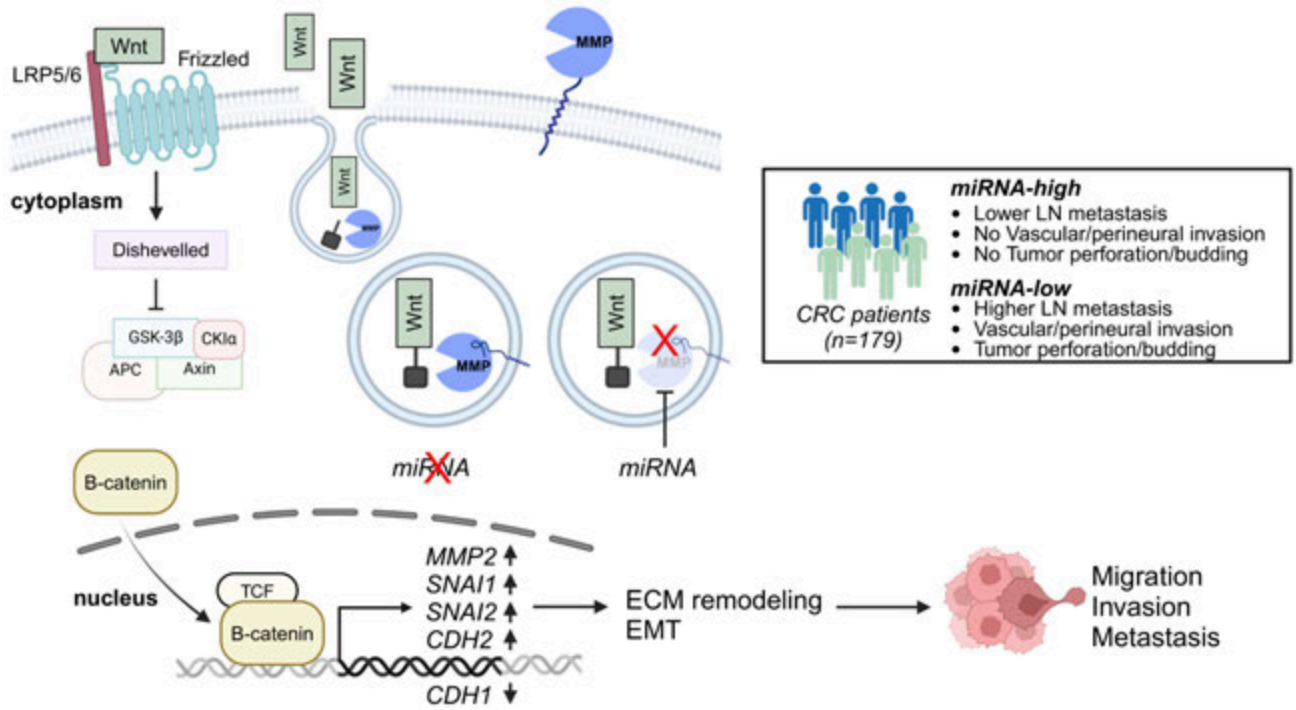
42nd Winter School on Proteinases and Their Inhibitors

Tiers am Rosengarten
March 12 - March 16, 2025

Program & Abstract Book



MicroRNA - metalloprotease axis controls metastatic behavior in colorectal cancer

Courtesy of Ayşe Seray Güzel, Koç University, Türkiye

The cover shows a view of Tiers am Rosengarten.

42nd Winter School on Proteinases and Their Inhibitors in Tiers (Italy)
March 12 - March 16, 2025

Organizing Committee

Klaudia Brix (Bremen)
Hans Brandstetter (Salzburg)
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PROTEOCURE

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Editors

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GENERAL INSTRUCTIONS

Accommodation

All Winterschool participants are accommodated in the hotels Paradies, Laurin or in nearby appartments, all of which are within a few minutes walking distance. If you are unsure, please ask at the registration of Hotel Paradies. They have a list of all participants and are happy to help you.

While accommodation and meals including mineral water are covered, **any extras are not**:

- Please pay all drinks (except mineral water) directly after the meal.
- Please do not forget to pay your good night-drinks before you leave for the night.
- Please do not forget to pay all your extras (personal orders, …) at your hotel before departure.

If you want to rent ski equipment, please ask at the Hotel Paradies.

Scientific lectures

All lectures will take place at the *Haus der Dorfgemeinde* (City Hall).

Practical trainings

• Wednesday night, 22:00 – 23:00	Hands-on session on proteases and hydrolases. An Introduction (Paradies)
• Thursday evening, 20:00 – 23:00	Intense training in proteolytic assays: Quantitative assessments and quality control (Paradies)
• Friday evening, 19:00 – 20:00	Sample preparation in protease research (City Hall)
• Friday evening, 20:15 – 23:45	Acoustics and kinetics in enzymology, featuring InhibiTIERS (City Hall)

Social events

• Saturday afternoon	Hiking tours, excursions
• Saturday evening, Castle Haselburg	Gala Dinner & Farewell

Disclaimer

To enable and foster the unrestrained presentation and discussion of new results and ongoing research, every Winter School attendee must respect that all contents presented and discussed at the Winter School are private communications, not allowed for public use. Consequently, recording of lectures or photographing/ copying of slides or presentations is prohibited.

Wednesday, March 12

18:00 DINNER

20:00 – 21:15 Wednesday evening session: CELL MEMBRANE-ANCHORED PROTEOLYSIS

20:00 – 20:05 Welcome: Hans BRANDSTETTER, Klaudia BRIX and Thomas REINHECKEL

20:05 – 20:15 Chairs: Christoph BECKER-PAULY and Walter STÖCKER

20:15 SHEIKHOUNY Farah (Christian-Albrechts-Universität zu Kiel) *Characterization of the ectodomain shedding of transferrin receptor I*

20:30 LÖK Necati (Christian-Albrechts-Universität zu Kiel) *Regulated ectodomain shedding of the polymeric immunoglobulin receptor (pIgR) by meprin metalloproteases influences mucosal cell immunity*

20:45 SANTOS Naiá (Paris Lodron University of Salzburg) *Processing of sAPPα/β by legumain has a regulatory effect on Aβ oligomerisation*

21:00 VOGEL Lotte (University of Copenhagen) *A Biochemical Difference between HAI-2 variants causing Syndromic and NonSyndromic Congenital Sodium Diarrhea*

21:30 Mandatory hands-on session on proteases and hydrolases. An Introduction (Paradies)

Please note:

Please consider time for discussion for your talk.
Standard talks are scheduled for **15 min**, i.e. **10 min presentation plus 5 min discussion**

Thursday, March 13

09:00 – 10:25 Thursday morning session I: SUBSTRATES, INHIBITORS, ABPs & INTERACTOME

09:00 – 09:10 Chairs: Marcin POREBA, Chris OVERALL and Esther SCHÖNAUER

09:10 PLOHL Lara (Jožef Stefan Institute) *Are there physiological consequences of the cleavage of STAT6 by cathepsins?*

09:25 URŠIČ Tadej (Jožef Stefan Institute) *Are there physiological consequences of the cleavage of deiodinase I by cathepsins?*

09:40 ZOLG Samuel (University of Freiburg) *An interplay between proteases: DPP9 as regulator of non-lysosomal cathepsins*

09:55 KLAUSHOFER Rupert (Paris Lodron University of Salzburg) *An SFTI-derived FRET peptide to monitor the ligase activity of legumain*

10:10 ZABIJAK Maksymilian (Wrocław University of Science and Technology) *Novel substrate-based probes with DNA-intercalating fluorophores for caspase activity analysis in CAR-T cells*

10:25 – 11:00 COFFEE BREAK

11:00 – 12:15 Thursday morning session II

11:00 SOMMER Alexander (Technical University of Munich) *Glycosylation-dependent interaction of TIMP-1 with its newly identified receptor Amyloid Precursor Protein (APP)*

11:15 DEFANT Pauline (Paris Lodron University of Salzburg) *Identification of cytosolic legumain and its interaction partners in HEK293T cells*

11:30 METZ Melina Melissa (University of Freiburg) *A structural analysis of non-enzymatic functions of the SUMO isopeptidase USPL1*

11:45 HÖLL Anna (Paris Lodron University of Salzburg) *Production of active proprotein convertase 7 (PC7) for crystallization studies*

12:00 WICHTERLE Filip (Czech Academy of Sciences) *Structural characterization of peptidomimetics targeting fibroblast activation protein*

12:30 LUNCH

14:30 – 15:55 Thursday afternoon: TISSUE GNOSTICS Cancer session I

14:30 – 14:40 Chairs: Achim KRÜGER, Tobias DREYER and Rama KHOKHA

14:40 GÜZEL Ayse Seray (Koç University & Graduate School of Health Sciences) *MicroRNA-metalloprotease axis controls metastatic behavior in colorectal cancer*

14:55 BICKENBACH Kira (Christian-Albrechts-Universität zu Kiel) *Targeted approach to determine the impact of cancer-associated protease variants (TACAP)*

15:10 KÖNEMANN Johanna (Christian-Albrechts-Universität zu Kiel) *The Role of Meprin ß and CD99 in Colorectal Cancer Progression*

15:25 KELLY Hannah Breege (University of Oxford) *Identifying adaptor proteins involved in KIF-mediated transport of MT1-MMP in invasive cancer cells*

15:40 BATRA Jyotsna (Queensland University of Technology) *Unravelling the Snipping Effect on KLK3 SNP on Prostate Cancer Aggressiveness*

16:00 – 16:30 COFFEE BREAK

16:30 – 18:00 Thursday afternoon: TISSUE GNOSTICS Cancer session II

16:30 MAJCHRZAK Martyna (Wrocław University of Science and Technology) *Development of antibody-drug conjugates activated by prolyl oligopeptidases for breast cancer treatment*

16:45 NGUYEN Julia (Wrocław University of Science and Technology) *The analysis of proteolytic landscape pediatric acute lymphoblastic leukemia by mass cytometry-compatible activity-based probes*

17:00 KUTSCHHEIT Kira (University of Freiburg) *Neutrophils and their protease ASPRV1 in murine breast cancer*

17:15 BIELESCH Sophia (Technical University of Munich) *Effects of cytokeratin 19 on tumor progression and chemoresistance in ovarian cancer*

17:30 MÖLLER Johanna (Technical University of Munich) *Exploring TREX1 as a modulator of immune response and resistance mechanism to PARP inhibition in ovarian cancer*

17:45 REINHECKEL Thomas (University of Freiburg) *Survive or Die: An Intro to Tiers Extreme Hiking*

18:15 DINNER

20:00 – 23:00 Intense training in proteolytic assays: Quantitative assessments and quality control (Paradies)

Friday, March 14

09:00 – 10:25 Friday morning session I: PATHOGENS

09:00 – 09:10 Chairs: Charaf BENARAFA, Juhi BAGAITKAR and Jan POTEMPA

09:10 GRIN Peter (University of Bern) *Cutting outside the edge: viral protease secretion and extracellular activity*

09:25 SCHMIDT Dorothea (Constructor University Bremen) *SARS-CoV-2 spike protein affects the integrity of the intestinal barrier independent of an actual viral infection*

09:40 KUMP Ana (Jožef Stefan Institute) *Investigating subcellular localization and anti-SARS-CoV-2 activity of novel protein cathepsin L inhibitors*

09:55 VON WYTTENBACH Stefano (University of Bern) *N-terminomic analysis of African swine fever virus infected macrophages reveals novel potential substrates for the viral protease pS273R*

10:10 OZCATALKAYA Nazli Ece (Jagiellonian University) *Exploring the Mechanisms and Activity of Nazolysin, a Novel Metalloprotease from Tannerella forsythia*

10:25 – 11:00 COFFEE BREAK

11:00 – 12:00 Friday morning session II: PATHOGENS

11:00 KOWALCZUK Weronika (Jagiellonian University) *Kgp disturbs interferon signaling pathway promoting HSV-1 replication*

11:15 KSIĄŻEK Mirosław (Jagiellonian University) *The unique α2-macroglobulin (A2M) from the human periodontopathogen Porphyromonas gingivalis*

11:30 SCHÖNAUER Esther (Paris Lodron University of Salzburg) *License to unwind: How bacterial collagenases process collagen*

11:45 GEISS-FRIEDLANDER Ruth (Albert-Ludwigs-University Freiburg) *The **IPS** – what it is, what it can do for you, and what you can do for IPS*

11:50 BRIX Klaudia (Constructor University Bremen) ***ProteoCure** – what it can do for you and what can do for ProteoCure*

12:00 MAGDOLEN Viktor (TU Munich) ***The HENNER GRAEFF Foundation** – Dedication to the next generation of biomedical scientists*

12:20 LUNCH (Paradies, Laurin)

14:30 – 15:15 Friday afternoon session: Frontier Methods

14:30 – 14:35 Chairs: Klaudia BRIX

14:35 TAMILSELVAN Raja (Albert-Ludwigs-University Freiburg) *DIA-HUNTER to study proteolysis in kidney health*

14:50 ECKER Rupert (TissueGnostics & Queensland University of Technology) *Update on AI-Empowered Spatial Biology*

15:20 – 15:45 COFFEE BREAK

15:45 – 17:00 Molecular sexuality, standard orientation, and much more - a tribute to Wolfram Bode (hybrid session)

15:45 – 15:50 Chairs: Dusan TURK and Boris TURK

15:50 BRANDSTETTER Hans (Paris Lodron University of Salzburg) *The influence of scientific giants on each of us at the Winter School*

16:00 HUBER Robert (Max-Planck-Institute of Biochemistry, Martinsried/Munich) *Wolfram's protease research at the MPI*

16:10 STÖCKER Walter (University of Mainz) *The sound of science*

16:20 GOMIS-RÜTH F. Xavier (Molecular Biology Institute of Barcelona) *High standards in protease research*

16:30 POTEMPA Jan (University of Louisville & Jagiellonian University) *Wolfram in Tiers in pictures*

16:40 KRÜGER Achim, DREYER Tobias (TU Munich) *In memoriam of Wolfram, a Researcher from Lower Saxony*

16:50 STUBBS Milton (Martin-Luther-Universität Halle-Wittenberg) *Happy people make a happy lab*

18:00 DINNER

19:00 – 20:00 SAMPLE PREPARATION IN PROTEASE RESEARCH (CITY HALL)

20:15 – 23:45 ACOUSTICS AND KINETICS IN ENZYMOLOGY FEATURING 'inhibiTIERS' (CITY HALL)

Saturday, March 15

09:00 – 11:15 Saturday morning session: IMMUNITY

09:00 – 09:10 Chairs: Klaus-Peter KNOBELOCH and Ruth GEISS-FRIEDLANDER

09:10 KÖHLING Vasco (Christian-Albrechts-Universität zu Kiel) *Pathological epidermal meprin α expression drives a psoriasis-like skin disease*

09:25 BECKINGER Silje (Christian-Albrechts-Universität zu Kiel) *Blocking IL-6 trans-signaling prevents lethality in a meprin α-mediated sepsis mode*

09:35 MANDEL Marcella (University of Freiburg) *Effects of ValBoroPro treatment on the myeloid compartment in a mouse breast cancer model*

09:50 CHAKRABORTY Pallab (University of Freiburg) *Caspases regulate type-I interferon signaling through the cGAS/STING and IRF3 pathways in steady-state conditions*

10:05 – 10:30 COFFEE BREAK

10:30 FERNÁNDEZ-FERNÁNDEZ María (Institute of Biomedical Research of Barcelona) *Cathepsin D expressed by tumour-associated macrophages sustains the immunosuppressive tumour microenvironment contributing to HCC progression*

10:45 WĄDRZYK Magdalena (Wrocław University of Science and Technology) *Neutrophil subsets based on amount and the activity of neutrophil serine proteases*

11:00 MOLES FERNÁNDEZ Anna (Institute of Biomedical Research of Barcelona) *Proteolytic enzymes driving macrophage phenotypical shift during fibrotic diseases*

11:20 Hans BRANDSTETTER (Paris Lodron University of Salzburg)
Closing remarks & end of scientific sessions

12:00 LUNCH

SATURDAY AFTERNOON: HIKING, EXCURSION, ROUND TABLE DISCUSSIONS

12:00 – 17:00 'Extreme' hiking tour (lunch package will be provided)
12:00 – 17:00 Locally organized skiing tours (skiing equipment can be rented at the Frommer Alm and lunch package will be provided)
12:09 – 16:51 Public bus to Bozen (Tour of Bozen, Visit Ötzi the Iceman & more)

17:45 Shuttle bus to Castle Haselburg (Bozen)

19:00 **GALA DINNER AT THE HASELBURG**

Sunday, March 16

09:09 Public bus to Bozen main station
10:09 Public bus to Bozen main station
11:09 Public bus to Bozen main station
12:09 Public bus to Bozen main station

ABSTRACTS

Characterization of the ectodomain shedding of transferrin receptor I

Farah Sheikhouny[1], Kira Bickenbach[1], Maximilian Keller[2], Tomas Koudelka[3], Patrick Kaleja[3], Lea Stahmer[1], Fred Armbrust[1], Andreas Tholey[3], Claus Pietrzik[2], Christoph Becker-Pauly[1]

1 Institute of Biochemistry, Christian-Albrechts-University Kiel, Otto-Hahn-Platz 9, Kiel, Germany
2 Institute of Pathobiochemistry, University Medical Center of the Johannes Gutenberg-University Mainz, Langenbeckstraße 1, Mainz, Germany
3 Institute for Experimental Medicine, Christian-Albrechts-University Kiel, Niemannsweg 11, Kiel, Germany

Transferrin receptor I (TfRI) is a ubiquitously expressed type II transmembrane protein that mediates cellular iron uptake. The ectodomain shedding of TfRI within its stalk region is already described in previous studies. The serum levels of soluble TfRI (sTfRI) in patients with iron deficiency anemia (IDA) are elevated and therefore sTfRI is considered a diagnostic biomarker for this condition.

Here, we identified the metalloproteases meprin β, ADAM10 and -17 as sheddases of TfRI. Western blot analysis revealed that cleavage of TfRI by these sheddases results in the release of the extracellular transferrin-binding part of the receptor from the plasma membrane. N-terminomics identified multiple and notably protease-specific cleavage sites of TfRI by meprin β, ADAM10 and-17. Consequently, cleavage of TfRI by theses sheddases resulted in reduced levels of TfRI at the cell surface, and decreased uptake of iron-bound transferrin, measured by flow cytometry.

Additionally, retention of meprin β in the endoplasmic reticulum or Golgi *via* retention using selective hooks (RUSH) system and a co-expression with TfRI revealed a potential shedding of TfRI by meprin β already within the secretory pathway.

In vivo relevance of TfRI shedding by meprin β was assessed in Mep1b$^{-/-}$ mice, where serum levels of iron, sTfRI, transferrin, and hepcidin were significantly altered compared to wild-type controls. In comparison, no such alterations were observed in ADAM17ex/ex mice, suggesting that the observed effects can be primarily attributed to meprin β.

Considering the elevated levels of sTfRI in IDA, we further aim to investigate the potential *in vivo* role of human meprin β in the progression of this disease by applying a neoepitope-specific antibody, specifically recognizing sTfRI generated by meprin β in human sera of IDA patients.

Regulated ectodomain shedding of the polymeric immunoglobulin receptor (pIgR) by meprin metalloproteases influences mucosal cell immunity

Necati Lök, Christoph Becker-Pauly, Cynthia Bülck

Institute of Biochemistry, University of Kiel, 24118 Kiel, Germany

Meprin metalloproteases are highly expressed in mucosal epithelia, particularly in the kidney and the gut. Under chronic inflammatory conditions meprins are significantly decreased as shown in intestinal tissue of inflammatory bowel disease (IBD) patients, which implicates that these enzymes have a protective biological function for mucosal homeostasis. In the small intestine, meprin β is responsible for cleavage and detachment of the major mucus component mucin-2 (MUC2), thereby preventing bacterial overgrowth and infection. While meprin β is primarily membrane-bound, meprin α is constantly secreted but is also able to form covalently linked heterodimers with meprin β tethering meprin α to the plasma membrane. These heterodimers are responsible for the constitutive cleavage of galectin 3, which on the one hand is regulated by the microbiome and conversely modulates the bacterial composition. Thus, the constitutive cleavage of galectin-3 by meprin α/β heterodimers may play a key role in colon host-microbiome homeostasis.

Employing mass spectrometry-based N-terminomics, we also identified the polymeric immunoglobulin receptor (pIgR) as an intestinal mucosal substrate of meprin α/β. Of note, pIgR is responsible for the transcytosis of dimeric IgA from the basolateral to the apical site of the gut enterocytes, where IgA protects the gut against pathogens. To reach the gut lumen, the pIgR bound to IgA must be shed from the apical cell surface, but the protease that is responsible for this proteolytic processing remains elusive. The identified cleavage site in pIgR between Glu_{589} and Glu_{590} is perfectly in line with the cleavage specificity of meprins, which show a strong preference for negatively charged amino acids. Preliminary *in vitro* data revealed that ectodomain shedding of pIgR can be conducted by meprin α/β. Hence, we will investigate if meprin α/β are the elusive proteases important for the release of the pIgR-IgA-complex.

Processing of sAPPα/β by legumain has a regulatory effect on Aβ oligomerisation

Naiá Porã Santos[1,2], Sven Dahms[1,2], Hans Brandstetter[1,2], Elfriede Dall[1,2]

1 Department of Biosciences and Medical Biology, University of Salzburg. Hellbrunner Straße 34, 5020 Salzburg, Austria
2 Center of Tumor Biology and Immunology, University of Salzburg, Hellbrunner Straße 34, 5020 Salzburg, Austria

The selective processing of the human amyloid precursor protein (APP) by γ-secretase after β- or α-secretases defines the so-called amyloidogenic and non-amyloidogenic pathways of APP, respectively. Amongst other products, the amyloidogenic pathway results in the formation of soluble amyloid precursor protein β (sAPPβ) and amyloid-β peptides (Aβ) with different lengths and aggregation propensities. The brain deposition of Aβ peptides as aggregates is a hallmark of Alzheimer's disease. Cleavage of APP by α-secretase generates soluble amyloid precursor protein α (sAPPα) and prevents Aβ formation. Besides γ-, β- and α-secretases, legumain (δ-secretase) has been shown to cleave the ectodomain of APP at two sites in mice, one of them within the E2 domain.

We investigated the processing of recombinant sAPPα, sAPPβ and the E2 domain of APP by legumain, and the effect of their respective cleavage products on Aβ40 and Aβ42 oligomerization *in vitro*. Using mass spectrometry and Edman sequencing we identified defined cleavage sites within the three substrates with stable fragments remaining after 2h of reaction. Using fluorescence-based experiments we monitored the oligomerization of the Aβ peptides after co-incubation with intact or legumain-cleaved sAPPα, sAPPβ and E2 domain. Interestingly, intact sAPPα, sAPPβ and E2 domain reduced Aβ oligomerization in a pH-dependent manner. This effect was even stronger after cleavage by legumain. Furthermore, we found different fibrillation sensitivities of Aβ40 and Aβ42 towards the presence of processed and unprocessed APP-derived proteins. Kinetic analysis suggested that the inhibition affected at least two independent oligomerization parameters, T50 and *tau*. Taken together, our experiments show that legumain-cleaved sAPPα/β proteins led to a delay in Aβ oligomerization, which may result in a neuroprotective effect. These findings set the basis for further *in vivo* experiments to validate the observed effects.

A Biochemical Difference between HAI-2 variants causing Syndromic and Non-Syndromic Congenital Sodium Diarrhea

Maiken Jaller Jensen[1], Monika Mrackova[1], Annika Weile Nonboe[1], Emilie Tina Lembke[1], Christine Schar[2], Jan Kristian Jensen[2], Asli Silahtaroglu[1], René Villadsen[1], Lotte Katrine Vogel[1]*

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2 Aarhus University, Gustav Wieds Vej 10C, Aarhus 8000, Denmark

Biallelic mutations in the SPINT2 gene, encoding HAI-2, cause autosomal recessive Congenital Sodium Diarrhea (CSD), a rare disease, often life-threatening characterized by early-onset, persistent diarrhea with elevated levels of sodium. Several mutations in HAI-2 are known to cause syndromic CSD. A newly discovered variant in SPINT2 encoding HAI-2 Y68C, was shown to cause non-syndromic CSD in an isolated case, displaying a milder phenotype compared to patients with the syndromic form of CSD. This investigation also includes two newly discovered variants, HAI-2 Y129C and R148H causing syndromic CSD. HAI-2 has two Kunitz type inhibitor domains targeting a range of serine proteases. HAI-2 and at least one of its target proteases, prostasin, are located on the apical plasma membrane of intestinal cells, which also harbours NHE3 and ENaC, mainly responsible for enterocytes uptake of Na+ across the apical plasma membrane. All CSD-causing variants of HAI-2 were expressed with a similar protein steady state level however, none of them were able to inhibit prostasin as efficiently as wild-type HAI-2. In addition, the HAI-2 Y68C variant is unable to inhibit the serine protease, matriptase. This constituting a clear biochemical difference between the variants causing syndromic and non-syndromic CSD. We hypothesize that CSD is caused by lack of inhibitory activity of apically located HAI-2 allowing a target protease, possibly prostasin, to degrade proteins like NHE3 or ENaC who are responsible for uptake of Na-ions from the gut lumen.

Are there physiological consequences of the cleavage of STAT6 by cathepsins?

Lara Plohl[1,2], Livija Tušar[1,3], Dušan Turk[1,3]

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Studies on extralysosomal cathepsins have shown that they are upregulated in pathological conditions and are involved in many diseases, such as cancer, viral infections, and neurological conditions. Therefore, cathepsins have untapped potential as drug targets.

Our previous study (Tusar et al., 2023) analyzed 30,000 cleavage sites of 3,147 proteins in cell lysates from the endoproteolytic activity of recombinant human cathepsins K, V, B, L, S, and F using the SAPS-ESI software platform. 941 proteins were cleaved only once by only one cathepsin.

In our study, we plan to focus on these 941 protein substrates to explore the potentially new physiological roles of cysteine cathepsins. As our first protein, we chose signal transducer and activator of transcription STAT6, which also plays a predominant role in virus-mediated signaling pathways. STAT6 has a partial structure determined (PDB code 4Y5U, UniProt code P42226). STAT6 was cleaved once by cathepsin V between A594 and K595 within the PFSA↑KDLS sequence string. The phosphorylated dimer of the STAT6 core fragment binds DNA in proximity of the cleaved sequence. We hypothesize that this cleavage disrupts the dimeric structure of STAT6, and may prevent the binding of DNA, and, subsequently, its transcription. We aim to show the consequences of the cleavage of STAT6 by cathepsin V and also other cathepsins and their estimated interactions at molecular and cellular levels.

Are there physiological consequences of the cleavage of deiodinase I by cathepsins?

Tadej Uršič[1,2], Livija Tušar[1,3], Dušan Turk[1,3]

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2 Jožef Stefan International Postgraduate School, Jamova cesta 39, 1000 Ljubljana, Slovenija
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Cathepsins are primarily found in the acidic environment of the endo-/lysosomal compartments. However, they have also been shown to be functionally active outside the lysosome, and their deregulated activity has been linked to various diseases such as cancer, metabolic syndrome and activation of viruses like coronavirus and Ebola. In a previous study, they used the SAPS-ESI (Statistical Approach to Peptidyl Substrate-Enzyme Specific Interaction) software platform to analyse the proteomic data from proteolytic cleavage of cell lysates by recombinant human cathepsins K, V, B, L, S and F. The analysis identified 30,000 cleavage sites on 3,167 proteins, 941 of which were only cleaved once. We hypothesize that at least some of the 941 cleavages may be physiologically significant.

To begin, we have selected deiodinase I, an enzyme responsible for the deiodination of thyroid hormones. Deiodinase I was cleaved once by cathepsin F between W169 and V170 within the GHRW↑VTDL sequence. The cleavage site is exposed to the solvent and may cause some conformational changes, as seen in used structure (PDB code 5YAK, UniProt Q6PHW0). Our aim is to show the consequences of the cleavage of deiodinase I by cathepsin F and also other cathepsins.

An interplay between proteases: DPP9 as regulator of non-lysosomal cathepsins

Samuel Zolg[1], Daniel Vogele[2], Larissa Meyer[2], Bettina Mayer[1],Oguz Bolgi[1], Thomas Reinheckel[1] , Oliver Schilling[2], Ruth Geiss-Friedlander[1]

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2 Department of Clinical Pathology, Breisacher Str. 115a, 79106 Freiburg, University of Freiburg

Intracellular dipeptidyl peptidase 9 (DPP9) has the rare ability to cleave its substrates post proline (X-P↓-Y). This cleavage exposes a degron at the N-terminus of the substrate that is further recognized by E3 Ligases, leading to the proteasomal degradation of the substrate via the N-degron pathway. DPP9 localizes to the cytoplasm and nucleus and is integral to various cellular processes, including survival, DNA repair and targeting of miss-localized ER and mitochondria proteins.

To better understand this broad spectrum of physiological functions, we performed a N-terminomic screen for DPP9 substrates. In this screen, we identified cathepsin Z as promising candidate, as it accumulates in DPP9KO cells, and shows a corresponding peptide with a proline in 2nd position that maps to the immediate N-terminus of mature cathepsin Z. Strikingly, this proline is conserved in the mature N-termini of 11 human cathepsins.

We show that active DPP9 affects the protein steady state levels of cathepsin Z, while differences in the transcriptional levels were excluded. Subsequently, we designed peptides corresponding to the mature N-termini of cathepsins. These peptides were further analyzed in in-vitro cleavage of DPP9 by mass-spectrometry, and in competition-based assays to map their affinity to the DPP9 substrate channel. By using reporter constructs, in which the first 22-amino acids of different mature cathepsins were fused to GFP, we can further show, that these cathepsin N-termini are transferable, DPP9 dependent degrons. Furthermore, close proximity between endogenous DPP9 and cathepsin Z as well as cathepsin V could be demonstrated and triggered by the induction of lysosomal membrane permeabilization (LMP). From this evidence, we conclude that DPP9 regulates the stability of non-lysosomal cathepsins to prevent their otherwise toxic accumulation in the cytoplasm and nucleus.

An SFTI-derived FRET peptide to monitor the ligase activity of legumain

Rupert Klaushofer [1,2], Hans Brandstetter [1,2], Elfriede Dall [1,2]

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2 Center of Tumor Biology and Immunology, University of Salzburg, Hellbrunner Straße 34, 5020 Salzburg, Austria

The cysteine protease legumain typically localizes to the endolysosomal system, where it is an important player in the immune system. Notably, besides its well characterized protease activity, legumain harbors an additional ligase activity. Both activities reside in the same active site and require substrates harboring an asparagine residue at the P1 position. Although both activities overlap, they show distinct pH preferences: legumain's protease activity is favored under acidic conditions, while the ligase activity requires neutral pH environments. Interestingly, translocation of legumain into compartments with neutral pH was observed under pathophysiological conditions, for example in the context of Alzheimer's disease or in different types of cancer. This observation raises questions about the role of legumain's ligase activity in diseases. However, tools to study legumain's ligase activity in real time are still missing.

Therefore, we set out to develop a FRET assay to monitor the ligase activity of legumain. To that end we use the sunflower trypsin inhibitor precursor peptide I (SFTI-I) as a model substrate. Using a positional scanning approach we developed an SFTI peptide sequence, optimized for cyclic product formation. Subsequently, we incorporated three different FRET pairs into the optimized peptide. Employing MALDI-ToF analysis, we monitored the cyclic product's formation. Importantly, the FRET peptides largely differ in the amount of cyclic peptide formed. Kinetic analysis reveals a K_M in the micromolar range for one of the FRET peptides. Structural analysis indicates a relatively small initial distance between the FRET pair in the non-cyclized peptide. Thus, further optimization of the FRET peptide still remains a challenge. In a next step, we aim to increase the distance of the FRET donor and acceptor pair by increasing the peptides flexibility via introducing single amino acid exchanges.

Novel substrate-based probes with DNA-intercalating fluorophores for caspase activity analysis in CAR-T cells

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Caspases play a crucial role in programmed cell death. Due to their involvement in various biological processes, they have proven to be important biomarkers for many diseases. Consequently, a wide range of methods for detecting and analyzing proteolytic activity have been developed over the years. One such approach involves substrate-based probes – chemical compounds consisting of an enzyme-selective, cleavable peptide sequence and a reporter group – typically a fluorophore. Fluorescence can be activated through structural restoration, FRET disruption or aggregation. Recently, novel probes containing DNA-binding dyes have been developed. In these probes, the fluorescence signal increases upon binding to DNA, which is possible only after bond cleavage. However, to the best of our knowledge, the structure of only one commercially available caspase-3/7 detection reagent has been published.

CAR-T cell therapy is an innovative strategy for cancer treatment. Preliminary studies have revealed robust activation of caspases following antigen-dependent CAR-T cell stimulation, suggesting that caspases could serve as biomarkers of CAR-T cell efficacy. This highlights the growing demand for new chemical tools to examine caspase activity.

Here, we present the design of novel substrate-based probes equipped with DNA-intercalating fluorophores. By replacing the peptide sequence with those derived from HyCoSuL data, the selectivity towards different proteases can be tailored. This, in turn, could allow for the identification of the most suitable caspases as potential biomarkers of CAR-T cell activity. The proposed strategies include enabling intercalation through structural restoration of the 3,8-diaminophenanthridine moiety or introducing a self-immolative linker which prevents fluorophore's DNA-binding properties via intramolecular charge transfer quenching.

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Glycosylation-dependent interaction of TIMP-1 with its newly identified receptor Amyloid Precursor Protein (APP)

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Glycosylation is a major post-translational modification of proteins, impacting on various cellular and molecular processes, including receptor-ligand interactions. Tissue Inhibitor of Metalloproteinases-1 (TIMP-1) is a glycosylated multifunctional protein known for its inhibitory effect on Metalloproteinases. Recently, TIMP-1 has also been identified to act as a cytokine affecting various cell types. This non-canonical cytokine function of TIMP-1 leads to activation of monocytes *via* Amyloid Precursor Protein (APP) interaction. TIMP-1 harbors two glycosylation sites at positions N30 and N78, but the impact on its interaction with APP is unknown. Recently, we showed that the C-terminal domain of TIMP-1 was necessary to induce a TIMP-1/APP interaction-dependent pro-inflammatory cytokine profile in monocytes. This study investigates the effect of macroheterogeneous glycosylation of TIMP-1 (i.e. double-, single-, and non-glycosylated TIMP-1) on its binding to APP using *in silico* molecular docking algorithms and subsequent wet lab validation. First, we developed a pipeline enabling us to assess glycosylation-dependent ligand-receptor interactions implementing the HADDOCK2.4-derived docking results, leading to a contact map elucidating the participation of residues in the TIMP-1/APP interaction. Notably, the occupation of N30 resulted in a shift of the predicted TIMP-1/APP interaction towards the C-terminal domain of TIMP-1. Exposure of the monocyte cell line THP-1 to different recombinant glycosylation variants of TIMP-1 revealed that non- or N78 glycosylated TIMP-1 was most potent in the induction of the pro-inflammatory cytokine profile, whereas occupation of N30 attenuated this effect. This is the first demonstration that glycan-modification in the N-terminal domain of TIMP-1 gradually modulates the C-terminal domain-dependent biological activity of TIMP-1 *via* APP. This highlights the importance of considering glycosylation as a modulator of multifunctional proteins.

Identification of cytosolic legumain and its interaction partners in HEK293T cells

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Primarily located to the endolysosomal system, overexpression and translocation of the cysteine protease legumain to the cytosol or extracellular space, was associated with a variety of severe pathologies including cancer and Alzheimer's disease. Remarkably, the active form of legumain, called 'asparaginyl-endopeptidase' (AEP), was shown to cleave substrates in the neutral environment of the cytosol even though AEP is conformationally unstable at pH > 6. In the last few years, we could show that the existence of active legumain in the extra-lysosomal space is possible if stabilized by intra- or intermolecular complex formation, e.g. with the prodomain, integrin avb3 or protein inhibitors. Within this study we aim to investigate whether legumain is present in the cytosol of non-cancerous HEK293T cells and to further identify potential binding and/or stabilization partners.

To that end, we performed co-immunoprecipitaiton assay (CoIPs) using cytosolic proteins of HEK293T cellsand antibodies that were raised against three different forms of legumain: (i) the inactive zymogen (prolegumain), (ii) the active AEP form, or (iii) the legumain stabilization and activity modulation (LSAM) domain. Samples were measured with mass spectrometry (DIA LC/MS) and spectra were assigned peptides using DIA-NN software in Fragpipe. Using this experimental setup, we provide evidence, that prolegumain is present in the cytosol of HEK293T cells. Furthermore, we identified several potential legumain interaction partners, which will be further validated in upcoming experiments. Taken together, this study sets the basis for further experiments investigating legumain's interactions in the cytosol of cancerous and non-cancerous human cells.

A structural analysis of non-enzymatic functions of the SUMO isopeptidase USPL1

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The ubiquitin-specific protease (USP) family's SUMO isopeptidase USPL1 is a low-abundance protein that has emerged as a crucial component of Cajal bodies (CB), a highly dynamic nuclear structure involved in various RNA processing events. Cajal bodies are associated with spliceosomal small nuclear (snRNPs) and small nucleolar RNPs (snoRNPs) biogenesis, telomere maintenance, and histone mRNA processing. Thus far it has been found that USPL1 depletion alters the localization of Coilin and impairs cell proliferation. These effects are not dependent on the enzymatic activity of USPL1.

USPL1 further seems to interact with at least two of the CB-associated proteins: interactor of little elongation complex ELL subunit 1 (ICE1) and the RNA polymerase II (RNAPII) associated LEC. Thus, USPL1 appears involved in snRNA transcription and RNA biogenesis. It has already been established that USPL1 depletion leads to reduced RNAPII-transcribed snRNAs and compromised downstream processes such as snRNP assembly and pre-mRNA splicing. Furthermore, it has been found that USPL1 associates directly with U snRNA loci and colocalizes with LEC components. These findings suggest a critical role for USPL1 in RNAPII-mediated snRNA transcription and highlight its potential interaction with the LEC, in regulating gene expression within Cajal bodies.

We hypothesize that USPL1 interacts with the Little Elongation Complex (LEC), specifically through its association with ELL. To investigate this hypothesis, we utilized mouse embryonic fibroblasts (MEFs) with a conditional USPL1 knockout system (USPL1 fl/fl Rosa26Cre ERT) and tamoxifen treatment, alongside USPL1 fl/fl MEFs as controls. Additionally, we extended our study to embryonic stem cells (ESCs) with the same USPL1 fl/fl Rosa26CreERT genotype. Our ongoing experiments aim to elucidate the functional relationship between USPL1 and LEC components, focusing on their roles in transcriptional regulation within Cajal bodies.

Production of active proprotein convertase 7 (PC7) for crystallization studies

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Proprotein convertase 7 (PC7) is the most ancestral and yet the least investigated member of the furin-like proprotein convertase (PC) family. PC7 mediates the maturation of several disease-associated substrates and is an interesting target to treat certain cancer types, infections, anxiety or cardiovascular diseases. Structure-based drug design typically requires mg-amounts of active protein for X-ray crystallographic investigations. Expression of the soluble PC7 ectodomain, however, results in secretion of the auto-inhibited protease still associated with the prodomain (refer to as proPC7*). Thus, proPC7* is not suitable for co-crystallization with inhibitors or enzyme kinetic measurements.

In this talk we describe the large-scale expression of proPC7* using a stably transfected HEK293S cell line. We obtained up to 5 mg recombinant proPC7* per litre of cell culture medium. To obtain active PC7, we established an *in-vitro* activation procedure using limited proteolysis. Thereby the PC7-prodomain is degraded by thermolysin while active PC7 remains stable under these conditions. This activation step is followed by inhibitor affinity chromatography to purify only active PC7 from the activation mixture containing thermolysin, degradation products and inactive PC7 species. This protein preparation was used to setup initial crystallization screens obtaining a promising condition with needle-like crystals. We could improve the size of PC7 crystals in fine screens to max. 10 x 500 µm. The diffraction quality of the crystals will be evaluated at a microfocus synchrotron beamline. In addition, we will test ligand soaking with PC7 crystals to resolve structures of the protease in complex with inhibitors. These structures could further guide *in-silico* based approaches to identify novel PC7-inhibiting scaffolds.

Structural characterization of peptidomimetics targeting fibroblast activation protein

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Fibroblast activation protein (FAP) is a membrane-bound serine protease that has emerged as a promising tumor marker. FAP is overexpressed in the tumor stroma of most carcinomas, and has been linked to promoting angiogenesis, tumor cell invasion, and immunosuppression. Despite great interest in FAP targeting, limited structural information on FAP–inhibitor complexes has hampered further elaboration of inhibitor structures through rational design. In our recent work, we conducted a structure–activity relationship study to explore the chemical space in the P1′ and P2′ positions and developed a new class of peptidomimetic inhibitors bearing an α-ketoamide warhead. Besides other lead-like properties, the compound I22AP446 outperformed the most potent inhibitor published to that date.

To gain insight into the binding mode of the α-ketoamide derivative, we determined a crystal structure of FAP in complex with I22AP446 at 1.75 Å resolution revealing key interaction features between the inhibitor and the enzyme. We thus present the first reported crystal structure of FAP bound to a peptidomimetic. Our findings provide a basis for structure-guided modifications of our lead compound and will fuel the development of FAP inhibitors with fine-tuned properties.

MicroRNA-metalloprotease axis controls metastatic behavior in colorectal cancer

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Colorectal cancer (CRC) is one of the most common cancer types worldwide. In spite of advances in the diagnosis and treatment of the disease, a better understanding of the molecular mechanisms of CRC formation, spread, stress, and drug resistance mechanisms is required. In search for new markers of CRC, we discovered a microRNA that was downregulated in CRC tumors compared to corresponding non-tumoral tissues in a large cohort of cancer patients. The role of the microRNA in CRC tumor formation, progression, cellular stress, and death responses was studied using KRAS mutant CRC cellular models. The microRNA deficient cells showed higher cell migration and invasion while cell growth, autophagy, and drug resistance phenotypes were unaffected. Furthermore, genes that were targeted by the microRNA and involved cell migration phenotype were investigated to enlighten the molecular mechanisms. We discovered a metalloprotease as a direct target of the CRC-related microRNA. Knock-out and rescue experiments showed that the metalloprotease is a rate-limiting target of the microRNA and it is responsible for the observed increased cell migration and invasion phenotype. Metastasis-related protease targets of this less studied metalloprotease and its connection to CRC metastasis are under investigation in our lab.

Targeted approach to determine the impact of cancer-associated protease variants (TACAP)

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Proteolytic activity is critically involved in several steps of cancer progression, from tumour initiation to metastasis. Proteases, such as MMPs, KLKs, cathepsins, and metalloproteases, for example degrade barriers by cleaving extracellular matrix components, basement membranes, and cell adhesion molecules. Genomic studies have identified numerous single nucleotide variants (SNVs) in protease genes that may impact protease function. To elucidate the role of proteases in cancer and as potential drug targets, it is particularly important to consider SNVs affecting the active site of proteases, thereby influencing cleavage specificity, substrate processing and thus cancer cell behaviour. To facilitate systematic studies, we have developed a **T**argeted **A**pproach to determine the impact of **C**ancer-**A**ssociated **P**rotease variants (TACAP). Starting with the semi-automated identification of potential specificity-modulating SNVs, TACAP comprises mass spectrometry-based cleavage specificity profiling and substrate identification, localisation and inhibitor studies, followed by functional analyses investigating tumor cell biological roles of variants of interest.

To demonstrate feasibility we analysed the meprin β R238Q variant. This amino acid exchange at S1' position leads to a loss of meprin β's characteristic cleavage preference for acidic amino acids at P1' position, accompanied with changes in substrate pool and inhibitor affinity compared to meprin β wildtype (wt). Employing N-terminomics and Western Blot analyses, differential processing of e.g. CD99, IL-6R, syndecan-1, fibronectin and collagen IV, were detected for meprin β R238Q compared to the wt enzyme. Cellular transendothelial migration and invasion assays as well as tumor spheroid invasion assays using meprin β variants expressing glioma cells, showed an increased pro-migratory effect for meprin β R238Q compared to meprin β wt.

TACAP is easily adaptable to other cancer-relevant proteases, to perform a systematic, comprehensive in vitro analysis of cancer-associated SNVs. This can assist to predict efficacies of protease inhibitors and to design effective variant-specific inhibitors for personalised anti-cancer therapy.

The Role of Meprin ß and CD99 in Colorectal Cancer Progression

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Meprin β, a membrane-bound zinc-dependent metalloprotease, is primarily expressed on enterocytes and has been shown to influence colorectal cancer cell proliferation and migration. CD99, a cancer-associated cell adhesion protein, is cleaved by meprin β within conserved regions, which influences transendothelial cell migration, thereby fostering a tumor-promoting environment.

We functionally characterized the high meprin β expressing adenocarcinoma cell lines Colo320 and NCI-H716 and compared wt and CRISPR-Cas9-generated MEP1B knockout cells. Notably, both Colo320 and NCI-H716 cells deficient for meprin β exhibited significantly increased CD99 levels and showed reduced transendothelial cell migration. This suggests a potential role of meprin β and CD99 in enhancing metastasis.

The activity and substrate specificity of meprin ß is influenced by its shedding from the cell surface through other metalloproteases, such as ADAM10 and -17. We observed only minor ectodomain shedding of meprin β in NCI-H716 cells compared to Colo 320 wild-type cells. The soluble form of meprin β is crucial for intestinal mucus detachment and maintaining host-microbiome homeostasis, which is dysregulated in pathological conditions. Of note, meprin β shedding in mouse intestine under germ-free conditions is fully abolished, highlighting microbiome-dependent regulation.

Our aim is to further characterize colorectal cancer cell lines regarding meprin β and CD99 expression, proteolytic processing, proliferation, and migration. Additionally, we will explore how bacteria and the microbiome influence meprin β shedding. To this end, we plan to treat cells with different bacteria to investigate regulation of the shedding mechanism.

Identifying adaptor proteins involved in KIF-mediated transport of MT1-MMP in invasive cancer cells

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Cancer invasion and metastasis represent major challenges in cancer treatment, and greatly increase the mortality of the disease. For cancer cells to invade tissue, they must first create a path for migration by degrading the extracellular matrix (ECM), which acts as a physical barrier to migrating cells. A crucial enzyme involved in this process is MT1-MMP (membrane-type 1 matrix metalloproteinase). MT1-MMP localises to the leading edge of invasive cancer cells. This localisation step is required for cells to degrade the ECM during invasion, and is achieved by targeted transport of MT1-MMP-containing vesicles along microtubules by kinesin superfamily proteins (KIFs). Our group previously showed that KIF3A and KIF13A transport MT1-MMP-containing vesicles to focal adhesions while KIF9v1 competes to transport these vesicles to other parts of the cell membrane, away from the leading-edge. However, it is still unclear how KIFs specifically recognise MT1-MMP-containing vesicles.

KIFs do not recognise MT1-MMP-containing vesicles directly. An adaptor protein (or multiple adaptors) likely mediates the recognition of vesicles by KIFs. These adaptors, therefore, must play a crucial role in the leading-edge localisation of MT1-MMP. We aim to identify adaptor proteins mediating the interaction of MT1-MMP-containing vesicles with KIF3A, KIF13A, and KIF9 and their roles in dynamic vesicle transport during cellular invasion. This presentation describes a BioID2 and miniTurbo (mTb)-mediated proximity labelling approach for identifying MT1-MMP, KIF3A, KIF13A, and KIF9v1 interactions with potential adaptor proteins. Our data showed that BioID2 or mTb-mediated proximity labelling is a viable approach to identifying adaptor proteins during dynamic vesicle transport in invasive cancer cells. I will also discuss our initial analyses of LC-MS/MS data. Identifying the adaptor proteins involved in MT1-MMP vesicle transport will increase understanding of MT1-MMP-mediated cellular invasion and may contribute to identifying novel therapeutic strategies to prevent cancer cell invasion and metastasis.

Unravelling the Snipping Effect on KLK3 SNP on Prostate Cancer Aggressiveness

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Genome-wide association studies (GWAS) have identified the 19q13.3 KLK locus as a key genetic region associated with prostate cancer risk. Within this locus, the rs17632542 SNP (c.536 T > C; Ile163Thr substitution in PSA) has been linked to reduced prostate cancer susceptibility. PSA (prostate-specific antigen), a glycoprotein enzyme encoded by KLK3, plays a critical role in semen liquefaction and is widely used as a biomarker for prostate cancer detection. However, the functional consequences of this SNP on PSA activity and prostate cancer progression remain unclear.

In this study, we investigated the impact of the rs17632542 variant using an in-vivo mouse model and biochemical assays. Mice injected with prostate cancer cells expressing the 'Thr' PSA variant developed significantly smaller subcutaneous tumours, suggesting a protective effect against tumour initiation. However, metastatic models revealed that the 'Thr' PSA variant exhibited enhanced osteolytic activity, indicating increased metastatic potential. Biochemical characterisation demonstrated that the 'Thr' variant had markedly reduced proteolytic activity, correlating with tumour burden differences.

Clinical analysis of three independent prostate cancer cohorts confirmed that this SNP is associated with an increased risk of aggressive disease and prostate cancer-specific mortality. Furthermore, carriers of this allele exhibited lower serum total PSA levels and a higher free-to-total PSA ratio, which may contribute to delayed biopsy decisions and later-stage diagnosis.

These findings highlight the dual role of the rs17632542 PSA variant in prostate cancer progression, underscoring its potential impact on screening strategies and clinical outcomes.

Development of antibody-drug conjugates activated by prolyl oligopeptidases for breast cancer treatment

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Breast cancer remains the most common malignancy among women worldwide, with its diverse subtypes presenting unique challenges that often require tailored therapeutic strategies. Current treatments, such as chemotherapy and surgery, are frequently associated with severe side effects. Antibody-drug conjugates (ADCs) have emerged as an innovative approach, combining the targeted specificity of monoclonal antibodies with the potent cytotoxicity of chemotherapeutic agents.

The efficacy of ADCs partially relies on peptide linkers, that upon hydrolysis by proteases release the drug within cancer cells, minimizing off-target effects. Traditional linkers, such as Val-Cit and Gly-Gly-Phe-Gly, are widely used but are cleaved by multiple proteases (e.g., cathepsins, elastase), which reduces their precision. Our research investigates the use of an alternative protease as a therapeutic target for ADC activation, supported by literature evidence highlighting the potential of the S9 family proteases.

To achieve this, we have synthesized a Hybrid Combinatorial Substrate Library (HyCoSuL) with the proline at the P1 position, and utilized it for profiling the substrate specificity of prolyl oligopeptidase (PREP) at the P4-P2 positions. This enzyme is abundantly expressed across various tumor types, making it a prime candidate for ADC development. Additionally, we have synthesized P1 libraries incorporating proline derivatives adjacent to the scissile bond. Leveraging chemical tools and kinetic data, we have designed peptide-drug conjugates (PDCs) with exceptional selectivity and activity for the enzyme PREP. Their substantial cytotoxicity in BT-474 breast cancer cells highlights their potential for further studies.

This research underscores the potential of HyCoSuL-guided ADC optimization to provide a more effective and less toxic alternative to traditional treatments.

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The analysis of proteolytic landscape pediatric acute lymphoblastic leukemia by mass cytometry-compatible activity-based probes

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Acute lymphoblastic leukemia (ALL) is the most common malignant tumor in children, originating in the bone marrow, the crucial site for producing and maturing blood cells such as erythrocytes, platelets, lymphocytes, and granulocytes. In ALL, malignant cells replace healthy hematopoietic cells, disrupting bone marrow function and blood cell production. Diagnostic procedures typically include bone marrow aspiration and detailed immunological and cytogenetic profiling. However, approximately 10% of pediatric patients exhibit resistance to standard treatments, despite an overall cure rate exceeding 90%.

This project aims to unravel the mechanisms of treatment resistance in ALL by investigating the activity of proteolytic enzymes implicated in disease progression, including MALT1, cathepsins B, L, S, legumain, and the 20S proteasome. Traditional methods, such as fluorescent activity probes, are limited to assessing individual enzymes and cannot simultaneously evaluate multiple enzymes at the single-cell level. To overcome this limitation, we propose developing activity probes incorporating stable lanthanide isotopes. These probes will be applied in mass cytometry, an advanced technique capable of analyzing over 50 biomarkers simultaneously in single cells. Mass cytometry is widely utilized in immunology and oncology for identification of cellular subsets, functional states, and protein expression levels. By enhancing mass cytometry with lanthanide-tagged probes, we aim to simultaneously monitor multiple proteolytic enzyme activities in individual leukemia cells. This comprehensive profiling will provide better insights into the biochemical pathways underlying drug resistance in pediatric ALL. Understanding these mechanisms is crucial for developing targeted therapies that could enhance treatment efficacy.

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Neutrophils and their protease ASPRV1 in murine breast cancer

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Measured by incidence, breast cancer is the second most common cancer type worldwide. In that context, recent research suggested that neutrophils potentially exert a more substantial influence on cancer pathogenesis than previously understood. Especially neutrophil proteases play crucial roles in many physiological as well as pathological processes. Thus, this research aims to shed light on a fairly unknown neutrophil protease in breast cancer, with a primary focus on its biochemical attributes and its functional significance.

A proteomic screen could identify a rather understudied protease in CD11b-enriched splenocytes of breast cancer-bearing mice, namely retroviral-like aspartic protease 1 (ASPRV1). In this screen, the levels of ASPRV1 were even higher than those of the common myeloid marker S100A8. To biochemically explore this protease, a reintroduction of ASPRV1 translation start mutants into an ASPRV1 knockout SCF ER-Hoxb8 cell line demonstrated that the translation start might be misannotated leading to an exclusion of a putative transmembrane domain, potentially resulting in cytoplasmic location. Finally, to investigate ASPRV1 functionally in a cancer context, an in vivo 4T1 breast cancer model was used. Here, we could reveal that ASPRV1 showed higher levels in CD11b+Ly6G+ bone marrow cells of breast cancer-bearing mice. Additionally, lung and tumour tissues of these mice indicated elevated levels of ASPRV1 when compared to their healthy counterparts.

Further studies will explore additional biochemical and functional aspects of ASPRV1 as well as its potential as a novel therapeutic target in breast cancer.

Effects of cytokeratin 19 on tumor progression and chemoresistance in ovarian cancer

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Ovarian carcinoma remains one of the deadliest malignancies in women, with high-grade serous ovarian carcinoma (HGSOC) accounting for more than 70% of cases. The major clinical challenges of HGSOC continue to be late diagnosis and therapy resistance, with over 80% of patients experiencing recurrence within five years due to limited responsiveness to standard chemotherapy. Addressing these challenges requires a deeper understanding of HGSOC biology to identify novel diagnostic markers and therapeutic targets.

Kallikrein-related peptidases (KLKs) have been implicated in the progression of multiple cancer types. In HGSOC, the upregulation of KLK 4-7 correlates with poor overall and disease-free survival, though the precise mechanisms remain unclear. Our study demonstrates that KLK 4-7 overexpression induces the upregulation of cytokeratin 19 (CK19) alongside their proteolytic activity. CK19, an intermediate filament, plays a key role in maintaining cellular integrity and polarity. Although CK19 is overexpressed in several tumor types, its specific contribution to ovarian cancer progression remains poorly understood.

To elucidate the role of CK19 in HGSOC progression, we established stable CK19-overexpressing ovarian cancer cell lines and assessed their impact on proliferation, migration, adhesion, and chemoresistance. Compared to vector controls, CK19-overexpressing cells exhibited increased motility and adhesion, accompanied by alterations in epithelialmesenchymal transition (EMT) markers, including E-cadherin and vimentin. Furthermore, CK19 overexpression conferred enhanced resistance to first-line chemotherapeutics used in ovarian cancer, without significantly altering baseline proliferation.

Our findings indicate that CK19 overexpression in HGSOC may contribute to poor prognosis by promoting tumor cell motility and chemoresistance. These results suggest that targeting CK19 could represent a promising strategy for adjuvant therapeutic interventions in ovarian cancer.

Exploring TREX1 as a modulator of immune response and resistance mechanism to PARP inhibition in ovarian cancer

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Ovarian cancer remains a highly lethal disease, with a 5-year survival rate below 50%. The primary challenges lay in early detection and frequent relapses. Poly-ADP-Ribose-Polymerase (PARP) inhibitors have significantly improved outcomes for patients with homologous repair deficiencies (HRD) by exploiting synthetic lethality to selectively target cancer cells with DNA repair defects.

PARP inhibitors not only induce double-strand breaks but also stimulate immune responses through the cGAS-STING pathway. This pathway enhances anti-tumor immunity by triggering the release of chemokines like CXCL10 and CCL5 upon accumulation of cytosolic DNA. However, immune responses need to be carefully regulated to avoid harmful autoimmunity. On the one hand, caspases responding to cellular stress can directly degrade components of the cGAS-STING pathway and therefore impact the effectiveness of PARP inhibitors. On the other hand, the STING-activating stimuli can be degraded upstream. The 3′→5′ exonuclease TREX1 is a critical negative regulator of the cGAS-STING pathway, degrading cytosolic DNA to prevent excessive inflammation. In cancer, its function may enable tumor cells to evade immune detection by decreasing the amounts of infiltrating lymphocytes. Therefore, TREX1's activity might impair the effectiveness of immune therapies, such as PARP inhibitors, by impeding cGAS-STING activation.

We show that PARP inhibitors like Olaparib upregulate TREX1 expression in a time- and dose-dependent manner, both *in vivo* and *in vitro*, while also depending on the HRD status. This upregulation may negatively affect immune responses by limiting the release of chemokines like CXCL10 through decreased cGAS-STING activity.

The project investigates the role of TREX1 in immune modulation and its contribution to resistance against PARP inhibitors. The modulation of TREX1 could impact the expression and availability of tumor-suppressive chemokines and the immune response in ovarian cancer. Targeting TREX1 to regulate the cGAS-STING pathway may provide a novel strategy to overcome resistance to PARP inhibitors in ovarian cancer.

Cutting outside the edge: viral protease secretion and extracellular activity

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Viral proteases are key drug targets due to their essential roles in cleaving viral polyproteins and host substrates to facilitate viral replication and evasion of antiviral host responses. We recently discovered that the main 3C-like protease ($3CL^{pro}$) of severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) is unconventionally secreted from infected cells through gasdermin-D and gasdermin-E pores. These gasdermin pores are activated by caspases and regulated by $3CL^{pro}$ to balance $3CL^{pro}$ secretion and throttle antiviral pyroptosis. In the extracellular environment, $3CL^{pro}$ cleaves and inactivates interferon-lambda 1 to facilitate viral evasion of innate immune responses. Moreover, $3CL^{pro}$ retains activity in serum despite the abundance of endogenous protease inhibitors, and blocks platelet activation and aggregation. Recent evidence suggests that extracellular $3CL^{pro}$ also binds to the cell surface or enters into naïve cells independently of virions or viral infection. Together these findings highlight the pleiotropic nature of SARS-CoV-2 $3CL^{pro}$ and improve our understanding of the complex biology of viral proteases.

SARS-CoV-2 spike protein affects the integrity of the intestinal barrier independent of an actual viral infection

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COVID-19 patients often suffer from gastrointestinal tract complications. It is therefore important to study the effects of SARS-CoV-2 interactions with the intestinal mucosa, which are not yet fully characterised.

We have previously shown that vesicular stomatitis virus particles pseudo-typed with the SARS-CoV-2 spike protein were able to enter CaCo-2 cells, which represent intestinal enterocytes, whereas the HT29-MTX cell line, as a surrogate for goblet cells, was not susceptible. Although proteolytic processing of the spike protein was limited, the interaction of pseudo-typed particles with the apical surfaces of the Caco-2 and HT29-MTX cell monolayers induced profound structural changes in the cytoskeleton. This suggests that priming of the spike protein before or upon SARS-CoV-2 entry into enterocyte and goblet cells may not even be necessary to induce gastrointestinal tract complications. Hence, we hypothesize that the interaction of enterocytes and goblet cells with SARS-CoV-2 spike protein alone can affect the integrity of the intestinal barrier. To test this proposal, we will examine the barrier integrity of monocultures and organotypic co-cultures of Caco-2 and HT29-MTX cells, respectively, after incubation with different spike protein variants from the apical side. Any cytoskeletal and cell-cell junction changes will be characterized by cell biological and biochemical approaches. A better understanding of cellular changes evoked by the contact of spike protein with cell surfaces may help to understand why SARS-CoV-2 patients develop gastrointestinal tract complications.

Investigating subcellular localization and anti-SARS-CoV-2 activity of novel protein cathepsin L inhibitors

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The SARS-CoV-2 pandemic highlighted that for development of effective therapeutics understanding viral infection mechanisms is mandatory. Viruses can enter cells through multiple pathways, with the endosomal route being critical for SARS-CoV-2. This entry depends on host cell proteases, particularly cathepsin L, which cleaves the viral spike protein within endosomes to facilitate membrane fusion and viral genome release. While chemical cathepsin inhibitors show therapeutic potential, their broad distribution and off-target effects limit their clinical use. We aimed to develop a more targeted approach by leveraging the potent inhibitory action of the endogenous cathepsin L inhibitor p41 and generating protein constructs targeted to specific subcellular compartments to increase their potency and efficiency in halting viral replication. We designed multiple GFP-tagged p41-based constructs with varying targeting sequences and linkers that specifically localize to endosomes, endoplasmic reticulum (ER), and cytoplasm. Confocal microscopy analyses of cell lines co-transfected with p41-encoding and appropriate reporter protein-encoding plasmids revealed distinct localization patterns of the constructs. Our findings highlighted the crucial role of linker sequences between the localization signal-transmembrane domain and the thyroglobulin inhibitory domain in achieving proper subcellular targeting. SARS-CoV-2 pseudovirus infection assays demonstrated a significant reduction in infection rates when constructs were targeted to endosomal compartments. These findings advance our understanding of virus-host cell interactions and provide valuable insights for developing targeted antiviral strategies. The implications of our study go beyond SARS-CoV-2 and can be applied to other viruses that rely on cathepsin L-dependent entry pathways, such as Ebola.

N-terminomic analysis of African swine fever virus infected macrophages reveals novel potential substrates for the viral protease pS273R

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African swine fever virus (ASFV) is a large, double-stranded DNA virus, which causes African swine fever (ASF), a devastating disease of domestic and wild pigs with up to 100% mortality. Currently, there are no commercial vaccines or antiviral therapies against ASFV leading to socioeconomic disruption in many countries worldwide. ASFV mainly infects macrophages in pig and wild boar and is transmitted through feed, contact, *Ornithodoros spp.* ticks, and infected meat products. The ASFV viral protease pS273R is a SUMO-1-like cysteine protease required in the cleavage and assembly of polyproteins in ASFV virions and is required for viral replication. pS273R is thought to have additional functions during infection in modulating host immune signaling. Overexpression studies of pS273R in vitro have shown the potential effects pS273R on pathogen recognition receptor signaling.

In our project, we have generated a recombinant pS273R and a proteolytic inactive mutant. We used the viral polyprotein pp62 and SUMO-1/2 substrates to confirm the proteolytic activity in vitro as well as its dose-dependent activity. Next, we infected primary porcine macrophages with the virulent ASFV Armenia 2008 strain. Cell lysates of infected and mock macrophages were collected at 5 and 30h post infection, labelled with tandem mass tags, and analyzed by mass spectrometry. Among the neo-N-terminal peptides, previously known cleavage sites of the viral polyprotein pp62 and pp220 were identified. Importantly, we identified a novel potential viral protein substrate and several host substrates cleaved at the GG/X consensus sequence. Additionally, many new N-termini with an aspartic acid at the P1 position were detected at the late timepoint of infection suggesting increased caspase activity. To validate the host protein targets of pS273R found in infected macrophages, macrophage lysates were incubated with active or inactive recombinant pS273R and analyzed by mass spectrometry. The functional consequences of the cleavage of viral and host protein substrates of pS273R on the virus life cycle and pathogenesis are under investigation.

Exploring the Mechanisms and Activity of Nazolysin, a Novel Metalloprotease from *Tannerella forsythia*

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Periodontal disease is one of the most prevalent infectious inflammatory conditions affecting teeth-supporting tissues, with proteolytic enzymes playing a critical role in the disease pathogenesis. In this study, we identified and characterized a new protease from the M43 family, named Nazolysin, identified in the re-sequenced genome of *Tannerella forsythia* ATCC 43037. The conserved KLIKK motif at the enzyme C-terminus classifies it as another KLIKK protease encoded in the *T. forsythia* genome.

Full-length Nazolysin consists of 499 amino acids, including a signal peptide, a unique for KLIK proteases catalytic domain of the M43 family of metalloproteases, and a C-terminal domain characteristic of proteins secreted via type 9 secretion system (T9SS). To investigate its properties, Nazolysin was expressed in *Escherichia coli* as a GST-fusion protein and purified as a 54.4 kDa recombinant enzyme. Spontaneous activation of recombinant Nazolysin involved the cleavage of 24-amino acid N-terminal profragment, maintaining the proenzyme's latency through a cysteine-switch mechanism independent of calcium ions. This mechanism was experimentally confirmed using the directed mutagenesis approach. A Cys27Ala variant of Nazolysin transformed into the mature ~36 kDa active enzyme through autoproteolytic cleavage. N-terminal sequencing of autocleavage products revealed that Nazolysin, unlike other M43 proteases, prefers aromatic residues at the P1' position and degrades various physiological proteins relevant to development of periodontal disease.

The enzymatic activity of Nazolysin was assessed using FITC-casein as a substrate and found to be inhibited by 1,10-phenanthroline and DTT but not by EDTA. Additionally, activity was inhibited by divalent cations such as Ca^{2+}, Zn^{2+}, and Mn^{2+}, but not by Mg^{2+}. Interestingly, Nazolysin was inhibited by Potempin N, the endogenous *T. forsythia* protease inhibitor encoded by the ORF directly downstream of the Nazolysin gene.

In conclusion, the preliminary characterization of Nazolysin highlights its unique features, justifying further structural and functional studies to elucidate its role in the pathophysiology of *T. forsythia* in periodontitis.

Kgp disturbs interferon signaling pathway promoting HSV-1 replication

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Periodontitis (PD) is a chronic inflammatory disease of the gingiva with a high prevalence. Clinical reports indicate the significant role of PD in the development of comorbidities, including *Herpesviridae* infections, but the molecular basis of this phenomenon has not been described. Possible explanations include modification of mucosal membranes and subversion of the antiviral response of epithelial cells. Therefore, the aim of this study was to determine the effect of *Porphyromonas gingivalis* infection, a bacterium that is a key etiological factor in PD, on the development of viral infections. We uncovered a novel molecular mechanism by which the global interferon-dependent antiviral response is tailored by a P. *gingivalis* cysteine protease - Kgp. Using gingival keratinocytes and the human gingival model, we showed that lysin-specific gingipain promotes the propagation of HSV-1 and enhances virus penetration into deeper layers of the gingival tissue. These results extend our knowledge of the mechanisms underlying mixed infections and may provide a basis for considering PD as a gateway to viral infection.

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The unique α2-macroglobulin (A2M) from the human periodontopathogen *Porphyromonas gingivalis*

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α2-macroglobulins (A2M) are a family of large inhibitors (>180 kDa) that inhibit a wide range of proteases using a unique trapping mechanism. The best-studied A2M, the human one, is a tetramer with a dimer as the functional unit. Hydrolysis of the free and unstructured loop called bait region in hA2M by the target protease leads to significant structural changes within the inhibitor molecule, leading to protease encapsulation. The protease trapped in the cage remains intact and active towards small substrates (<5-10 kDa). In stark contrast, monomeric (m) A2M barely inhibit proteases, and the key step in the interaction with the protease is not encapsulation but the formation of a covalent protease-inhibitor linkage.

Here, we show that A2M from the human periodontopathogen *Porphyromonas gingivalis* (PgA2M) was active in its monomeric form, but its inhibition efficiency was similar to that of hA2M. PgA2M inhibited prey proteases with an SI of 1.5–2 and an association constant higher than 10^6 $M^{-1}s^{-1}$. In contrast to all known mA2Ms, PgA2M does not require the formation of a covalent complex with a proteolytic enzyme for inhibition. Moreover, PgA2M does not even possess the conserved CXEQ motif, which is responsible for forming a hyperreactive thioester bond. However, PgA2M forms inhibitory complexes stable for even 48 h at 37°C. PgA2M prevents the trapped protease from hydrolyzing even 3.5 kDa peptide substrates. The solution of spatial structures using cryogenic electron microscopy provided the answer to the unique properties of PgA2M. Hydrolysis within the bait region of native PgA2M leads to significant structural changes, forming a compact, symmetric cage inside which the prey protease is located. Very narrow tunnels leading to the cage's interior explain the inhibition of proteases by PgA2M observed even on peptide substrates.

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License to unwind: How bacterial collagenases process collagen

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Collagens form the resilient scaffold of the mammalian extracellular matrix. Only idiosyncratic proteases can process collagen because of its tight triple-helical fold and high content of imino acids. Among these few are the bacterial collagenases of the metalloprotease family M9 which can efficiently degrade triple-helical collagen into small peptides. They are secreted by several pathogenic *Clostridium*, *Bacillus*, *Spirochaetes*, and *Vibrio* species as virulence factors. Yet, their mechanism of action is not well understood.

Based on biochemical and mutational studies, we show that collagenase G (ColG) from *Hathewaya histolytica* recognizes and processes collagen substrates differently depending on their nature (fibrillar *vs*. soluble collagen). Based on the substrate type distinct dynamic interactions between the activator and peptidase domain are required. Using biochemical and circular dichroism studies, we identify the presumed non-catalytic activator domain as the single-domain triple helicase that unwinds collagen locally, transiently and reversibly, enabling its subsequent cleavage by the peptidase domain. This unwinding mechanism differs markedly from the one employed by human matrix metalloproteinases (MMPs). This discovery discloses a new avenue for the development of highly selective inhibitors of bacterial collagenases.

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DIA-HUNTER to study proteolysis in kidney health

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The kidney glomeruli are essential for waste filtration and fluid balance in the body. Damage to the slit diaphragm, a critical component of the glomeruli, impairs kidney function and leads to chronic kidney diseases (CKD). While structural changes in the glomeruli have traditionally been attributed to transcriptional and translational alterations, emerging evidence highlights the pivotal role of proteolysis—particularly in cytoskeletal and cell adhesion proteins—in driving disease progression (Rinschen et al., 2017).

To investigate the proteolytic events underlying slit diaphragm effacement, we will employ discovery proteomics strategies, including N-terminomics, with High-Efficiency Undecanal-based N-Termini Enrichment (HUNTER). We have optimized the HUNTER protocol to improve the sensitivity and coverage of proteolytic cleavage site detection in complex biological matrices such as glomeruli. Furthermore, we integrated Data-Independent Acquisition (DIA) with HUNTER, leveraging its superior sensitivity and reproducibility over traditional Data-Dependent Acquisition (DDA)-MS methods.

Benchmarking experiments using a triple-organism proteome mix demonstrate that the DIA-HUNTER workflow significantly enhances N-termini coverage and data completeness compared to DDA-MS. To streamline the analysis of semi-specific database searches, we have developed a Python/R-based pipeline compatible with FragPipe, enabling automated data pre-processing, quality control, differential analysis, and visualization of proteolytic events.In addition to DIA-based terminomics, we will employ complementary structural studies, including LiP-MS and complexome profiling with Blue-Native MS, to explore how proteolysis impacts slit diaphragm integrity and podocyte function. Together, with these integrative proteomics strategies we aim to unravel the proteolytic mechanisms underlying kidney disease progression, offering new insights and potential therapeutic avenues.

Update on AI-Empowerd Spatial Biology

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INTRODUCTION: While flow cytometry has been available for researchers and clinicians for decades to perform functional analyses on single cells and determine cellular phenotypes of large cell populations in blood, technologies to perform a similar analysis in situ – ie. in the tissue, the actual localization of most immune responses – are relatively new.

METHODS: Our research teams at TissueGnostics and Queensland University of Technology have joined forces to combine TissueGnostics' existing tissue cytometry technology platform and established knowhow with innovative AI solutions to establish The Virtual Histopathologist. This represents a tissue cytometry platform that allows to quantify immune responses where they happen – in the tissue.

RESULTS: Tissue Cytometry permits to determine the in-situ phenotype of individual cells as well as histological entities, like glands, vessels or tumor foci. Applications include but are not limited to the exploration of immune responses in situ and the tumor microenvironment and/or the spatial organization of cellular subpopulations. Earlier attempts to analyse single cells in tissue have mostly been subject to visual estimation, or – at best – to manual counting for decades. To better understand the function of inflammatory cells in tumor development, type and number of inflammatory cells and their proximity to glandular/tumor structures have to be analyzed in-situ and correlated with disease state. Using TissueFAXS™ Cytometry the time-consuming and error-prone human evaluation of stained histological sections can be approached with an observer-independent and reproducible technology platform, offering a high degree of automation, paired with user interaction at relevant points of the analytical workflow (Fig. 1).

Machine & Deep Learning are essential methodologies in contemporary research in general and for image analysis in particular. We will present preliminary data from the Virtual Histopathologist project on prostate cancer classification.

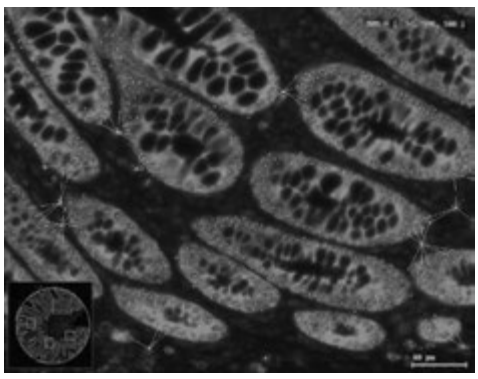

Fig.1: Spatial immune-phenotyping in-situ incl. neighbourhood analysis. CD8+/PD1+ T-cells in the vicinity of tumor cells.

DISCUSSION & CONCLUSIONS: The TissueFAXS Cytometry platform incorporates Machine & Deep Learning algorithms. It can do end-point assays as well as live-cell imaging and time-kinetic experiments to measure enzyme activity. It also promotes tissue cytometry to a new level of quality, where complex cellular interactions, intracellular expression profiles and signal transduction cascades can be addressed on the single-cell level but still in histological context, empowering precision diagnostics.

Pathological epidermal meprin α expression drives a psoriasis-like skin disease

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Meprin α is a zinc-dependent metalloprotease, with a cleavage preference for negatively charged amino acids around the scissile bond, expressed by keratinocytes in the *stratum basale* of the epidermis. In inflammatory skin diseases, like psoriasis vulgaris and Netherton syndrome, epidermal meprin α expression is increased and mislocalized into the upper more differentiated layers of the epidermis.

To investigate whether reported epidermal alterations in meprin α expression might be causative for inflammatory skin diseases, we generated the tamoxifen-inducible mouse C57BL/6J-KRT5tm1.1-CreERT2;ROSA26-Stop-Mep1a (K5Mα) to mimic epidermal expression of pathology-associated meprin α levels. After atopic induction of the epidermal meprin α overexpression, the animals developed within six days a step-wise pathological skin phenotype progressing from hyperproliferation-induced acanthosis to an inflammation-associated hyper- and parakeratosis, accompanied with massive leukocyte infiltration. Subsequent, mass spectrometry analyses revealed progressive alterations in the abundance of proteins that regulate epidermal barrier defect responses. Specifically filtering Terminal amine isotopic labeling of substrate (TAILS) results, revealed the Na^+/K^+-ATPase subunit β (Atp1b1) and SPARC-related modular calcium-binding protein 1 (SMOC1) as putative substrates. Moreover, the phenotype during the first 3 days was further characterized.

Blocking IL-6 trans-signaling prevents lethality in a meprin α-mediated sepsis model

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The substrate pool of the metalloprotease meprin α comprises key regulators of inflammatory processes, including interleukin-6 (IL-6) and its receptor (IL-6R). IL-6 can act via two main signaling pathways, the classic- and trans-signaling, both require signal transduction via the transmembrane co-receptor gp130. IL-6 trans-signaling is relevant in pathological and chronic inflammation. The serum concentrations of IL-6 are greatly increased in septic patients compared to healthy patients. Numerous therapeutic biologicals clinically target the IL-6 signaling cascade in both acute and chronic inflammatory diseases, however, these inhibitors only block both IL-6 signaling pathways so far.

We have generated an inducible cre-based mouse model (KRT5^{CreERT2};Rosa26$^{Mep1A-HA}$, short K5Mα) to mimic pathology-associated meprin α expression in the skin. Our model develops within three weeks a lethal sepsis-like systemic inflammatory syndrome. Phenotype progression correlates with increasing levels of IL-6 in the serum. In order to examine the pathological relevance of IL-6 signaling, especially trans-signaling, we crossed KRT5^{CreERT2}; Rosa26$^{Mep1A-HA}$ mice with mice that constitutively overexpress the soluble fusion protein gp130Fc (short K5Mα sgp130Fc), to block the proinflammatory IL-6 trans-signaling but not the classic IL-6 signaling. This therapeutic mouse model develops inflammation-associated characteristics that have already been described for the K5Mα model, but to a much lesser extent and with a slower progression. Interestingly, K5Mα sgp130Fc mice do not develop a lethal systemic inflammatory syndrome.

These findings indicate that pathological meprin α expression *in vivo* impacts IL-6 trans-signaling, thereby enhancing inflammatory processes, which represents a crucial factor for developing the fatal phenotype in our mouse model.

Effects of ValBoroPro treatment on the myeloid compartment in a mouse breast cancer model

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ValBoroPro (VbP) is an inhibitor of the Dipeptidyl peptidase IV family. It has previously been reported that treatment with VbP leads to cell death in multiple cell lines derived from the monocyte-macrophage lineage. Due to the inhibition of DPP8/9, treatment with VbP leads to the activation of the NLRP1 inflammasome, resulting in the activation of pyroptosis, characterized by the activation of caspase-1, processing and release of IL-1β and IL-18, and Gasdermin-D cleavage.

To further investigate the cell populations affected by VbP, *ex vivo* treatment of healthy bone marrow cells was performed, revealing that VbP treatment specifically targets monocytic cells which are characterized by high Ly6C expression, while $Ly6G^+$ cells remain unaffected. *In vitro* studies using the ER-Hoxb8 cell system supported this observation, showing that during differentiation, VbP specifically affects Ly6C-expressing cells, while undifferentiated progenitor cells and fully differentiated cells remain unaffected.

Several studies described a beneficial effect of DPP8/9 inhibition in different cancer models. In line, VbP treatment also reduced the tumor size in the 4T1-tumor cell mouse model for breast cancer. However, the exact mechanisms by which VbP affects tumor progression, and the tumor immune cell response are unknown. Yet monocytic premature cells which have immunosuppressive function, also called myeloid derived suppressor cells, are among the cells strongly affected by VbP-induced pyroptosis. Loss of this cell population could benefit the anti-tumor immune response and be an underlying reason for the anti-tumor effect of VbP. To comprehensively analyze the immune reaction in the tumor tissue, multiplex imaging of tumor samples was performed using the Akoya Phenocycler system, enabling the staining and single-cell resolution analysis of >30 biomarkers. This high-dimensional approach allows for an extensive characterization of the tumor and its microenvironment, offering deeper insights into the effects of VbP treatment on the myeloid compartment in this cancer model.

Caspases regulate type-I interferon signaling through the cGAS/STING and IRF3 pathways in steady-state conditions

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Caspases, a group of cysteine-aspartate proteases, regulate apoptosis by cleaving a variety of substrates. Cell surface 'death receptors' receptors (extrinsic) or the release of mitochondrial cytochrome *c* (intrinsic) can trigger apoptosis by activating caspase-8 or -9 (initiator caspases), which further activate caspase-3 and -7 (effector caspases). Recent work has demonstrated that the mitochondrial apoptosis pathway can be active in a sub-lethal manner, characterized by low caspase activity. This low-level activity maintains diverse signaling pathways without causing cell death. However, it is still unclear how individual caspases and their substrates contribute to sub-lethal signaling.

Here we demonstrate that apoptotic caspases, mainly caspase-3 and -9, are active in steady-state conditions. This appears to occur through the proteolytic cleavage of a small portion of activated cGAS. The basal caspase activity downregulates type-I interferon signaling through the cGAS-STING-IRF3 signaling pathway. Blocking caspase activity by deletion of any essential component of the mitochondrial apoptosis pathway enhances the type-I interferon response. We propose that such low-level caspase activity is important to fine-tune the innate immune response, which may help to maintain the balance between immune activation and undesired inflammation.

Cathepsin D expressed by tumour-associated macrophages sustains the immunosuppressive tumour microenvironment contributing to HCC progression

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Background and Aims: Hepatocellular carcinoma (HCC) is the 3rd leading cause of cancer-related deaths worldwide. Tumour-associated macrophages (TAMs) secrete factors, including cathepsins, that promote tumour malignancy and resistance in breast and pancreatic cancer; however, their contribution to HCC development is unknown. Thus, this study aimed to investigate Cathepsin D (CtsD) as an immunosuppressive molecule in TAMs during HCC.

Method: HCC was established in CtsDF/F or CtsDΔMyel (CtsD-myeloid cell KO) mice by DEN injection. Pepstatin A (PepstA) treatment was initiated 20 weeks post-DEN. Tumour number and histopathological features were evaluated at the endpoint. Serum AFP was measured by ELISA. Gene expression and immune cells were evaluated by RT-qPCR and IHP. Macrophage polarization and autophagic state were assessed by RT-qPCR and WB, respectively. Growth with CtsDF/F or CtsDΔMyel BMDM conditioned media (CM) or mixed BMDM-HepG2 spheroids was monitored over 14 days. ScRNAseq analysis was performed using GSE151530.

Results: ScRNAseq and CtsD IHP demonstrated high CtsD expression in liver macrophages from HCC patients. Growth of HepG2 spheroids was reduced in the presence of CtsDΔMyel BMDM or their CM. In vitro analysis of CtsDΔMyel macrophages revealed decreased expression of markers associated with TAMs accompanied by defective autophagy, suggesting a phenotypical shift. Next, CtsD deletion in macrophages led to reduced HCC development in a DEN-carcinotoxic model, as evidenced by decreased tumour number, circulating AFP levels, and liver expression of HCC markers. Additionally, CtsDΔMyel mice showed reduced cell proliferation. Similar results were obtained when inhibiting CtsD pharmacologically using PepstA. Although there were no differences in the number of liver macrophages, CtsDΔMyel livers exhibited decreased expression of immunosuppressive markers and cytokines. Finally, CtsDΔMyel livers showed an enhanced anti-tumoral T cell response and decreased T-cell exhaustion.

Conclusion: CtsD deletion in macrophages modulates the tumour microenvironment, enhancing the anti-tumoral immune response, reducing tumour growth and slowing down HCC progression.

Neutrophil subsets based on amount and the activity of neutrophil serine proteases

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Neutrophils are the most abundant leukocytes in the blood and play a key role in the innate immune system by enabling a rapid and nonspecific response to infections. Upon activation, they employ diverse mechanisms for host defense, including the release of serine proteases (NSPs) such as neutrophil elastase, proteinase 3, cathepsin G, and neutrophil serine protease 4. These enzymes, primarily stored in azurophilic granules in their active form, are crucial for pathogen destruction. Beyond their role in host defense, neutrophils contribute to various pathological conditions, indicating their functional heterogeneity. Our data suggest that neutrophils not only perform multiple functions but also exhibit heterogeneity based on the amount and activity of NSPs. This underscores the significant role of these enzymes in neutrophil function and their potential implications for health and disease.

In our study, neutrophil subsets were defined using fluorescently labeled antibodies targeting both surface markers and intracellular NSPs. Additionally, we employed internally-quenched fluorescent substrates to assess enzymatic activity of NSPs. To ensure specificity, we designed probes for NSPs containing a selective peptide sequence with a donor-acceptor fluorophore pair positioned at opposite ends of the peptide chain. This approach enables the precise identification of neutrophil subpopulations based on NSP activity. Importantly, it preserves enzyme function, preventing alterations in cell phenotype.

Using these tools, we demonstrated that neutrophil heterogeneity depends not only on NSPs quantity but also on its enzymatic activity. This finding highlights the complexity of neutrophil functions and suggests that variations in NSPs activity may play a critical role in immune responses, including phagocytosis and disease mechanisms.

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Proteolytic enzymes driving macrophage phenotypical shift during fibrotic diseases

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Background and Aims: Liver fibrosis is caused by an excessive accumulation of extracellular matrix (ECM) proteins. Macrophages are important effectors for ECM remodelling through recycling of the ECM within acidic compartments and can contribute to liver fibrosis resolution. Proteases, such as cathepsins, are essential for lysosomal proteolytic activity; however, their contribution to ECM remodelling within the macrophages is unknown. Thus, the aim of this study was to investigate the proteolytic and degradative signalling pathways associated to macrophages during liver fibrosis.

Method: A macrophage-specific cathepsin D knockout ($CtsD^{\Delta Myel}$) mouse was generated by crossing LysMCre with CtsD-floxed mice. Fibrosis was induced via CCl_4 or bile duct ligation and assessed using histological, molecular and proteomic techniques. Reversion by collagen degradation (R-CHP staining) was assessed 72h post-challenge in a 4-week CCl_4 model. Macrophage polarization and proteolytic secretome was assessed by RT-qPCR and protease array, respectively. Collagen degradation and endocytosis was determined by FACS in liver macrophages. Single-cell RNA sequencing analysis was performed using GSE136103 dataset.

Results: ScRNAseq analysis and CtsD IHP demonstrated high expression of CtsD in liver macrophages from cirrhotic patients. Next, $CtsD^{\Delta Myel}$ mouse was validated by FACS and WB in KC and dual IHP (F4/80-CtsD) in liver. CtsD deletion in macrophages enhanced liver fibrosis with enriched matrisome proteomic signatures in chronic CCl_4 and BDL models. Analysis of KC isolated from 72h-CCl_4-treated livers demonstrated significantly lower expression of markers associated with resolutive macrophages (CD206, TREM-2 and TGF-β) and defective proteolytic secretome profile in $CtsD^{\Delta Myel}$ KC. In addition, $CtsD^{\Delta Myel}$ KC displayed defective proteolytic processing of collagen I without impairment of the Endo180 receptor-mediated endocytosis demonstrated by FACS. Analysis of CtsD macrophage subclusters in control and cirrhotic human livers, confirmed cirrhotic CtsD-expressing subclusters were differentially enriched in ECM degradation and organization signalling pathways. In addition, it revealed a decrease in the number of CtsD expressing macrophage subclusters in cirrhotic livers, which could contribute to inadequate ECM recycling, perpetuating fibrosis and hampering resolution. Indeed, $CtsD^{\Delta Myel}$ mouse was unable to remodel collagen *in vivo* when subjected to a fibrosis reversion model determined by both percentage of HP and fluorescent intensity of collagen hybridizing peptide (CHP) binding to liver tissue.

Conclusion: CtsD is essential in regulating the collagenolytic activity of macrophages during liver fibrosis and is part of a novel and currently unknown degradome landscape of restorative macrophages.

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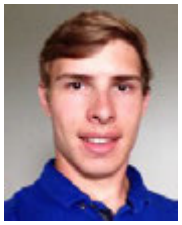

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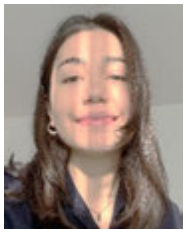

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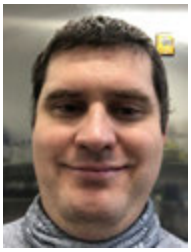

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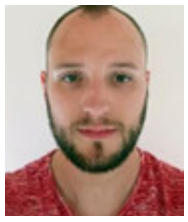

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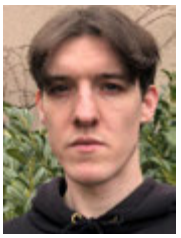

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