in this workflow are the low abundance of crosslinked peptides and high sample complexity, particularly for in situ crosslinking. Cleavable crosslinkers, such as DSSO and DSBSO, are commonly used for these applications due to their distinctive doublet peaks observed in MS/MS spectra. In this study, we developed XL-MS methods for the Orbitrap Astral Zoom mass spectrometer with fast stepped collision energy (SCE) HCD MS/MS analysis. We compared NCE charge dependent method to methods using two and three SCEs, with and without FAIMS. We achieved a highest increase of 20% in the identification of DSSO crosslinks, a 74% increase in CSMs, and a 28% increase in monolink peptides compared to results obtained with the Orbitrap Astral instrument and three SCEs method. The quality of the spectra also improved, showing a 15% enhancement in the in the XlinkX score. Furthermore, to test the instrument's sensitivity and dynamic range, we spiked DSBSO-crosslinked protein mix (64 human proteins) into DSBSO-crosslinked E. coli cells at various ratios, ranging from 1: 50 to 1:1. With FAIMS on the Orbitrap Astral Zoom MS, we successfully detected DSBSO crosslinks from human protein mix down to the ratio of 1:50. In summary, we

optimized the XL-MS workflows for cleavable crosslinkers on the Orbitrap Astral Zoom mass spectrometer, demonstrating enhancement in both duty cycle and spectrum quality.

Characterizing the Intrinsically Disordered Protein PIF4 by Cross-linking Mass Spectrometry, Hydrogen-Deuterium Exchange and Native Mass Spectrometry

DAY 1 – POSTER 15

Ilnaz Soleimani Mashhadi[1,2], Jian Xia[3], Lennart Eschen-Lippold[3], Marcel Quint[3], Andrea Sinz[1,2]

[1] Department of Pharmaceutical Chemistry and Bioanalytics, Martin Luther University Halle-Wittenberg, Halle (Saale), Germany, [2] Center for Structural Mass Spectrometry, Martin Luther University Halle-Wittenberg, Halle (Saale), Germany, [3] Institute of Agricultural and Nutritional Sciences, Martin Luther University Halle-Wittenberg, Halle (Saale), Germany

Plants, as sessile organisms, have developed intricate sensory systems to adapt their growth and development to dynamic environmental cues, such as temperature and light. Thermomorphogenesis, a specific type of phenotypic plasticity, involves morphological changes triggered by ambient temperature fluctuations [1]. The bHLH transcription factor PHYTOCHROME INTERACTING FACTOR 4 (PIF4) serves as a central hub in thermosignaling, regulated by factors, such as PHYTOCHROME B (phyB) and EARLY FLOWERING 3 (ELF3). PIF4 and ELF3 are intrinsically disordered proteins and currently, no structural data exist for these proteins [2]. Recent studies highlight the role of liquid-liquid phase separation (LLPS) in the dynamic compartmentalization of PIF4 and ELF3. In Arabidopsis thaliana, ELF3 undergoes temperature-sensitive LLPS driven by its prion-like domain, influencing thermoresponsive growth [3]. In this study, a detailed structural analysis of PIF4 is being performed by different methods of structural mass spectrometry, namely cross-linking MS (XL-MS), hydrogen-deuterium exchange MS, and native MS. XL-MS is conducted using the amine-reactive MS-cleavable cross-linker DSBU that bridges Cα- Cα distances up to 30 Angstrom. The high disorder content of both PIF4 and ELF3 results in a low reliability of the structural models generated by Alphafold. Our initial XL-MS data clearly indicate that PIF4 is more compact. We will derive novel structural models based on these cross-linking constraints. Additional experiments will be performed with different cross-linkers (different spacers, different reactivities). Our aim is to provide insights into the molecular mechanisms how PIF4 exerts its specific functions, also considering single nucleotide polymorphisms. This will advance the understanding of plant thermo-responsiveness and adaptation mechanisms. [1] Box, Mathew S., et al. "ELF3 controls thermoresponsive growth in Arabidopsis." Current biology 25.2 (2015): 194-199. [2] Quint, Marcel, et al. "Molecular and genetic control of plant thermomorphogenesis." Nature plants 2.1 (2016): 1-9. [3] Hutin, Stephanie, et al. "Phase separation and molecular ordering of the prion-like domain of the Arabidopsis thermosensory protein EARLY FLOWERING 3." Proceedings of the National Academy of Sciences 120.28 (2023): e2304714120

Using native MS and DIA-PTCR to resolve the spectral congestion of complex engineered biotherapeutic

DAY 2 – POSTER 15

Rafael Melani, Christopher Mullen, Weijing Liu

Thermo Fisher Scientific

The growing complexity of engineered biotherapeutics, with diverse biomolecular modalities in drug candidates, challenges traditional analytical methods for intact mass analysis. Conjugated and fusion molecules, exceeding conventional antibody formats, hinder control of molecular heterogeneity during manufacturing. This inherent molecular diversity impedes thorough characterization using only mass spectrometry or other conventional techniques. Current approaches to analysis involve fragment measurements or partial digestion of glycans, complicating human-compatible glycosylation or drug-to-antibody ratio reporting. These challenges impact biotechnology companies striving for consistent drug safety and efficacy. A streamlined workflow has been established for the direct intact analysis of complex, heterogeneous proteins that have previously posed challenges for intact mass spectrometry (MS) characterization. This innovative method, termed "DIA-PTCR," employs native MS and a data-independent approach to analyze intact glycoproteins, conjugated molecules, and non-covalent complexes using proton-transfer charge-reduction tandem mass spectrometry. Notably, DIA-PTCR provides mass information on spectrally unresolvable proteins without the need for denaturation, upfront digestion, or separation. The effectiveness of DIA-PTCR has been significantly enhanced through the integration of a high mass quadrupole on the Orbitrap Ascend Structural Biology Tribrid mass spectometer. Prior to the incorporation of this Native MS option, ion isolation beyond 2000 would necessitate the use of the ion trap. With the new high mass quadrupole, ions up to 8000 m/z can now be isolated within an exceptionally narrow Thompson window (5-10), compared to the previous range of greater than 50-100 Th in the ion trap. This advancement has led to improved experimental reproducibility and optimization of acquisition parameters, such as achieving interpretable spectra with a minimum number of scans, ensuring optimal overlap of neighboring isolation windows, and automating the entire method for an 8-minute run. Noteworthy applications of DIA-PTCR include successful analysis of complex biotherapeutics, such as highly glycosylated Fc fusion proteins and a noncovalent protein-ligand complex, showcasing the "something from nothing" advantage of this technique. We tested the approach on various biotherapeutic molecules, including Fc-fusion, VHH-fusion, and peptide-bound MHC class II complexes. For glycoproteins, this method offers insights into glycan composition when coupled with a suitable bioinformatic strategy. Notably, we inferred the glycoform distribution for hundreds of molecular weights for the eight-times glycosylated fusion drug IL22-Fc. This enabled correlations between glycoform sub-populations and the drug's pharmacological properties. The IL22-Fc fusion protein, developed for epithelial repair, consists of two IL22 cytokines dimerized by Fc domains that increase bioavailability. The fusion protein contains eight N-linked glycosylation sites, resulting in a highly complex MS1 spectrum with no discernable m/z peak series with mass information. Despite the

complexity, the DIA-PTCR method detected masses corresponding to the fully assembled and glycosylated molecule and to partial constructs missing the VHH domain, demonstrating the method's capability to resolve molecular heterogeneity stemming from glycosylation and other attributes, such as domain mis-assembly. The native MS DIA-PTCR platform can resolve molecular heterogeneity from glycosylation and other post-translational modifications, such as phosphorylation and acetylation, without denaturation, upfront digestion, or separation, retaining information about non-covalent structures and co-occurrence of glycans. In summary, our method provides an unprecedented window into the molecular structural heterogeneity of biotherapeutics, addressing the growing challenge of controlling molecular heterogeneity during manufacturing and ensuring optimal pharmacological properties.

Mapping Protein-Protein Interactions in Bacillus subtilis Infected with AR9 Phage through In-cell Crosslinking and Mass Spectrometry

DAY 1 – POSTER 16

Bhavna Menon[1], Barbara Steigenberger[2], Maria Sokolova[1]

[1] Research Group Bacteriophages, Max Planck Institute of Biochemistry, Munich, Germany, [2] Mass Spectrometry Core Facility, Max Planck Institute of Biochemistry, Munich, Germany

Cross-linking mass spectrometry (XL-MS) is a powerful technique for mapping the topology of protein-protein interactions, especially for purified proteins. Recently developed in-cell XL-MS allows stabilization of weak or transient protein-protein interactions that are often lost during cell lysis, thereby providing a more complete view of molecular interactions inside the cell. However, the use of in-cell XL-MS in complex biological systems, such as bacteriophage-infected bacterial cells, has not yet been established. In this work, we aim to establish in-cell XL-MS to study protein-protein interactions during phage infection cycle. We will focus on the Bacillus subtilis jumbo phage AR9, which has DNA containing uracil and encoding two RNA polymerases (RNAP) that are homologous to bacterial RNAP but have unique properties. Using this method, we expect to identify missing subunits of AR9 virion RNA polymerase (vRNAP), explore interactions between both AR9 RNAPs and regulatory proteins, find out if transcription by the phage RNAPs is coupled with translation, and get insight into how the phage protects its uracil-containing DNA genome during infection. Furthermore, this work will help to advance the application of in-cell XL-MS to phage-bacteria systems and provide new insights into the molecular mechanisms underlying phage infection.

Analyzing the orientation of membrane proteins in proteoliposomes by mass spectrometry

DAY 2 – POSTER 16

S. Meyer[1], F. Uliana[1], M. Alfes[2], C. Schmidt[1]

[1] Johannes Gutenberg University Mainz, Germany, [2] Martin Luther University Halle-Wittenberg, Germany

Membrane proteins mediate important processes in cell and organelle membranes and are, therefore, essential for cellular function. However, due to missing high-resolution structures, the understanding of their function is still limited. An ideal model system for structural and functional studies of membrane proteins are proteoliposomes. However, to perform those assays, reconstitution of proteins into the liposome membrane requires the correct orientation of the proteins in the liposome membrane. Importantly, the orientation of the proteins might be affected by sterical properties of the proteins and should carefully be evaluated. While this is difficult to assess with available biochemical methods, mass spectrometry (MS) provides the opportunity to gain (quantitative) information on the solvent accessibility of the proteins. Here, we develop an MS-based chemical labelling workflow, using light (d0) and heavy (d6) acetic anhydride to calculate the degree of inwards and outwards oriented membrane proteins in proteoliposomes. For this, we make use of Synaptotagmin-1, a membrane protein of synaptic vesicles containing a transmembrane helix as well as a small luminal and a large cytoplasmic domain. To assess the orientation of the protein in the liposome membrane, we label the protein with the two labelling reagents in two consecutive steps: (i) after incorporation of the protein into the liposomes (d0-acetic anhydride), and (ii) after dissociation of the liposome using detergents (d6-acetic anhydride). Comparing the intensity ratios of light versus heavy labelled amino acid residues of Syntaptotagmin-1 delivers information on the degree of inward and outward orientation. Having established the general workflow, we will challenge our approach by swapping the labels and by using additional membrane proteins containing soluble and luminal domains of varying size. Our workflow will provide a robust and simple method to assess the orientation of a membrane protein in a liposome membrane and, therefore, contribute to the generation of reliable functional models to study membrane proteins.

Enhancing XL-MS sampling using non-standard acid modifiers in LC-MS/MS

DAY 1 – POSTER 17

Andrew R. M. Michael, Sascha Vatic, Jan Rasl, Jaraj Lenčo & Petr Novák

Department of Analytical Chemistry, Charles University, Prague, Czech Republic

Introduction: Crosslinking mass spectrometry (XL-MS) has become a powerful tool for structural and systems biology. However, the identification rates of crosslinked peptides remain noticeably low despite constant efforts to improve methodology, instrumentation, and analysis tools. The standard approach to identify crosslinks is through bottom-up analysis where proteins are proteolytically digested into peptides. Digestion of crosslinked samples generates a mixture of peptides with crosslinked-peptide pairs being the least abundant. The sub-stoichiometric nature of crosslinked peptides in digest mixtures presents a glaring problem with identification as the higher abundant non-crosslinked peptides occupy most of the signal and can supress the signal of co-eluting low-abundant peptides. Because crosslinked peptides tend to occupy higher charge states and larger collisional cross-sectional areas, compared to linear peptides, ion mobility-based enrichment strategies have been implemented seeing improvements with crosslink identifications. However, sampling remains low. We reasoned that the problem of low crosslink signal in the mass spectrometer primarily stems from poor ionization efficiency. In this presentation we establish the value of changing the acidic modifier of the mobile phase to improve ionization efficiency of crosslinked peptides to increase crosslink identification. Methods: A biological quadruplicate of 3 µM bovine serum albumin (BSA) was crosslinked using either DSS or DSBU at 1 molar equivalence with respect to lysine content for 30 minutes at 25°C. Reaction products were analyzed using LC-MS/MS on an Evosep One connected to a TimsTOF SCP. Different mobile phases were prepared for RP-LC separation of peptides to contain either 0.1% formic acid, 0.5% acetic acid, or 0.5% propionic acid. Crosslink data analysis was performed using xiSEARCH in conjunction with xiFDR. Preliminary data: Prior to acquiring crosslinked BSA data, a non-crosslinked BSA standard was run for quality control analysis of the different acid modifiers. Several XICs from RP-LC traces of non-modified BSA peptides were profiled across a range of retention times for all three acid modifiers within each respective mobile phase. Reduced retention times were observed across all XICs when acetic or propionic acids were used. In addition, the XICs from acetic acid and propionic acid mobile phases displayed an average of 10-13% reduced peak widths and less chromatographic tailing when compared to the standard 0.1% formic acid control. Several XICs of BSA peptides in 0.5% acetic acid also displayed higher signal intensity compared to the standard mobile phase. We next crosslinked BSA with two common crosslinkers to explore how crosslinked peptides behave during LC-MS/MS when subjected to different acid modifiers in the mobile phase. Protein was crosslinked in a way that limited reagent interactions thereby limiting overall crosslink signal to emulate higher complexity systems. For both crosslinkers, an increase of precursor charge state was observed when the acid modifier was changed from formic acid. Additionally, DSBU crosslink precursors had increased charge state coalescence

when the mobile phase contained these alternative acid modifiers. For both crosslinkers, changing the acid modifier increased the number of unique crosslink identifications by 20-60% with most crosslinks identified from the formic acid control being shared across acid modifiers. Novel aspect: Changing acid modifier of mobile phase from formic acid to acetic or propionic acid improves crosslinked peptide identification rates.

Protein Characterization with CIU and MSn eXd on the timsOmni platform

DAY 2 – POSTER 17

S. Pengelley, A Smymakis, A. Grigoriadis, M. Kosmopoulou, **C. Albers**, F. Busch, A. Apalategui, C. Gebhardt, E. Carrascosa, D. Papanastasiou

Bruker Corporation

Protein characterization is a very wide-reaching discipline which can encompass a broad range of techniques and approaches. Mass spectrometry is making an increasingly significant contribution to this field as the technology matures and top-down workflows are improved upon with new hardware and software innovations. The new timsOmniTM platform combines trapped ion mobility spectrometry (TIMS) with the Omnitrap® multidimensional MSn ion processor, providing the necessary analytical flexibility required for the increasingly complex studies aimed at sequencing and structural characterisation of biopharmaceuticals.

The configuration of the timsOmni instrument contributes greatly to the experimental options that are available, including a variety of ion activation methods which can be applied in multiple different locations along the ion path. Firstly, the protein desolvation unit (PDU) is positioned upstream of the TIMS cell and can be used for desolvation or unfolding of protein ions. Here, we demonstrate collision induced unfolding (CIU) of proteins in the PDU prior to mobility analysis in the TIMS analyzer. This was then combined with electron capture dissociation (ECD), a soft fragmentation technique, which was applied to proteins at differing levels of unfolding in the Omnitrap section Q5 to reveal which sequence regions become more flexible as the protein unfolds.

In-source collision induced dissociation (isCID) can be applied directly downstream of the TIMS analyzer and is typically used for desolvation or all-ion CID. For MS3, fragment ions produced by isCID can be isolated in the quadrupole ion filter, accumulated in the Omnitrap section Q2 and fragmented again by electron activated dissociation (eXd) in Q5. For pseudo MS4 experiments, eXd products can be isolated and accumulated prior to fragmentation by CID in the collision cell. The MSn eXd workflow is demonstrated for NISTmAb, producing up to 88% sequence coverage for N-terminus light and heavy chain fragments produced by isCID.

Enhanced Sensitivity and Quantitation in Crosslinking Mass Spectrometry Using Orbitrap Astral DDA and DIA Workflows

DAY 1 – POSTER 18

Fränze Müller[1], Micha J. Birklbauer[23], Julia Bubis[1], Karel Stejskal[4], Viktoria Dorfer[2], Karl Mechtler[145]

[1] Research Institute of Molecular Pathology (IMP), Vienna BioCenter (VBC), Vienna, Austria, [2] Bioinformatics Research Group, University of Applied Sciences Upper Austria, Hagenberg, Austria, [3] Institute for Symbolic Artificial Intelligence, Johannes Kepler University Linz, Linz, Austria, [4] Institute of Molecular Biotechnology (IMBA), Vienna BioCenter (VBC), Vienna, Austria, [5] Gregor Mendel Institute of Molecular Plant Biology (GMI), Vienna BioCenter (VBC), Vienna, Austria

Crosslinking mass spectrometry (CL-MS) is increasingly applied for structural biology and interactome mapping, yet quantitative applications remain technically challenging due to the low abundance of crosslinked peptides and software availability. Here, we present advances in quantitative XL-MS using both data-dependent acquisition (DDA) and data-independent acquisition (DIA) on the Thermo Scientific Orbitrap Astral mass spectrometer. Here, we show that the Orbitrap Astral outperforms the Orbitrap Eclipse, yielding up to 40% more unique crosslink identifications across a range of sample inputs. This improvement is driven by enhanced MS1-level detection, particularly for low-abundance precursors, enabling access to crosslinks in internal or less accessible regions of proteins, whereas high-abundance crosslinks predominantly map to surface-exposed sites. Single higher-energy collisional dissociation (HCD) consistently outperformed stepped HCD on the Astral, with up to 35% more crosslinks identified at low sample amounts, likely reflecting its higher scan speed for single HCD. These improvements translate into more robust MS1-level quantitation of crosslinked peptides, particularly in the low-abundance range that often carries the highest structural relevance. Complementary to this, we present preliminary data-independent acquisition (DIA)-based CL-MS data, which highlight the potential of DIA to improve quantitative reproducibility and precursor sampling across crosslinking experiments. These findings highlight that sensitivity, scan speed, and tailored method optimization are key determinants of CLMS performance.

Analysis of androgen receptor phosphorylation state in muscle cells: effects of the poly-glutamine expansion

DAY 1 – POSTER 19

Marta Marchioretto[1], Daniele Peroni[2], Romina Belli[2], Maria Pennuto[34], Manuela Basso[5], **Laura Tosatto[16]**

[1] Institute of Biophysics, CNR, Italy, [2] Proteomics and Mass Spectrometry Core Facility, Department CIBIO, University of Trento, Italy, [3] Department of Biomedical Sciences, University of Padua, Italy, [4] Veneto Institute of Molecular Medicine, Italy, [5] Department CIBIO, University of Trento, Italy, [6] Institute of Clinical Physiology, CNR, Italy

Androgen receptor (AR) is the mediator of testosterone effects in humans at genetic level. It is a 920 residues multidomain protein belonging to the nuclear receptors family, with a large intrinsically disordered regulatory N-terminal domain (NTD), a conserved DNA binding domain (DBD) and a well characterised ligand binding domain (LBD). Loss of function of the protein is the cause of Androgen Insensitivity Syndrome, for which an individual has a male genotype but a female phenotype, while gain of function yields pathological conditions like prostate cancer and Spinal and Bulbar Muscular Atrophy (SBMA). The latter is caused by the expansion of a poly-Glutamine (polyQ) stretch in AR NTD, leading to neuromuscular degeneration in male adults. Patients are affected by muscle atrophy characterized by oxidative to glycolytic fibre type switch and the presence of AR aggregates.

AR is a heavily phosphorylated protein, with Serine 96 (S96) being the only constitutively phosphorylated residue. Other Serines are phosphorylated in response to androgens. Recent work demonstrated that removal of S96 phosphorylation rescue toxicity in neuronal cells and that S96 is more phosphorylated in presence of the polyQ expansion. Skeletal muscles are one of first tissues to be affected by SMBA, still the link between AR phosphorylation and pathology is poorly characterised.

In this work, we used a biochemical and a mass spectrometry approach to analysed the phosphorylation of AR in muscle cells. Samples from C2C12 cells, with stable or transient expression of AR has been analysed to identify phosphorylation sites. Moreover, samples from a transgenic mouse model for SBMA has been submitted to proteomics analysis, to identify key modifications in the proteome leading to oxidative to glycolytic fibre type switch. Results indicate differences in the expression of cytoskeleton and adhesion proteins, oxidative phosphorylation, myogenesis and metabolism.

Collectively, these results indicate that there is an alteration in the metabolic state of the muscles due to polyQ expansion of AR. We hope that these data and methodology will enable a validation on more clinically relevant samples to finally identify key players involved in muscle atrophy and identify potential therapeutical strategies.

Leveraging crosslinking mass spectrometry data for the structural characterisation of the interaction landscape of the chaperone LRPAP1 onto its cognate receptor LRP2

DAY 2 – POSTER 19

Karthik Ramanadane

Fondazione Human Technopole, Milan, Italy

The low-density lipoprotein receptor-related protein 2 (LRP2 or megalin) is a multiligand endocytic receptor implicated in the homeostasis of multiple organs, and mutations in its gene are associated with severe systemic disease. It is well-known that the low-density lipoprotein receptor-related protein-associated protein 1 (LRPAP1 or RAP) canonically interacts with LRP2 by modulating its function. Our cryo-EM data revealed the architecture of the LRP2 receptor and the molecular basis of the interaction of two LRPAP1 copies with 10 complement-type (LA) repeats within LRP2. However, it is remarkable that no more copies of LRPAP1 would interact with LRP2, as the latter is composed of 36 LA repeats. Thus, suggesting that multiple copies of LRPAP1 could associate with the receptor. Using Alphafold and crosslinking mass spectrometry data, we unravel a broader interaction landscape of the LRPAP1 onto the LRP2 receptor. Our integrative approach reveals that LRP2 interacts with at least 7 copies of LRPAP1, of which five overlap with ligand binding sites. This finding, validated by competitive in vitro binding assays, supports the hypothesis that LRPAP1 acts as a direct modulator of LRP2's ligand-binding activity. Additionally, we highlight the presence of one copy of LRPAP1, which would compromise the oligomeric state of LRP2, thus raising questions on the functional role of the dimeric state. Taken together, our study complements the cryo-EM data and reveals a broader molecular landscape of LRP2-LRPAP1 interactions, providing insights into its clinical and functional role.

Structural Analysis of Alpha-Synuclein Using Singlet Oxygen

Jan Rasl[1,2], Zuzana Kalaninová[1], Karel Holada[3], Michael Volný[2,4], Petr Man[2], Petr Novák[1,2]

[1] Faculty of Science, Charles University, Prague, Czech Republic, [2] Institute of Microbiology, Czech Academy of Sciences, Vestec, Czech Republic, [3] First Faculty of Medicine, Charles University, Prague, Czech Republic, [4] Department of Analytical Chemistry, University of Chemistry and Technology, Prague, Czech Republic

Footprinting methods coupled with liquid chromatography–mass spectrometry (LC-MS) have emerged as powerful tools for elucidating protein structures and interactions under various conditions. These techniques employ chemical probes to modify solvent-accessible regions of proteins in a manner that reflects their native conformations or binding environments. Subsequent LC-MS analysis enables precise identification and quantification of modified sites, providing detailed insights into structural changes, ligand binding, and conformational dynamics. Synucleinopathies, such as Parkinson's disease, are neurodegenerative disorders characterized by the abnormal accumulation of misfolded alpha-synuclein into fibrillar aggregates that disrupt normal cellular function. In this study, we employ singlet oxygen, generated under mild conditions, as a novel footprinting probe to monitor structural and ligand-binding changes in proteins. In alpha-synuclein, singlet oxygen selectively modifies methionine, tyrosine, histidine, and phenylalanine residues. Using LC-MS, we distinguish between monomeric and aggregated forms based on differences in the extent and pattern of these modifications. This approach offers a valuable addition to existing techniques for monitoring structural changes in alpha-synuclein.

Deciphering Munc13 assembly and dynamics through mass spectrometry

DAY 2 – POSTER 20

Zahra Riazimand, Carla Schmidt

Johannes Gutenberg University Mainz

Neurotransmission takes place at synapses, which are specialised contact sites between neurons that enable precise transfer of information. Synapses contain two functionally important membrane-less compartments within the presynaptic neuron that assemble from specific scaffold proteins: (i) The synaptic vesicle reserve pool stores neurotransmitter-filled vesicles, and (ii) the active zone, a highly organised region of the presynaptic membrane, controls vesicle docking, priming and their fusion with the presynaptic membrane thereby releasing the neurotransmitters. The active zone includes key proteins, some of which have been found to form condensates that organise voltage-gated Ca2+-channels and synaptic vesicles. However, mechanistic and quantitative insights into their assembly and regulation remain missing. Munc13 is a scaffold protein of the active zone and, due to its intrinsically disordered regions (IDRs), represents an interesting candidate for studying condensate formation and dynamic protein interactions. In this study, a variant of Munc13 including the intrinsically disordered C-terminal domain was expressed in E. coli and purified through an affinity tag and ion exchange chromatography. Its identity was then confirmed by mass spectrometry. Currently, we are employing different techniques including microscopy and cross-linking mass spectrometry to investigate condensate formation of this Munc13 variant, and to study protein interactions at residue level. We will further combine these experiments with native mass spectrometry to gain insights into the oligomeric state of the protein.

Employment of a DIA-diGly methodology for the identification of ubiquitination sites related to the novel E3 ligase MEX3A, prior polyUb structural studies.

DAY 1 – POSTER 21

Diego Fernando Garcia-del Rio[1,2], Marcell Lederer[2], Stefan Hüttelmaier[2], Andrea Sinz[1]

[1] Center for Structural Mass Spectrometry, Department of Pharmaceutical Chemistry & Bioanalytics, Institute of Pharmacy, Martin Luther University Halle-Wittenberg, Halle (Saale), Germany, [2] Charles Tanford Protein Center, Faculty of Medicine, Institute of Molecular Medicine, Section for Molecular Cell Biology, Martin Luther University Halle-Wittenberg, Halle (Saale), Germany

A primary objective of proteomics studies is the elucidation of functions and molecular mechanisms, in which a protein plays a role. This is of particular importance when comparing pathological and non-pathological states. Post-translational modifications (PTMs) contribute to the complexity of proteins by altering their function. In some cases, PTMs can alter the "known" function of the protein they modify. Among the most prevalent PTMs, ubiquitinylation has been extensively described as one of the primary drivers of protein fate, particularly involving proteasomal degradation. Despite its low molecular weight of 8.6 kD, ubiquitin (Ub) displays a notable capacity for self-association, resulting in the formation of Lys-linked and N-terminal Met -linked chains. This process gives rise to a multitude of Ub-Ub structural modifications, collectively termed "The Ubiquitin Code"1. It has been reported that diversity of these linkages is related to different cellular processes or mechanisms. For instance, K33 linkage has been associated with protein trafficking, TCR signaling, and protein kinase modification2. Due to the highly complex nature of these arrays of polyUb linkages, research in this field is predominantly conducted by antibody-based techniques and middle and top-down proteomics. It should be noted that these studies are performed on proteins with prior evidence of ubiquitination. Consequently, the selection of pertinent targets is imperative. This objective can be accomplished by implementing a high-throughput approach known as “diGly approach”. This approach is an antibody-based enrichment technique, which recognizes the diglycine modified lysine residue remanent after tryptic digestion. Therefore, the primary objective was to develop an extensive diGly experimental spectral library. This will facilitate the identification of variations in the ubiquitination profiles under diverse cellular conditions. These findings will serve as a foundation for conducting polyUb structural studies. The generation of this experimental spectral library was achieved through high pH fractionation of diGly-enriched samples from eight distinct human cancerous cell lines originating from six different organs. Data from 128 samples were acquired on a Bruker TIMS-ToF Ultra 2 mass spectrometer with a data dependent acquisition-parallel accumulation serial fragmentation (DDA-PASEF) method. Following acquisition, data were analyzed with FragPipe3 using a workflow comprising the DDA+ MSFragger search engine, MSbooster, Percolator, and ptmProphet as validation tools. Consequently, a total of 10,186 proteins and 38,030 diGly sites were identified. From these identifications, an experimental spectral library containing 156,226 precursors was generated. The capacity of the experimental library to identify diGly sites was assessed by measuring six distinct amounts of diGly

enriched peptides using a data independent acquisition (DIA)-PASEF method. The analysis was performed in DIA-NN employing the experimental library, three in-silico predicted libraries (1, 2, and 3 missed cleavages), and the library-free approach in FragPipe. The experimental library yielded a higher number of diGly identifications in all tested sample amounts. For instance, 250 ng of injected peptides allowed us to identify 6,193 diGly sites in a 60 min diaPASEF run. The next objective of this study is to utilize the established methodology to identify ubiquitination targets associated with the novel E3 ligase MEX3A. It is notable that this protein has been found to be dysregulated in various types of cancer4. This finding presents an intriguing research opportunity as it suggests the potential for exploring the protein as a biomarker or therapeutic alternative. Notably, this protein is comprised of two KH domains, two intrinsically disordered regions (IDRs), and a RING domain. Consequently, this protein may possess a dual function, RNA binding and E3 ligase. Therefore, we will compare the ubiquitination profiles of two distinct eGFP-MEX3A-induced cell lines (HUH7 and H1975) in comparison with their non-induced counterparts. These results will allow for the identification of the dysregulation of different Ub sites. This dysregulation may be related to the overexpression of MEX3A. This examination may allow for the determination of whether the different effects observed when MEX3A regulation is disturbed are related to these targets and their polyUb patterns.

[1] Komander, D.; Rape, M. The Ubiquitin Code. Annu. Rev. Biochem. 2012, 81 (1), 203–229.

[2] Dang, F.; Nie, L.; Wei, W. Ubiquitin Signaling in Cell Cycle Control and Tumorigenesis. Cell Death

[3] Yu, F.; Teo, G. C.; Kong, A. T.; Fröhlich, K.; Li, G. X.; Demichev, V.; Nesvizhskii, A. I. Analysis of DIA Proteomics Data Using MSFragger-DIA and FragPipe Computational Platform. Nat. Commun.

[4] Lederer, M.; Müller, S.; Glaß, M.; Bley, N.; Ihling, C.; Sinz, A.; Hüttelmaier, S. Oncogenic Potential of the Dual-Function Protein MEX3A. Biology 2021, 10 (5), 415.

AI-driven Prediction of Circular Dichroism Spectra of Proteins

DAY 2 – POSTER 21

José Manuel Robles-Aguilar, Mauricio Demetrio Carbajal-Tinoco, Damian Jacinto-Méndez

Cinvestav

Circular Dichroism (CD) spectroscopy is a versatile and rapid method for characterizing the secondary structure, conformation, and folding of proteins under near-native conditions. While not a high-resolution technique, it is invaluable for analyzing protein-ligand interactions, assessing the effects of mutations, and validating computationally predicted structures. The ability to accurately predict CD spectra from high-resolution structural information is crucial for these applications. The Knowledge-based Circular Dichroism (KCD) method has demonstrated superior accuracy in predicting far-UV CD spectra compared to other well-established approaches. It uses a model based on the classical theory of optical activity with a complex set of atomic polarizabilities derived from a database of SRCD spectra and PDB structures. However, its predictive power can be further enhanced by refining these polarizabilities. In this work, we present a novel approach to enhance the accuracy of the KCD method. We have developed and implemented deep neural networks to optimize the weights of the complex polarizabilities used in the KCD algorithm. We will demonstrate how this data-driven approach significantly improves the accuracy of far-UV CD spectral predictions when compared to the original KCD method and other state-of-the-art prediction tools. This refined computational model offers a powerful new tool for the structural characterization of proteins.

Rescoring of Crosslink Mass Spectrometry Matches Using Machine Learning

DAY 1 – POSTER 22

Falk B. Schimweg[1], Lutz Fischer[1], Mostafa Kalhor[2], Mathias Wilhelm[2,3], Juri Rapsilber[1,4,5]

[1] Bioanalytics, Institute of Biotechnology, Technical University Berlin, Germany, [2] Computational Mass Spectrometry, Technical University of Munich, Germany, [3] Munich Data Science Institute, Technical University of Munich, Germany, [4] Wellcome Centre for Cell Biology, University of Edinburgh, UK, [5] Si-M "Der Simulierte Mensch", Technical University Berlin, Charité, Germany

Crosslink mass spectrometry (MS) search engines often identify crosslink spectrum matches (CSMs) by calculating first multiple sub-scores and then aggregating these into a final score to evaluate the quality of a CSM. This aggregation is often a hand-crafted function, which may not accurately capture the optimal weighting of the sub-scores. We propose xiRESCORE, a crosslinking MS focused machine learning based approach, to rescore CSMs based on the sub-scores of crosslinking MS search engines. We noticed an intricate challenge for machine learning, resulting from CSMs consisting of pairs of peptides. Training on crosslinking MS datasets introduces a higher risk of information leakage. We developed a novel approach to split the training data such that information overlap between test and training data is prevented. This aggregation improves separation of false-positive CSMs, leading to more matches after FDR cutoffs are applied. We show that our method can double the discovered protein-protein interactions (PPIs) in a whole-cell crosslinking experiment, for the three search engines tested (Kojak, Protein Prospector, xiSEARCH). Our method also allows incorporating features from external tools. For example, adding spectra intensity predictions from Prosit-XL (Kalhor et al. 2025) pushes the discovered PPIs for xiSEARCH results from a 1.9-fold to a 2.4-fold increase compared to the original scoring approach.

Adaptive CDK Pathways Underpinning Therapy Resistance

DAY 2 – POSTER 22

Alessia Schirripa[1], Helge Schoeppe[2], Elisabeth Gamper[1], Mark Steinlechner[1], Thorsten Klampfl[1], Veronika Sexl[2], Eduard Stefan[2], Ulrich Stelzl[3], Teresa Kaserer[2], Karoline Kollmann[1]

[1] University of Veterinary Medicine Vienna, Austria, [2] University of Innsbruck, Austria, [3] University of Graz, Austria

Cyclin-dependent kinases (CDKs) are master regulators of cell-cycle progression and transcriptional control, with CDK6 playing a role in both processes. CDK6 is a key regulator of both cell-cycle progression and transcription. Unlike other cell-cycle CDKs, CDK6 possesses an unusually long and structurally unresolved C-terminus. To study its function, we reconstituted CDK6-deficient leukemic cells with either full-length CDK6 or a C-terminally truncated variant (CDK6 ΔC, lacking the last 32 amino acids). Our results revealed that the C-terminus is essential for nuclear localization, chromatin binding, and interaction with canonical regulators such as Cyclin D, INK4 inhibitors, and p27Kip1. Structural modeling confirmed reduced protein flexibility due to truncation, disrupting conformational dynamics critical for partner binding. Surprisingly, interaction with Cyclin B1 remained intact, an unexpected finding suggesting a distinct, C-terminal-independent interaction mode, potentially linking CDK6 to later cell-cycle phases or alternative functional pathways. Preliminary data suggest that CDK8 represents another example of a CDK that bridges transcriptional regulation and cell cycle control. The combination of CDK8 mutant mouse models, RNA-seq analysis and CDK8 Co-IP-MS data of p185BCR-ABL+ cells, revealed a direct involvement of CDK8 during mitosis, suggesting a novel potential combinatorial strategy: pairing CDK8 inhibition with agents targeting mitotic progression to enhance anti-leukemic efficacy. These data highlight the need to explore cell-cycle-phase specific atypical CDK complexes which might contribute to resistance against CDK inhibitors, like CDK4/6 inhibitors, particularly in breast cancer and leukemia.

Redesigning the crosslinking reaction for high-fidelity cellular interactomics

DAY 1 – POSTER 23

Bruno C. Amaral, Andrew R.M. Michael, D. Alex Crowder, Pauline Douglas, Morgan F. Khan, David C. Schriemer

University of Calgary

Crosslinking mass spectrometry should be the ideal method for mapping protein-protein interactions in situ, however conventional crosslinking methods can distort the structural proteome and they appear to suffer from poor yield. We can overcome these problems by prestabilizing the cell prior to crosslinking, which preserves cellular ultrastructure and offers full control over crosslinker yield via a two-stage crosslinking protocol. We will describe the concept and illustrate that as much as 30% of the total lysine content of the cell can be crosslinked, presenting new analytical challenges that must be met before practical, ultra-deep interactome sampling will truly be possible.

Resolving the spatio-temporal organization of the tryptophan catabolism pathway

DAY 2 – POSTER 23

Filip Sente, Sara Marchese, Jonatan Caroli, Francesca Gasparini, Matteo Re, Andrea Mattevi

Department of Biology and Biotechnology 'L. Spallanzani', University of Pavia, Italy

In order to adapt to organism's repressive environment, cancer cells can rewire their metabolism and divert the immune system from its antitumor effects, thereby performing an immune escape. A putative pathway involved in this immune evasion strategy is the kynurenine pathway of tryptophan catabolism. Since kynurenine and its associated metabolites are known to bind aryl hydrocarbon receptor (AhR) and consequently dysregulate immune response, our project is interested in investigating possible spatial and temporal reorganization of the enzymes in the pathway, and how this recruitment might differ between healthy and cancer cells. In order to elucidate this tentative structural metabolic reprogramming, we applied different techniques and approaches for exploring and validating protein-protein interactions (PPIs). We started with in cellula pairwise PPI screening by performing bimolecular fluorescence complementation (BiFC) assay split-YFP, thereby exploiting E. coli cells as a host for protein co-expression and employing flow cytometry for measuring cell fluorescence. This approach is expected to give us an idea of how these enzymes might come together sequentially, but it could also be repurposed for identifying potential allosteric modulators of their interactions. Furthermore, to extend the scope of tested interaction partners and validate results obtained so far, we also performed native-PAGE, pull-down, and liquid chromatography-mass spectrometry experiments (LC-MS), which revealed possible new interactors. On the other hand, we are also interested to assess whether effects such as macromolecular crowding and liquid-liquid phase separation (LLPS) play an important role in regulating the kynurenine pathway. By producing synthetic phase-separated liquid droplets, thus mimicking crowded intracellular environment in vitro, and utilizing them for enzymatic and biochemical studies with the proteins of the pathway, we would like to address the possibility of reconstituting a transient multi-enzyme formation conceivably resembling a metabolon, which could take advantage of weak PPIs and ultimately lead to aforementioned immunomodulation. Taken altogether, the main objective of this project is to shed light on the molecular mechanisms underpinning metabolic flexibility and adaptability of human cancer cells. By understanding the processes that govern enzyme dynamics in metabolic pathways, and orchestrate cell's metabolic 'decision-making', our aim is to explore and uncover new frontiers for fighting cancer.

Leveraging HDX-MS and AI for discovery of a transient druggable pocket in TNFa family members

DAY 1 – POSTER 24

Anjana Shenoy, Nitzan Simchi , Joseph Rinberg, Alon Shtrikman, Kirill Pevzner, Eran Seger

Protai Bio, Ramat Gan, Israel

The Tumor Necrosis Factor alpha (TNFα) superfamily plays a central role in inflammation, immune regulation, and cell death. Dysregulated TNFα signaling is linked to autoimmune disorders, chronic inflammation, and cancer. Current therapies mainly rely on monoclonal antibodies that block ligand-receptor interactions. Although clinically successful, these biologics have notable drawbacks, including requirement of injections, high production costs, limited tissue penetration, and immunogenicity highlighting the need for small-molecule modulators as more convenient therapy in daily pill form, and brain penetration enabling indication expansion. Small-molecule development for TNFα family members has been challenging due to the absence of well-defined druggable pockets in static structures. These proteins often function as oligomers, and many allosteric or cryptic sites remain hidden without understanding their dynamic landscape. To address this, we applied an integrative structural proteomics approach combining molecular dynamics (MD) simulations, pocket identification method, and Hydrogen–Deuterium Exchange Mass Spectrometry (HDX-MS). Enhanced sampling methods across multiple TNFα family members revealed a previously uncharacterized transient pocket distal to the receptor binding interface. Some of these pockets were shared between other family members. AI-driven analysis indicated favorable physicochemical properties for small-molecule binding. MD simulations also reveal that the bound ligand stabilizes the protein in a conformation that is unfavorable for receptor binding, thereby reducing its affinity. HDX-MS validated these findings by showing localized increases in deuterium uptake in the predicted region, confirming enhanced flexibility and transient solvent exposure. This study demonstrates the power of combining protein dynamics and biophysical validation to expand therapeutic strategies for historically challenging targets. Our work enables rational design of small-molecule modulators for the TNFα superfamily, offering a promising alternative to antibody-based therapies and advancing treatment options for immune-mediated diseases.

Structural characterization of the full-length tumor suppressor p53 upon DNA binding

DAY 2 – POSTER 24

E. Siefke, A. Sinz, C. Arlt

Martin Luther University Halle Wittenberg, Center for Structural Mass Spectrometry, Halle, Germany

The tumor suppressor p53, also known as the "guardian of the genome", plays a central role in cellular stress response. As a transcription factor, p53 targets a wide variety of genes that are involved in controlling cell cycle, DNA-repair, and apoptosis. Mutations in p53 are reported for more than 50% of all tumor cases, classifying p53 as one of the most important targets in cancer [1] . p53 is one of the key examples of intrinsically disordered proteins (IDPs), which are challenging to study using the classical methods for 3D-structure analysis. While extensive research has been focused on p53's DNA-binding, molecular-level understanding has so far been largely restricted to the structured DNA-binding domain of p53. This study aims to explore the conformational changes and structural dynamics involved in the DNA-binding process of p53, with a particular focus on the N- and C-terminal intrinsically disordered regions. We employed full-length human wild-type p53 tetramer, along with the dimeric p53 mutant L344A [2]. For DNA-binding experiments, we generated a response element DNA (RE-DNA) library derived from the endogenous p21 RE-DNA, with variations in length, complexity, and specificity. P53-DNA complexes were identified using native MS and were analyzed in detail by cross-linking mass spectrometry (XL-MS) and hydrogen-deuterium exchange mass spectrometry (HDX-MS). XL-MS was conducted with photo-methionine, disuccinimidyl dibutyric urea (DSBU) or other cross-linkers. These complementary techniques of structural MS provided insights into the 3D-structure, the conformational changes of full-length p53 upon DNA binding, and the structural dynamics of p53's DNA-binding process. Changes in the structural dynamics were compared for the interaction of p53 with specific and non-specific DNA. In addition, we also established a Python workflow that exploits XL-MS data, incorporating both COMPASS (COMPetitive PAiring StatisticS) [3] analysis and quantitative XL-MS data. By combining these data, the method allowed refining the identification of conformational states and enhanced discrimination between protein ensembles in different functional states, such as DNA-bound versus DNA-free for p53. Our study provides conformational data on p53 upon DNA binding and gives important insights into the role of the N- and C-termini of p53 in DNA recognition and site-specific binding.

[1] Jörger, Fersht et al. 2008

[2] Fernandez-Fernandez et al., 2005

[3] Ubbiali et al., 2022

Hydrogen-deuterium exchange for analysis of blood serum model systems

DAY 1 – POSTER 25

Tomáš Smrčka[1,2], Dmitry Loginov[2], Petr Novak[2], Petr Pompach[3], Pavla Vanková[3], Alan Kádek[2], Petr Man[2]

[1] Faculty of Science, Charles University, Prague, Czech Republic, [2] Institute of Microbiology (BIOCEV), The Czech Academy of Sciences, Prague, Czech Republic, [3] Institute of Biotechnology, The Czech Academy of Sciences, Prague, Czech Republic

Hydrogen-deuterium exchange mass spectrometry has proven its importance in structural biology over the years. The advantages of HDX, in contrast with some other structural techniques, are the ability to study proteins directly in a solution and nearly no protein size limitations. These capabilities make HDX an excellent candidate for developing a method that can examine proteins directly in their natural complex matrices. To achieve this goal, we took human serum as an environment in which we can address relevant questions regarding binding of protein, peptide or small molecule ligand. For these purposes, two protein systems were selected – the haptoglobin-haemoglobin complex and α-1-acid glycoprotein, a known binder and transporter of numerous drugs. Our goal was to firstly characterise these systems individually and subsequently dive into the complex systems. Haptoglobin exists in three different phenotypes, namely 1-1; 1-2 and 2-2. The X-ray structure of human haptoglobin dimer (1-1) in complex with haemoglobin and additional stabilizing proteins has been already revealed, but the structure dynamics of oligomeric haptoglobins (2-1, 2-2) and their binding to haemoglobin remains unknown and was investigated in this work. Next, the interaction of α-1-acid glycoprotein with its famous interactor, warfarin, was probed together with its formerly suggested pH-dependency that may provide insight into the dynamics of α-1-acid glycoprotein binding cavity. Overall, the work presented here provided valuable insights in two isolated model systems that could be further applied in complex experiments in heterogeneous environment of the whole human serum. Financial support: This project is financed by Charles University Grant Agency (GA UK).

Fluorinated MS-Cleavable Cross-Linker for Enhanced Cellular Penetration in Structural Proteomics

DAY 2 – POSTER 25

Oleksandr Sorokin[1,2,], Frank Hause[1,2,3], Christian H. Ihling[1,2], Tomáš Vranka[4], Václav Matoušek[4], Andrea Sinz[1,2]

[1] Department of Pharmaceutical Chemistry and Bioanalytics, Martin Luther University Halle-Wittenberg, Halle (Saale), Germany, [2] Center for Structural Mass Spectrometry, Martin Luther University Halle-Wittenberg, Halle (Saale), Germany, [3] Institute of Molecular Medicine, Section for Molecular Cell Biology, Faculty of Medicine, Martin Luther University Halle-Wittenberg, Halle (Saale), Germany, [4] CF Plus Chemicals s.r.o., Brno, Czechia

Cross-linking mass spectrometry (XL-MS) has revolutionized our understanding of protein three-dimensional structures and protein-protein interactions (PPIs) over the past two decades. This powerful technique has emerged as a valuable tool for probing complex biological systems, offering unique insights into protein conformational dynamics and macromolecular assemblies. While XL-MS has proven beneficial for studying proteins in their native environments, significant challenges remain for in-cell and in vivo applications. The technique's ability to capture transient and weak protein interactions, which are often lost during traditional purification procedures, has made it particularly attractive for systems biology approaches. However, a critical limitation is the poor membrane permeability of current cross-linking reagents, restricting comprehensive mapping of PPIs in intact cells. The development of cross-linkers capable of efficient cellular penetration represents a crucial advancement for expanding XL-MS applications to the physiologically relevant cellular context where proteins function within their natural regulatory networks and environmental constraints. In this study, the development and characterization of bis(pentafluorophenyl) ureido-4,4′-dibutyrate (DPFU), a novel fluorinated MS-cleavable cross-linker, is presented. DPFU has been designed to overcome membrane permeability limitations. It is structurally based on the MS-cleavable cross-linker disuccinimidyl dibutyric urea (DSBU), which has demonstrated reliable performance in numerous structural proteomics applications. A strategic exchange of the N-hydroxysuccinimide (NHS) ester leaving groups with pentafluorophenyl groups, while keeping the central urea moiety, increases lipophilicity and maintains the MS cleavability and signature fragmentation patterns under collision-induced dissociation (CID) conditions. The design rationale centers on balancing hydrophobicity and aqueous solubility to optimize membrane permeability while preserving cross-linking specificity. The pentafluorophenyl groups provide enhanced lipophilicity compared to NHS esters, potentially facilitating improved cellular uptake while preserving the cross-linking reactivity toward primary amines in lysine residues and protein N-termini. Given DPFU's increased hydrophobicity relative to traditional water-soluble cross-linkers, we systematically evaluated its solubility behavior in aqueous environments using various detergent systems to identify optimal formulation conditions. Cross-linking experiments were conducted with bovine serum albumin (BSA) as a well-characterized model protein across multiple experimental parameters, including temperature variations (4°C and 20°C), reaction times (30, 90, and 120 minutes), and cross-linker concentrations (20-, 50-, and 100-fold molar excess). These systematic

variations allowed for a comprehensive assessment of reaction kinetics and efficiency under different conditions that might be encountered in cellular applications. Cross-linked samples underwent tryptic digestion followed by LC-MS/MS analysis using state-of-the-art instrumentation (Ultimate RSLC nano-HPLC coupled to timsTOF Pro mass spectrometer), employing optimized chromatographic gradients and data acquisition methods specifically tailored for cross-linked peptide identification. The analytical workflow incorporated advanced computational tools for cross-link identification and validation, ensuring high confidence in structural assignments. Under optimized conditions, DPFU successfully cross-linked BSA, with all identified cross-links being consistent with the known three-dimensional structure of the protein, validating the cross-linker's ability to capture authentic protein contacts. Importantly, the characteristic fragmentation patterns of the urea bond were preserved, enabling unambiguous identification of cross-linked peptides through database searching algorithms. This study represents the first systematic investigation of fluorinated cross-linkers for structural proteomics applications, opening new avenues for cross-linker chemistry optimization. Despite current limitations in cross-linking efficiency compared to conventional reagents, the successful demonstration of enhanced solubility properties and maintained MS-cleavable characteristics provides valuable insights for future fluorinated cross-linker development. The incorporation of fluorinated groups represents a promising strategy for addressing the long-standing challenge of cellular permeability in cross-linking reagents. Continued optimization of this approach, potentially including modifications to spacer length, reactivity, and fluorination patterns, holds significant promise for enabling comprehensive PPI mapping within the cellular environment, ultimately advancing our understanding of protein function in native biological contexts and facilitating the study of cellular proteomes under physiologically relevant conditions.

HDX-MS and limited proteolysis unravel the conformational features of human transthyretin (TTR) oligomers suggesting a role in the process of TTR fibril formation

DAY 1 – POSTER 26

Barbara Spolaore, Daniele Peterle, Anna Pagotto, Edoardo Viero, and Vincenzo De Filippis

Department of Pharmaceutical and Pharmacological Sciences, University of Padova, Padova, Italy

Human TTR is a homo-tetrameric protein involved in systemic TTR amyloidosis, characterized by polyneuropathy and cardiomyopathy phenotypes. Upon aging wild-type TTR deposits in the form of fibrils preferentially in the heart (ATTRwt) causing a life-threatening cardiomyopathy which affects about 10% of the elderly. Variant amyloidogenic transthyretin (ATTRv) amyloidosis is instead caused by mutation of the TTR gene and manifests earlier in life with variable phenotypes. In both forms of ATTR amyloidosis, TTR fibrils are mainly composed of proteolytic fragments, especially fragment 49-127 [1]. Hence, the amyloidogenic potential of TTR in vivo is strongly dependent on its susceptibility to proteolysis. Importantly, recent studies demonstrated the presence of circulating non-native oligomeric TTR in plasma samples of patients affected by ATTRwt [2] and ATTRv [3, 4] amyloidosis, opening to questions on the role of these soluble aggregates in the pathogenesis of ATTR amyloidosis. Here, we studied the conformational features of TTR oligomers (TTR-O) under physiological conditions by HDX-MS and limited proteolysis. Oligomers were produced in vitro from wild-type human TTR [5]. Global HDX-MS analyses evidenced the coexistence of protein states with different levels of flexibility, with oligomers showing a looser structure. Local HDX-MS indicated that TTR-O display a significant increased exposure of regions involved in the stabilisation of the native tetramer. TTR-O were much more susceptible than native TTR to proteolysis by digestive, coagulative and fibrinolytic proteases, and the initial sites of proteolysis occurred at the level of flexible regions as determined by HDX-MS. Importantly, proteolysis reactions on TTR-O generated the amyloidogenic fragment 49-127 already found in ATTR fibrils in vivo. Overall, our results suggest an involvement of TTR oligomers in the generation of amyloidogenic fragments in vivo and in turn of fibril deposition, in agreement with recent findings showing that the presence of circulating TTR aggregates is correlated with disease progression.

[1] Bergström et al. (2005) J. Pathol. 206, 224–232

[2] Pedretti et al., (2024) JACC Basic Transl. Sci. 9, 1088-1100

[3] Schonhoft et al., (2017) Sci. Transl. Med. 9, eaam7621

[4] Jiang et al., (2021) Proc. Natl. Acad. Sci. USA 118, e2016072118

[5] Dasari et al., (2019) Sci. Rep. 9, 33

Impact of Aging on Proteasome Composition and Activity

DAY 2 – POSTER 26

Shriya Srivastava, Michal Sharon

Weizmann Institute of Science, Israel

Background: Aging is characterized by the gradual accumulation of damaged macromolecules. This accumulation is due to malfunctions of cellular maintenance, repair and removal systems. One such system is the proteasome degradation machinery that is responsible for disposal of damaged proteins to maintain proteostasis. Proteasome displays remarkable reduction in proteolytic activity upon aging of tissues contributing to the development of plethora of disorders such as neurodegenerative disorders. Researchers have claimed this reduction in proteasome activity is a result of factors such as decreased expression of proteasome subunits, disassembly of the complex and/or aberrant posttranslational modifications. Despite these observations, the direct relationship between structural and functional alterations in the proteasome and the aging process remains poorly understood. Introduction: The proteasome complex is a 2.5 MDa complex, responsible for proteolytic function of the ubiquitin (Ub)-proteasome degradation system. It is composed of two components, a catalytic particle (CP) known as the 20S proteasome that performs proteolytic activity by cleaving the proteins into peptides and a 19S regulatory particle (RP), which together form the 26S proteasome. The RP recognizes and unfolds polyubiquitinated substrates by translocating them into the 20S core in an ATP-dependent manner. In addition to the 19S, other regulatory particles such as PA200, PA28γ, and the cytosolic PA28αβ (also known as the 11S) can associate with the 20S core, broadening the range of substrates targeted for degradation. Notably, the 20S proteasome can also function independently of regulatory particles, degrading partially unfolded or oxidized proteins in an ATP- and ubiquitin-independent manner. Structurally, the CP consists of four stacked-heptameric rings, two outer α-rings and two inner β-rings. The β-rings contain seven subunits, three of which exhibit distinct catalytic activities: chymotrypsin-like, trypsin-like, and peptidyl-glutamyl peptide-hydrolyzing activities. Under conditions of immune challenge, these catalytic subunits can be replaced by inducible counterparts, resulting in the formation of immunoproteasomes that support antigen processing. Although proteasomes have been extensively studied, the impact of aging on the catalytic properties, structural integrity, and functional dynamics of these subunits remains elusive. Methods: In this research, I combine fundamental biochemical techniques such as SDS-PAGE and Native-PAGE activity assay with advanced native mass spectrometry techniques to gain a multidimensional understanding on the complex properties. The primary objective is to investigate age-related structural and functional changes in the proteasome during the aging process, using a mouse model. Analyses were conducted on tissues from the brain, liver, kidney, lung, and heart of healthy mice across three age groups: infants (7 days old), adults (~3–6 months old), and aged mice (1–1.5 years old). Results: Analysis of mice revealed that all three catalytic activities of the

30S and 26S proteasome complexes were highly active across all examined tissues. In contrast, the 20S proteasome, while abundantly expressed, exhibited low activity, indicating that under normal physiological conditions, the ubiquitin- and ATP-dependent degradation machinery is the predominant pathway. Following infancy, a marked decline in the activity of the 30S and 26S proteasome populations was observed across tissues, with a more gradual decrease occurring between adulthood and old age. Notably, the expression levels of the 20S proteasome remained stable throughout the aging. The decline in age-dependent activity of proteasome subpopulation varied among tissues. For example, chymotrypsin-like activity of the 30S and 26S proteasome showed pronounced reduction after infancy in the brain, kidney, lung, and heart while activity in liver remained largely unaffected by age. Similarly, trypsin-like activity of 20S proteasome decreased with age in the brain and heart but increased in the kidney and liver. These findings highlight the tissue-specific heterogeneity in both activity and expression of proteasome subpopulations during aging. Comparing immunoproteasome subunit expression in various tissues, revealed consistent levels of immuno-catalytic subunits at all ages. However, the brain exhibited lower expression of these subunits, potentially contributing to its heightened vulnerability to age-related neurodegenerative diseases. To explore whether reduced proteasome activity during aging could be attributed to diminished expression of regulatory particles, the levels of common RPs such as 19S and 11S were analyzed. These analyses showed no significant age-related changes in RP expression. Discussion: These findings highlight the unique, tissue-specific properties of the proteasome system, suggesting that proteasome subpopulations undergo functional specialization to meet the distinct biological requirements of each tissue at different stages of life. The observed heterogeneity in proteasomal activity and subunit expression, particularly across the 30S, 26S, and 20S complexes, underscores the dynamic and adaptive nature of the proteasome machinery during aging. Additionally, the noticeable inter-animal variability points to an inherent biological "noise" in proteasome regulation, which may influence individual susceptibility to age-related decline.

Native mass spectrometry probes structures of DNA origami

DAY 1 – POSTER 27

M. Manceau, C. Saint-Pierre, **C. Tymen**, C. Masselon, V. Agache, D. Gasparutto, E. Boeri Erba

Nucleic acids (DNA, RNA and artificial analogues) have the capacity to form intricate three-dimensional structures that are not only pivotal for biological processes but also have potential for nanobiotechnology applications. In DNA nanotechnology, these nucleic acids can be engineered into static or dynamic assemblies with wide-ranging implementations in biotechnology, nanomedicine, and nanoelectronics. Thus, understanding and characterizing the size, shape and assembly routes of these oligomeric nucleic acids (like cages, Legos or DNA origami) is crucial for improving their rational design. Toward this end, conventional methods such as gel electrophoresis and dynamic light scattering assess the average size of nanoassemblies, whereas atomic force and electron microscopy provide high-resolution visualization at the nanometre scale. Nevertheless, these techniques do not offer insights into subunit composition and stoichiometry nor about assembly pathway intermediates or homogeneity of biomolecular assemblies across different formulations. To address these limitations, the MultiMASS project is developing a comprehensive, multi-scale analytical framework for native Mass Spectrometry (MS) characterization of nucleic acid assemblies. The project employs multiple MS approaches including nano-ESI-MS, Suspended NanoResonator (SNR), and NanoElectroMechanical Sensor (NEMS) in order to span an extensive mass range from hundreds of Daltons to hundreds of GigaDaltons. This wide mass coverage and complementarity of techniques enables the characterization of a vast array of DNA assemblies and hybrid constructs (DNA-organic molecules, DNA-particles, DNA-proteins). Moreover, we aim to synthesize functionalized DNA architectures to serve as reference standards, reinforcing mass measurement reliability for multi-megadalton assemblies. Taken together, this multi-scale mass spectrometry framework will allow precise characterization of DNA nanostructures by providing valuable information on composition, oligomeric state and morphology.

Protein Secondary Structure Patterns in Short-Range Cross-Link Atlas

DAY 2 – POSTER 27

A. Vetrano[1], A. Di Ianni[1,2], N. Di Fonte[1], G. Dell'Orletta[1], S. Reale[1], I. Daidone[1] and C. Iacobucci[1]

[1] University of L'Aquila [2] Human Technopole

Cross-linking mass spectrometry (XL-MS) has become an essential tool in structural biology, as it provides valuable information on protein conformations and interactions. While long-range cross-links are widely used to define tertiary and quaternary structures, short-range cross-links (those spanning fewer than 20 residues) have often been overlooked and considered of little structural significance.

In this study, we systematically analyzed these short-range cross-links to explore their relationship with the secondary structure of proteins. We introduce X-SPAN, a software designed to integrate publicly available XL-MS datasets from system-wide experiments with AlphaFold-predicted protein structures for entire organismal proteomes. Specifically, we analyzed 12 publicly available system-wide XL-MS datasets, comprising a total of 658432 cross-links from 4 organisms. By applying our filtering criteria, selecting only unique intra-protein cross-links with a maximum spacing of 20 amino acids in the primary sequence, we identified 78985 cross-links, of which 48748 were found in continuous motifs (α-helices, β-strands coils and mixed elements).

Our analysis unveils distinct cross-linking patterns that depend on the spatial constraints of secondary structure elements, with α-helices, β-strands, and coils exhibiting characteristic behaviors. We demonstrate that α-helices display a periodic cross-linking pattern consistent with their helical pitch, whereas coils and β-strands show nearly monotonic distributions.
Additionally, our analysis highlights context-dependent cross-linking grammar, where the local amino acid environment modulates cross-linking efficiency.

By comparing experimental cross-linking data with AlphaFold-predicted structures, we find that cross-link distributions are strongly influenced by the predicted local confidence score (pLDDT). This work provides a novel framework for benchmarking AlphaFold's local accuracy.
Beyond these structural insights, our study demonstrates the potential of short-range cross-links as quality control metrics for XL-MS experiments to distinguish between native distributions from potential artifacts.

We anticipate that our short-range cross-link database will serve as a valuable resource for studying local secondary structure switches and their potential roles in protein function and allosteric regulation.

Impact of natural allelic variation on structure and function of the immune proteases SAP1 and SAP2

DAY 1 – POSTER 28

Christian Wal[1,2], Dirk Tänzler[1,2], Diego F. Garcia del Rio[1,2], Mariana Schuster[3], Andrea Sinz[1,2], Christian Ihling[1,2]

[1] Department of Pharmaceutical Chemistry and Bioanalytics, Department of Pharmacy, Martin Luther University Halle-Wittenberg, Halle (Saale), Germany, [2] Center for Structural Mass Spectrometry, Martin Luther University Halle-Wittenberg, Halle (Saale), Germany, [3] Leibnitz Institute of Plant Biochemistry, Halle (Saale), Germany

Diseases caused by plant pathogens threaten ecosystems and crop productivity worldwide. Plants have evolved a complex immune system to cope with pathogen attack. One defense mechanism involves the secreted aspartic proteases 1 and 2 (SAP1 and SAP2) of Arabidopsis thaliana. Both immune proteases cleave evolutionary conserved bacterial protein MucD, thereby directly inhibiting the growth of Pseudomonas syringae. This project aims to generate insights into the molecular operating mechanisms of SAPs and to determine the effect of single amino acid mutations on their structures and conformations by utilizing complementary structural MS techniques in combination with computational modeling. GST-tagged variants of wild-type SAP1 and SAP2 were expressed in E. coli and purified via affinity chromatography. Following tag removal by thrombin cleavage, cross-linking experiments were conducted on SAP1, SAP2, and a 1:1 mixture of both proteins. The cross-linking reactions utilized a 50-fold molar excess of disuccinimidyl dibutyric urea (DSBU) and were incubated for 30 minutes at room temperature. The resulting samples were analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) using a 90-minute gradient on a U3000 nano-HPLC system (Thermo Fisher Scientific) coupled to a timsTOF Pro mass spectrometer (Bruker Daltonik). A total of six cross-links were identified across SAP1, SAP2, and their mixture, including one homotypic cross-link consistently observed in all samples, supporting the hypothesis of SAP dimer formation. For further cross-linking analysis, unlabeled and isotopically labeled (^{15}N) variants of SAP1 and SAP2 will be produced in A. thaliana. Additionally, native MS experiments are planned to determine the stoichiometry of SAP-MucD complexes, providing initial insights into the molecular mechanism underlying substrate proteolysis mediated by SAP1 and SAP2.

Hydroxyl Radical Footprinting MS data submission to PRIDE

DAY 2 – POSTER 28

Marek Zakopcanik[1], Dmitry S. Loginov[1], Juan Antonio Vizcaíno[2], Petr Novak[1]

[1] Institute of Microbiology, The Czech Academy of Sciences, Prague, Czech Republic, [2] European Molecular Biology Laboratory, European Bioinformatics Institute (EMBL-EBI), Wellcome Genome Campus, Hinxton, Cambridge, UK

The advances in mass spectrometry (MS)-based techniques have propelled proteomics to provide in-depth analyses of proteins, delivering both qualitative and quantitative information on protein-protein interactions, signalling pathways, and post-translation modifications. Similarly to other omics fields, proteomics has established open data policies, involving the PRIDE database and the other ProteomeXchange resources, forming the basic infrastructure for data exchange. Still, the lack of comprehensive metadata annotation and tailored data deposition standardisation for different proteomics disciplines present a major setback for structural proteomic data re-usability both within the field and in other life sciences, halting the full utilisation of its potential. Indeed, structural proteomics brings information about the higher-order structure and dynamics of proteins, vital for our understanding of their function. This knowledge has direct impact on elucidation of the molecular machinery of living organisms, translating into applications in pharmaceutical and biotechnological industry. Among the approaches at structural proteomics' disposal, hydroxyl radical footprinting (HRF) represents a valuable tool to study the protein surface accessible area. The increasing popularity of hydroxyl radical footprinting is mainly due to the water-like properties of the ·OH and its wide reactivity towards amino acid side chains. Together with recent advancements on the instrumental side, HRF is currently gaining attention in the structural proteomics community. As a part of ODEEP-EU project, we aspire to set examples of how to make HRF proteomics data publicly available in a manner that enables re-usability, inter-field collaboration and data processing algorithm development. We have then prepared guidelines for HRF data submission to PRIDE. In addition to data deposition standardisation, the future aim is to establish processing pipelines between PRIDE and PDBe, and between PDBe-KB and AlphaFoldDB to support the integration of MS-based protein structural information with the experimentally derived or predicted structures. Therefore, to fully leverage the information on surface accessibility provided by HRF, the data needs to be deposited in well-defined and reliable manner suitable for both human and machine reading. These guidelines represent a first step in this direction. This work was supported by the Technology Agency of the Czech Republic (ODEEP-EU TH86010001).

Mapping the Networks of Intracellular Transport with Crosslinking Mass Spectrometry

DAY 2 – POSTER 29

d'Amico EA[1], Chen ZA[2], Rappsilber J[2], Carter AP[1]

[1] MRC Laboratory of Molecular Biology, Cambridge, UK, [2] TU Berlin, Berlin, Germany

Intracellular transport is a fundamental process in eukaryotic cells that ensures the delivery of cargoes to their correct cellular destinations. Cargoes recruit motors through networks of adaptors and regulatory proteins, but despite extensive literature, it is not clear what combinations of components are required to get an organelle moving. We are currently setting up in cellulo crosslinking to capture the transient, native contacts that motors make with adaptors and cargoes. Using NHS-diazirine crosslinkers, we stabilize the interaction between the minus-end directed motor dynein and its activator complexes dynactin and LIS1. Preliminary structural information from crosslink analysis is consistent with published literature. We plan to extend our analysis by investigating a broader library of adaptors and cargo markers to understand the networks that determine organelle transport in neurons.

Chemical Proteomic Investigation of Fatty Acid Decarboxylase Activation

DAY 1 – POSTER 30

F. Mercuri, A. Vetrano, G. Dell'Orletta, I. Daidone and C. Iacobucci

University of L'Aquila

Fatty acid photodecarboxylase (FAP) has recently been identified in the microalgae Chlorella variabilis NC64A.[1] FAP is a photoenzyme belonging to the glucose-methanol-choline (GMC) class. It is involved in lipid metabolism and it catalyses the decarboxylation of fatty acids to alkanes in response to light exposure.[1] FAP is a photoenzyme with great potential for technological applications in green chemistry, as hydrocarbon synthesis plays a crucial role in fuel and solvent production[2].
Beisson et al. investigated the elementary steps responsible for the catalytic activity of FAP[1,2]. According to their hypothesis, the illumination of FAP triggers an electron transfer from the fatty acid to the cofactor flavin adenine dinucleotide (FAD). This generates a semi-quinone FAD radical (FAD•-) and a carboxyl radical. The latter undergoes a rapid decarboxylation, forming an alkyl radical (R•). The final alkane may be generated through a hydrogen atom transfer from a cysteine or asparagine residue present in FAP active site.
The problem arises when the FAP is not bound to the substrate. Under this condition, light illumination triggers a rapid and irreversible FAP deactivation. This makes economically unprofitable the industrial application of FAP. FAP deactivation has never been investigated at a molecular level, but understanding this process could unlock the potential for real-world FAP applications.

Holmann et al. hypothesised that FAP deactivation may involve covalent modification of amino acid residues near the active site[3]. We aim to investigate this hypothesis by leveraging mass spectrometry-based chemical proteomics. High-resolution tandem mass spectrometry could reveal both the specific amino acid residues undergoing photoinduced covalent modifications and the precise nature of these modifications. These chemical modifications will be incorporated into molecular dynamics simulations of FAP to elucidate their mechanistic role at the molecular level.

[1] D. Sorigué, B. Légeret, S. Cuiné, S. Blangy, S. Moulin, E. Billon, P. Richaud, S. Brugière, Y. Couté, D. Nurizzo, P. Müller, K. Brettel, D. Pignol, P. Arnoux, Y. Li-Beisson, G. Peltier, F. Beisson; Science, 357, 903-907 (2017)
[2] D. Sorigué, K. Hadjidemetriou, S. Blangy, G. Gotthard, A. Bonvalet, N. Coquelle, P. Samire, A. Aleksandrov, L. Antonucci, A. Benachir, S. Boutet, M. Byrdin, M. Cammarata, S. Carbajo, S. Cuiné, R. B. Doak, L. Foucar, A. Gorel, M. Grünbein, E. Hartmann, R. Hienerwadel, M. Hilpert, M. Kloos, T. J. Lane, B. Légeret, P. Legrand, Y. Li-Beisson, S. L. Y. Moulin, D. Nurizzo, G. Peltier, G. Schirò, R. L. Shoeman, M. Sliwa, X. Solinas, B. Zhuang, T. R. M. Barends, J.P. Colletier, M. Joffre, A. Royant, C. Berthomieu, M. Weik, T. Domratcheva, K. Brettel, M. H. Vos, I. Schlichting, P. Arnoux, P. Müller, F. Beisson; Science, 372, 148 (2021)
[3] Y. Wu, C. E. Paul, F. Hollmann; ChemBioChem, 22, 2420-2423 (2021)

Higher Order Structure Characterization on ADCs using Capillary Flow Hydrogen/Deuterium Exchange Mass Spectrometry (HDX-MS)

DAY 2 – POSTER 30

Yuqi Shi[1], **Ulrik H. Mistarz[2]**, Cong Wang[3], Peter Krueger[3], Thaís de Faria[4], Rosa Viner[1], Thomas Moehring[3]

[1] Thermo Fisher Scientific, San Jose, CA, USA, [2] Thermo Fisher Scientific, Copenhagen, Denmark, [3] Thermo Fisher Scientific, Bremen, Germany, [4] Thermo Fisher Scientific, Eugene, OR, USA

Introduction

Antibody-Drug Conjugates (ADCs) are an innovative class of therapeutics that merge the high specificity of mAbs with the potent cytotoxic capabilities of small-molecule drugs, to offer a more precise and effective treatment. ADCs present unique challenges in development, particularly in understanding their complex structural and functional attributes. Any structural perturbations during conjugation, formulation, or storage could impact the ADC's ability to bind its target antigen and maintain its therapeutic profile. To address these challenges, HDX-MS shows as a powerful and sensitive tool for studying the higher order structure (HOS) of ADCs. In this study, we showed the ability to use HDX-MS to probe the HOS change on the antibody upon drug binding at both peptide and residue levels, using an automated data processing workflow.

Method

Unconjugated and conjugated forms of Rituximab were analyzed by HDX-MS using a fully automated HDX-MS workflow using Thermo Scientific™ Vanquish™ Binary Pumps N/F and a Thermo Scientific™ Orbitrap™ Excedion™ Pro MS coupled to a CHRONECT HDX robot (Trajan). Labelled samples were incubated in D2O at multiple time points in triplicates, quenched, digested with a Nepenthesin-2 protease column (AffiPro), and separated using capillary flow and a 0.3x5 cm column with a 6 min gradient for improved sensitivity. MS1 and DIA spectra were used to identify regions of deuterium incorporation.

Preliminary data

Peptide mapping experiments were conducted using unconjugated Rituximab across a dilution series. At the highest injection amount of 1.5 µg, 570 and 234 peptides were identified for HC and LC, respectively, achieving 100% sequence coverage for both. At the lowest injection amount of 300 ng, 445 and 186 peptides were identified for the HC and LC, respectively, with >98% sequence coverage. Based on these results, 300 ng was selected for labeling experiments, and the identified peptides served as the peptide library.

Peptide-level HDX-MS experiments were performed using both MS1 full scan and MS2 DIA modes, with data analyzed using HDExaminer and HDExaminer PRO (Trajan). The

drug-binding site was identified at the CH2 domain of the antibody, and allosteric changes were detected across the antibody, particularly at antigen-binding sites within the CH1 domain, to evaluate structural alterations that could affect antigen recognition. Both the binding site and allosteric changes were mapped onto a homology model for structural interpretation. ed onto a homology model for structural interpretation.

Molecular Recognition of Thyroglobulin by Sortilin: An Integrated Structural Biology Approach

Irene Boniardi, Giorgia Tanzi, Francesca Coscia

Human Technopole, Milan, Italy

DAY 1 – POSTER 31

Thyroglobulin (TG) is a large glycoprotein essential for thyroid hormone synthesis and storage. Synthesized and secreted by thyrocytes, TG forms a dimeric structure within the thyroid follicle lumen, where it undergoes iodination and hormonogenesis before being retrieved by endocytosis for hormone release. Despite its central role in thyroid physiology, the mechanisms governing TG uptake remain poorly understood. Sortilin, a ubiquitous receptor involved in protein sorting and endocytosis, has been proposed as a key mediator of TG internalization, particularly for its iodinated forms. However, the molecular basis of this interaction has remained elusive.

Here, we elucidate the mechanisms of TG recognition by sortilin using an integrated structural biology approach combining cryo-electron microscopy, crosslinking mass spectrometry, biophysical characterization, and endocytosis assays in human thyroid cells. We demonstrate that sortilin preferentially binds the monomeric form of TG over its native dimeric state, suggesting conformational selectivity in cargo recognition. Crosslinking mass spectrometry, combined with AlphaFold predictions, was critical in narrowing down the search for potential binding sites and pinpointing TG regions that contact sortilin. This strategy revealed an interaction involving an unstructured region of TG, shown here for the first time in a structural context bound to sortilin.

Furthermore, an in vitro iodination assay revealed that iodination does not affect sortilin binding, indicating that recognition is independent of TG's hormone content. Instead, the receptor appears to target partially unfolded or degraded TG. Structural analysis of sortilin in complex with a TG-derived C-terminal peptide (TGpep) revealed a binding mode conserved across sortilin ligands, relying on peptide accommodation within the β-propeller domain. Guided by our results, we computationally identified a shared recognition motif that may explain sortilin's broad ligand repertoire.

Overall, this work elucidates a molecular mechanism for TG endocytosis, identifies sortilin as a monomer-selective receptor, and provides a structural basis for their interaction. Importantly, it highlights how crosslinking mass-spectrometry can be used not only to map transient or heterogeneous protein–protein interactions but also to guide structural studies by refining hypotheses and directing complementary techniques. These findings expand our understanding of thyroid physiology and regulation while also illustrating the power of integrative structural biology approaches in uncovering dynamic aspects of receptor–cargo recognition.

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

DAY 2 – POSTER 31

Giuseppe Ciossani[1], Silvia Monzani[1] and **Luigi Scietti[1]**

[1] Biochemistry and Structural Biology Unit, Department of Experimental Oncology, IRCCS European Institute of Oncology (IEO), Via Adamello 16, 20139 Milan, Italy.

The Biochemistry and Structural Biology Unit (BSU) at the European Institute of Oncology (IEO – Milan, IT) offers a comprehensive range of services in protein biochemistry, biophysics, and structural biology. We work closely with researchers to facilitate the production and structural characterization of protein macromolecular complexes. The BSU employs optimized protocols and SLIC-based plasmid libraries for recombinant protein expression in various hosts, including E. coli, insect cells (Sf9 and High5), and suspension HEK293 cells. Together with multiple AKTA systems, this forms a robust platform for protein expression and purification. Our biophysical instruments encompasses SEC-SLS, mass-photometry, micro-ITC, BLITz, and FP plate readers. We have a mosquito nanoliter dispenser for crystallization screenings, all the necessary equipment for hit optimization, regular access to synchrotron facilities and computational resources for structure determination.

Additionally, we provide training and support in cryoEM, from sample preparation to data processing. We recently established a cryoEM lab, including a glow discharger a Vitrobot Mark IV and a BioComp gradient maker. We have regular access to 200 kV and 300 kV cryoEM for screening and data collection. For data processing, we operate a multi-GPU workstation that will be soon integrated with a multi-GPU high-performance cluster.

Insights into the Molecular Mechanisms of α-Synuclein/HSP70-1 Interaction Upon Liquid-Liquid Phase Separation

DAY 1– POSTER 32

Wiebke F. Günther, Oleksandr Sorokin, Marta Fratini, Marcel Köhn, Andrea Sinz

Martin Luther University Halle

Liquid-liquid phase separation (LLPS) is a mechanism that drives the formation of condensates or "membrane-less organelles" in cells through noncovalent interactions between macromolecules [1]. Intrinsically disordered proteins (IDPs), such as α-synuclein, play a central role in LLPS due to their high structural flexibility. The ability of α-synuclein to undergo LLPS can ultimately lead to aggregation and the formation of Lewy bodies, which are a hallmark of Parkinson's disease[2].

In our previous work, we showed by cross-linking mass spectrometry (XL-MS) that α-synuclein undergoes a conformational shift from a "hairpin-like" structure to a more extended state during the initial 24 hours of LLPS [3]. However, the molecular mechanisms that initiate and regulate α-synuclein LLPS remain unclear.

Chaperone proteins, such as the heat shock protein 70-1 (HSP70-1), are essential for preventing pathological protein aggregation by modulating protein folding. Interestingly, HSP70-1 has been observed to co-phase separate with α-synuclein, suggesting a regulatory role during LLPS [4].
In this study, we investigate the structural dynamics and interactions between α-synuclein and HSP70-1 during the initial stages of LLPS. By integrating XL-MS, HDX-MS, and native MS with light microscopy, we correlate the molecular details of the α-synuclein/HSP70-1 interactions, namely topology, dynamics, and binding interfaces, with LLPS. Our findings are important for a detailed understanding of α-synuclein's phase separation behavior. They will highlight the role of molecular chaperones in modulating LLPS and yield insights into the early events of protein misfolding.

[1] S. Alberti and D. Dormann, 'Liquid-Liquid Phase Separation in Disease', Annu Rev Genet, vol. 53, pp. 171–194, 2019.
[2] K. Hou, T. Liu, J. Li, M. Xian, L. Sun, and J. Wei, 'Liquid-liquid phase separation regulates alpha-synuclein aggregate and mitophagy in Parkinson's disease', 2023, Frontiers Media SA.
[3] D. Ubbiali et al., 'Direct Observation of "Elongated" Conformational States in α-Synuclein upon Liquid-Liquid Phase Separation', Angewandte Chemie - International Edition, vol. 61, no. 46, Nov. 2022.
[4] F. A. Aprile, E. Källstig, G. Limorenko, M. Vendruscolo, D. Ron, and C. Hansen, 'The molecular chaperones DNAJB6 and Hsp70 cooperate to suppress α-synuclein aggregation', Sci Rep, vol. 7, no. 1, Dec. 2017.