
# SFB –Retreat 2016

## Osnabrück 14. - 15.07.

UOS SUMMER SCHOOL

***Biomembranes & Cellular Microcompartments***

# Time table

| | | |
|---|---|---|
| 14.7. | 09:00-09:15 | Registration |
| | 09:15 – 9:30 | Opening Remarks |
| | | Chair: E. Schüßler |
| | 09:30 – 9:45 | Regulation of aminoacid permeases by lateral segregation and turnover (A. Elting) |
| | 09:45 –10:00 | Structure of the TSC2 N-terminus provides insight into TSC complex assembly and tuberous sclerosis pathogenesis (R. Zech) |
| | 10:00 - 10:15 | Analysis of the contribution of distinct protein domains to the interactions between cardiac ECM components in *Drosophila melanogaster* (Y. Post) |
| | 10:15 – 10:30 | Coffee Break |
| | | Chair: J. Rose |
| | *10:30 -11:10* | *Gating in the $K^+$ transporter KtrAB is mediated by an elongated α-helix of KtrB that protrudes into KtrA (I. Hänelt)* |
| | 11:10 – 11:25 | Quantitation of *Escherichia coli* phospholipids by MALDI TOF/TOF mass spectrometry (R. Scheffer) |
| | 11:25 - 11:40 | The role of the Cpx-system within the envelope stress systems in *Escherichia coli* analyzed by SRM and localization studies (E. Ćudić) |
| | 11:40 - 11:55 | Unraveling the working mechanism of a tumor suppressor lipid (S. Dadsena) |
| | 12:00 – 13:00 | Lunch |
| | | Chair: K. Fitzian |
| | *13:00 - 14:00* | *Assembly, structure, and function of a bacterial nanosyringe (S. Wagner)* |
| | 14:00 - 14:15 | Molecular analysis of the adhesion-invasion microcompartment formed during interaction of *Salmonella enterica* with polarized epithelial cells (S. Geißelsöder) |
| | 14:15 - 14:30 | Analyzing Membrane Ruffle Formation during Invasion of *Salmonella* Typhimurium in Polarized Epithelial Cells with Correlative Light and Electron Microscopy (CLEM) (C. Kommnick) |
| | 14:30 - 15:00 | Coffee Break |
| | | Chair: J. Schoppe |
| | *15:00 - 15:40* | *Genetic analysis of GET pathway components in Arabidopsis thaliana (C. Grefen)* |

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|---|---|---|
| | 15:40 - 15:55 | Nuclear ROXY interactions with TGA transcription factors (L. Poleratzki) |
| | 15:55 - 16:10 | In *situ* single cell pull-down for probing stability and stoichiometry of cytosolic protein complexes (T. Wedeking) |
| | 16:10 - 16:25 | Coffee Break |
| | | Chair: J. Flesch |
| | *16:25 - 17:05* | *The Role of Ubiquitin in Host-Pathogen Encounters (C. Behrends)* |
| | 17:05 – 18:35 | Poster Session 1 (odd numbers) |
| | 18:35 | Dinner and come together |
| 15.7. | | Chair: S. Geißelsöder |
| | *9:30 – 12:00* | *PhD Workshop "Career entry"*<br>*Dr. Carsten Roller and Christian Lange* |
| | 12:00 – 13:00 | Lunch |
| | | Chair: P. Hansmann |
| | *13:00 - 13:40* | *Proteomics of ADP-ribosylation (I. Matić)* |
| | 13:40 - 15:10 | Poster Session 2 (even numbers) and Coffee break |
| | | Chair: M. Rierola |
| | *15:10 - 15:50* | *Autophagosome transport via an endocytic adaptor mediates neuronal complexity and prevents neurodegeneration (N. Kononeko)* |
| | 15:50- 16:05 | Analysis of Mechanism of Autophagosome-Vacuole Fusion in Yeast (J. Gao) |
| | 16:05 – 16:20 | Dissecting the behaviour of RNA binding proteins in neuronal stress granules with super-resolution microscopy (B. Niewidok) |
| | 16:20 – 16:35 | Single vesicle recording in hippocampal 'xenapses' reveals diffusional dispersion of SV proteins after fusion (J. Trahe) |
| | 16:35 – 16:50 | Closing Remarks |

# Abstracts invited speaker

**Gating in the $K^+$ transporter KtrAB is mediated by an elongated α-helix of KtrB that protrudes into KtrA**

Inga Hänelt

Institute of Biochemistry, Biocentrum of the Goethe University, Frankfurt

Potassium ions ($K^+$) are the main cations in living cells from all biological kingdoms of life. Within the cell, $K^+$ is responsible for a plethora of tasks, including the regulation of pH homeostasis, cell growth, maintenance of osmolarity and cell volume, movement and electrical signaling. Consequently, $K^+$ translocation across the membrane is a highly regulated process, facilitated by integral membrane proteins.

KtrAB is a $Na^+$- and ATP-dependent bacterial $K^+$ uptake complex. The complex is composed of the $K^+$-translocating subunit KtrB and the regulatory subunit KtrA. The translocation of cations is, among other factors, regulated by nucleotide binding-induced conformational changes in the KtrA ring. However, the mechanism by which conformational changes within the so-called RCK (regulator of $K^+$ conduction) domain KtrA control the activation of the pore domain KtrB remained elusive.

I will present data that reveal the direct regulation of the pore domain by ligand-induced conformational changes in the RCK domain and that suggest a yet unknown homologous but modified mechanism of gating of one-pore RCK channels.

**Assembly, structure, and function of a bacterial nanosyringe**

Samuel Wagner

Interfakultäres Institut für Mikrobiologie und Infektionsmedizin; Eberhard Karls Universität Tübingen

Many bacteria that live in contact with eukaryotic hosts, whether as symbionts or as pathogens, have evolved mechanisms to manipulate host cell behavior to their benefit. One such mechanism, the type III secretion system, is employed by Gram-negative bacterial species to inject effector proteins into host cells. This function is reflected by the overall shape of the machinery, which resembles a molecular syringe. Despite the simplicity of the concept, the type III secretion system is one of the most complex known bacterial nanomachines, incorporating one to more than hundred copies of up to twenty different proteins into a multi-MDa transmembrane complex. I will review what we recently learned about the orchestration of the assembly of type III secretion systems, the stoichiometry and structural interactions of its components, and the coordination of the secretion of substrates.

**Genetic analysis of GET pathway components in *Arabidopsis thaliana***

Christopher Grefen

Zentrum für Molekularbiologie der Pflanzen; Eberhard Karls Universität Tübingen

SNARE proteins catalyse the final step in membrane fusion with their cognate SNARE partners through tight interaction via their cytosolic N-terminal domains. Their C-terminal membrane anchor pulls the opposite membranes together, overcoming the strong dehydration forces associated with the lipid bilayer and ultimately leading to fusion of the two membranes. This important function is prerequisite to a multitude of vital cellular functions such as trafficking of cargo to the outside of the cell or adding additional membrane material to the plasma membrane for expansion.

In yeast and mammals integration of tail-anchored (TA) membrane proteins seems to be facilitated via cytosolic components in an ATP-dependent fashion. This 'Guided-Entry of TA proteins' (GET) pathway has not been described in plants where research focusses on the import pathways into chloroplasts and mitochondria. How the abundance of SNARE and other important TA proteins are integrated into the ER membrane in plants is currently entirely unknown.

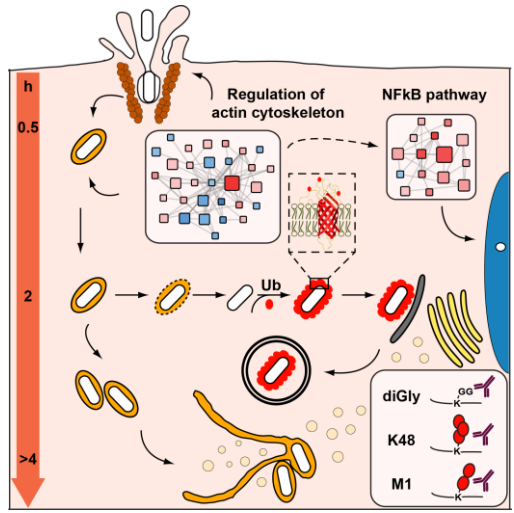
We have identified the candidates involved in a putative GET pathway of Arabidopsis. Our data show that plants have evolved multiple orthologues of specific GET pathway components, albeit in a compartment-specific manner. In contrast, others seem to be absent in plants suggesting differences in the protein insertion mechanism or the development of alternative pathways. The latter hypothesis is supported by highly specific rather than general phenotypes associated with loss-of-function lines highlighting the plant's need for backup insertion mechanisms.

## The Role of Ubiquitin in Host-Pathogen Encounters

Christian Behrends

Institute of Biochemistry II, Medical School Goethe University Frankfurt

Ubiquitination serves as a critical signal in the host immune response to infection. Many pathogens have evolved strategies to exploit the ubiquitin (Ub) system to promote their own survival through a complex interplay between host defense machinery and bacterial virulence factors. Recently, we reported dynamic changes in the global ubiquitinome of host epithelial cells and invading pathogen in response to *Salmonella Typhimurium* infection. The most significant alterations in the host ubiquitinome concerned components of the actin cytoskeleton, NFκB and autophagy pathways and the Ub and RHO GTPase systems. Specifically, infection-induced ubiquitination promoted CDC42 activity and linear ubiquitin chain formation, both being required for NFκB activation. Conversely, the bacterial ubiquitinome exhibited extensive ubiquitination of various effectors and several outer-membrane proteins. Moreover, we revealed that bacterial Ub-modifying enzymes modulate a unique subset of host targets, affecting different stages of *Salmonella* infection.




*Reference*
*Fiskin E, Bionda T, Dikic I, Behrends C. Global Analysis of Host and Bacterial Ubiquitinome in Response to Salmonella Typhimurium Infection. Mol Cell. 2016 May 18. pii: S1097-2765(16)30065-X*

**Proteomics of ADP-ribosylation**

Ivan Matić

Max Planck Institute for Biology of Ageing, Cologne

Mass spectrometry has established itself as a key technology for unbiased and direct analysis of PTMs. Recent breakthroughs in proteomic technology are not only constantly extending the list of known PTMs, but have also made it possible to map and quantify PTMs on a system-wide scale. Currently, tens of thousands of phosphorylation, acetylation and ubiquitination sites can be almost routinely profiled in a single experiment. This has greatly accelerated progress in many fields of research by fuelling studies of the functional significance of these modifications at an unprecedented scale. However, studies on the regulation of cellular processes by other biologically important PTMs have been hampered by a lack of data on substrates and their modification sites. This has been especially problematic for ADP-ribosylation, which are particularly complex and labile modifications. We are developing and implementing methods for unbiased (on all possible amino acids) proteomic investigations of ADP-ribosylation by advanced mass spectrometry, including high resolution ETD. This has allowed us to discover novel amino acid specificities of ADP-ribosylation in vivo.

**Autophagosome transport via an endocytic adaptor mediates neuronal complexity and prevents neurodegeneration**

Natalia Kononenko

CECAD; University Cologne

Autophagosomes are thought to primarily mediate turnover of cytoplasmic proteins or entire organelles to provide nutrients and eliminate damaged proteins. In neurons, autophagosomes form in distal axons and are trafficked retrogradely to eventually fuse with lysosomes in the soma. Although defective neuronal autophagy is associated with neuronal cell death and neurodegeneration the function of neuronal autophagosomes has remained largely enigmatic.

Here we show that conditional loss of the endocytic adaptor AP-2μ in neurons leads to defective retrograde transport of autophagosomes that contain active TrkB receptors and convey brain-derived neurotrophic factor (BDNF) signals. Retrograde transport of TrkB-containing autophagosomes is mediated by the association of AP-2 with the ubiquitin-like microtubule-associated protein 1A/1B light chain 3 (LC3) protein and with p150Glued/ dynactin to promote neuronal complexity and to prevent early-onset neurodegeneration in vivo. These data establish a mechanism for retrograde axonal transport of BDNF/ TrkB-containing autophagosomes via an endocytosis-independent function of AP-2 and suggest a causative link between autophagy and BDNF/ TrkB signalling in brain function.

**BioPerspectives – the first employment agreement**

Dr. Carsten Roller, VBIO

The major topics of the presentation:

- Statistics

  *The majority of biologists finds employment in:*
  - *25% R&D*
  - *20% Administration and public institutions*
  - *18% Teaching (universities)*
  - *12% Public Health*

  *Also in:*
  - *Pharmaceutical industry*
  - *Laboratories*
  - *Associations*
  - *Whole sale*
  - *Trade*

  *Rarely in:*
  - *Consulting*
  - *Museums*
  - *Zoological and botanical gardens*
- Orientation
  - *The hardest part is to get „the foot in the door"*
  - *Practical experience is a good way to get the „foot in the door"*
  - *If you apply in industry you should a have a very good idea of what you want and why you want it*
  - *Job interviews in companies have a very different quality from interviews at universities etc.*
- Examples for various careers
  - Academia, research institutes
  - Teaching, education
  - „Around nature"
  - Lab, diagnostics
  - Communications
  - Industry
- Questions and answers

  Which field of specialty is interesting for you?

  If you decide to stay in research, are you really cut out for it, do you have original ideas?

  If you decide to leave R&D, what is your other strong suit?

  Do you like to communicate, to write, to teach, to administrate, to organise, to travel, to sell… ?

  How willing are you to move around?

How important is financial security?

How much responsibility do you want to have/do you need?

Do you prefer to work self-employed?

Do you want a family?

How much can you compromise?

Dr. Roller is head of education&career at VBIO – German Life Science Association (Verband Biologie, Biowissenschaften und Biomedizin in Deutschland e.V.).

He studied Biology at the TU Munich and graduated in microbiology. After postdocs in the field of environmental toxicology and later on bioweapons he is engaged in life science lobbying since over 17 years.

VBIO is Germany's largest association for the life sciences. It combines about 30 000 members which represent individual members, life science organisations, companies and other institutions. VBIO aims to be a strong, unifying voice for scientists, teachers, schools, universities, industries, administrations, research institutions and free-lancers. Our members represent the entire spectrum of life sciences, from the molecular and cellular to the organismic and ecological level, also including the biomedical field.

http://www.vbio.de/jobs

http://www.vbio.de/firmen

www.jobvector.com

www.biologie-bilingual.de

**Career entry – the first employment agreement**

Christian Lange, VAA Führungskräfte Chemie

The major topics of the presentation:

- Your rights inside the application procedure
- Which questions are allowed during a job interview?
- The essential topics of an employment agreement
- How can the employer change the conditions of employment, e.g. relocation
- Benefits and compensation, salary at beginning of your career

Mr. Lange is lawyer for labor law. He studied in Osnabrück and is engaged in labour law since over 13 years. He advices the members of VAA (Verband Angestellter Akademiker). VAA is an association of employed academics and executives, which represents its members in the material, legal and social interests by giving them advice on all aspects of working life and providing better labour conditions.

Members of VAA belong to a wide range of occupational groups, usually scientists, engineers, managers, economists and lawyers – all working in responsible positions for their respective companies.

# PhD Talks Abstracts

## Regulation of aminoacid permeases by lateral segregation and turnover

J. V. Busto, F. Spira, D. Haase, A. Elting, J. Kuhlmann, M. Schäfer-Herte, N. Müller, C. Schuberth, R. Wedlich-Söldner

Institute of Cell Dynamics and Imaging and Cells-In-Motion Cluster of Excellence (EXC1003-CiM), University of Münster, Münster.

The Plasma Membrane (PM) of the budding yeast *S. cerevisiae* is laterally segregated into a patchwork of overlapping network domains. These intricate patterns are likely required to orchestrate the manifold functions of the PM and are regulated through multiple mechanisms. We study the lateral segregation of PM components using state o

f the art fluorescence microscopy techniques. This approach is facilitated by the exceptionally slow lateral diffusion within the yeast PM - several orders of magnitude slower to that in mammalian cells. However, this slow diffusion also poses a particular need to study how PM proteins are delivered to and removed from the PM, and to what extent lateral compartmentalization is affected by membrane turnover.

In the present work we studied the lateral distribution, endocytic regulation and function of the yeast methionine specific permease Mup1. Using TIRF microscopy we observed a very precise regulation of Mup1 at the PM that can be divided into three distinct steps. At low substrate supply, Mup1 is highly expressed at the PM and concentrated within the patchy MCC/eisosomal domain, where it is functionally active. Upon substrate binding and transport, Mup1 exits the MCC and re-locates into a network-like domain that is distinct from the previously described MCP domain that mainly contains the ATPase Pma1. Once outside the MCC, Mup1 becomes ubiquitinated via the arrestin Art1 and the E3 ligase Rsp5. Ubiquitinated Mup1 is then recruited to endocytic foci, internalized and finally degraded.

In summary, we have thoroughly characterized the spatio-temporal organization of a yeast amino acid transporter. We found an elaborate connection between lateral compartmentalization in the PM, biochemical function of the transporter and endocytic downregulation. The concentration of a variety of amino acid permeases and PM transporters within patch-like domains suggests that the identified 3-step mechanism could be valid for a variety of PM components.

**Structure of the TSC2 N-terminus provides insight into TSC complex assembly and tuberous sclerosis pathogenesis**

Reinhard Zech[1], Stephan Kiontke[1], Uwe Mueller[2], Andrea Oeckinghaus[3] & Daniel Kümmel[1]

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[2]Macromolecular Crystallography (BESSY-MX), Helmholtz Zentrum Berlin für Materialien und Energie, 12489 Berlin, Germany
[3]Institute of Molecular Tumor Biology (IMTB), Medical Faculty of the WWU Münster, 48149 Münster Germany

The tuberous sclerosis complex phenotype is a genetic disorder characterized by the formation of benign tumor like lesions designated as hamartomas. The tuberous sclerosis complex (TSC) gene products form a heterodimeric tumor suppressor consisting of TSC1/hamartin (130 kDa) and TSC2/tuberin (200 kDa), the latter acts as GTPase activating protein (GAP) for Ras homolog enriched in brain (Rheb) and negatively regulates mammalian target of rapamycin complex 1 (mTORC1). The large size of the TSC1-TSC2 complex is puzzling in contrast to the size of other known GAP proteins. Additionally, TSC1-TSC2 complex assembly and molecular architecture of the complex remains unknown.

We solved the structure of the N-terminal half of TSC1 (TSC1-N) in a novel crystal form [5] and tested its role in membrane recruitment. We also show that the TSC2 N-terminus interacts with the TSC1 C-terminus to mediate complex formation and analyze the molecular requirements for TSC1-TSC2 interactions. Furthermore, the structure of N-terminal TSC2 is solved and was applied to further analyze pathological point mutations. The majority of point mutations produce improperly folded protein, which explains their role in pathology. However, one point mutation was identified to abolish specifically complex formation. Altogether, this study provides the first structural and functional insight about TSC2/tuberin.

**Analysis of the contribution of distinct protein domains to the interactions between cardiac ECM components in *Drosophila melanogaster***

Yanina Post, Bárbara Rotstein, Jürgen Heinisch, Ariane Wilmes, Heiko Harten, and Achim Paululat

University of Osnabrück, Department of Zoology and Developmental Biology

The cardiac extracellular matrix (ECM) of *Drosophila melanogaster* is of great relevance regarding heart integrity, stability and maintenance. In a broad genetic screen, a new mutation was discovered that causes a heart phenotype characterized by age-dependent luminal collapse concomitant with a progressive disintegration of the cardiac ECM network. The affected gene was named *lonely heart (loh)* and encodes for a secreted tissue-specific adapter protein that assembles into the cardiac ECM and plays a crucial role in the recruitment of the collagen-like protein Pericardin (Prc) (Drechsler et al., 2013).

Utilizing the UAS-Gal4 system, we will express different constructs of Lonely heart, which all hold specific deletions of single domains or motifs. The respective constructs were generated in cooperation with Prof. Dr. Jürgen Heinisch and are currently tested for their ability to become secreted and incorporated into the ECM. Subsequently, the individual capacities to recruit Pericardin into the ECM will be analyzed.

Here I will report on the first observations made and the conclusions we were able to deduce so far.

*References*

*Drechsler, M., Schmidt, A. C., Meyer, H. and Paululat, A. (2013). The conserved ADAMTS-like protein Lonely heart mediates matrix formation and cardiac tissue integrity. PLoS Genet. 9, e1003616.*

## Quantitation of *Escherichia coli* phospholipids by MALDI TOF/TOF mass spectrometry

Ramona Scheffer[1], Stefan Walter[1] and Gabriele Deckers-Hebestreit[1]

[1]Arbeitsgruppe Mikrobiologie, Fachbereich Biologie/Chemie, Universität Osnabrück

The inner membrane of *E. coli* consists of roughly 70-75% phosphatidylethanolamine (PE), 20-25% phosphatidylglycerol (PG) and 5-10% cardiolipin (CL) with mainly three different fatty acid chains, C16:0, C16:1 and C18:1 [1]. Using MALDI TOF/TOF mass spectrometry, a semi-automatic set-up was designed that allows a quantitation of *E. coli* glycerophospholipids, extracted from intact cells, in a single step and enables a detailed analysis of the distribution of lipid classes, fatty acids as well as a comparison of the fatty acid composition of individual phospholipids.

Lipid standards of phospholipids present in *E. coli* membranes were used in defined amounts to determine the parameters essential for mass spectrometry (MALDI target plate, matrix, laser intensity, peak resolution, etc.) and to analyze the different phospholipid species. Whereas the variation in head groups has a high impact on the detection level within the MALDI TOF/TOF, the fatty acid composition of phospholipids naturally occurring in *E. coli* has no influence. To compensate the different characteristics, specific *flight factors* were defined for each lipid class. However, in dependence on the lipid mixture analyzed, individual phospholipid species reveal differences in miscibility and, therefore, different flight characteristics. To identify an internal standard that can be used independent of the composition of lipid extracts, a screening was performed for a phosphorylated compound with optimal flight properties. This internal standard now enables a quantitation of all *E. coli* phospholipid species by including so-called *miscibility factors* adapted to the corresponding lipid mixture.

To verify the method, phospholipid classes as well as fatty acid compositions of *E. coli* K-12 cells were determined at different growth phases and different growth temperatures. For the identification of the different phospholipid species a *lipid identification filter* was performed additionally to the accuracy of the molecular weight by fragmentation of the relevant molecules by post source decay. In addition, *E. coli* mutant strains carrying defects in the phospholipid head group biosynthesis were analyzed to confirm lipid identification. Interestingly, some of the mutant lipid extracts showed enrichment in precursors, which were also identified and quantified. The results obtained will be discussed in detail.

In summary, after extraction of *E. coli* phospholipids with an aqueous organic solvent mixture, all lipid species were identified and quantified by MALDI TOF/TOF mass spectrometry. Determination of phospholipid concentrations, head group *flight factors* and *miscibility factors*, if required, combined with the generation of a *lipid identification filter* now allows a rapid, detailed and comprehensive *lipidome* analysis for *E. coli*.

*References*

*[1] de Mendoza DD, Cronan JE (1983) Thermal regulation of membrane lipid fluidity in bacteria. Trends Biochem Sci 8, 49-52.*

## The role of the Cpx-system within the envelope stress systems in *Escherichia coli* analyzed by SRM and localization studies

Emina Ćudić[1], Kristin Surmann[2], Elke Hammer[2], Rainer Kurre[3], Jacob Piehler[3], Sabine Hunke[1*]

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[2]University Medicine Greifswald, Department of Functional Genomics, Interfaculty Institute of Genetics and Functional Genomics, Friedrich-Ludwig-Jahn Straße 15A, 17475 Greifswald, Germany
[3]University Osnabrück, Department of Biology/Chemistry, Division of Biophysics, Barbarastraße 11, 49076 Osnabrück, Germany

In order to acclimate in response to environmental changes and therefore to different stimuli, bacteria rely on two-component systems (TCS) [1]. These systems make use of a phosphorylation cascade from a transmembrane sensor kinase (SK) to a cytoplasmic response regulator (RR) and are set back to the initial state via dephosphorylation of the RR [2]. The Cpx envelope stress TCS consists of the membrane-bound SK CpxA, the cytosolic RR CpxR and the periplasmic accessory protein CpxP, which inhibits the autophosphorylation activity of CpxA [3, 4].

For a better understanding of the Cpx-TCS we were interested in whether the absolute amounts of all Cpx-components contribute to its activity after activation of the system. Therefore, we absolutely quantified CpxA, CpxR and CpxP by single reaction monitoring (SRM) under different growth conditions. Furthermore, we monitored changes in the whole cell proteome of *E. coli* after Cpx-activation and could identify acid stress as a new target of the Cpx-system [5].

In addition, we analyzed whether the cellular localization of CpxA and CpxP changes upon Cpx-activation or Cpx-inhibition. For this purpose, we generated chromosomally encoded, C-terminal fusions of CpxA and CpxP with the HaloTag® or SnapTag®. CpxA and CpxP were localized by TIRF-microscopy under Cpx-non activating conditions (WT), various Cpx-activating and Cpx-inactivating conditions in living cells. Furthermore, we investigated if CpxA and CpxP co-localize since it was shown that CpxA and CpxP interact in order to keep the Cpx-TCS in an OFF-state [3; 6; 7].

Altogether, our results provide a deeper insight into regulation mechanisms within the Cpx-TCS and its role within the stress network of *E. coli*.

*References*
*[1] Stock, A. M.; Robinson, V. L. and Goudreau, P. N. (2000). Annu. Rev. Biochem.* ***69****, p. 183-215.*
*[2] Parkinson, J. S. (1993). Cell* ***73****, p. 857-871.*
*[3] Fleischer, R; Heermann, R.; Jung, K, and Hunke, S. (2007). J. Biol. Chem.* ***282****, p. 8583-8593.*
*[4] Hunke, S.; Keller, R., and Müller, V.S. (2012). FEMS Microbiol.* ***326****, p. 12–22.*
*[5] *Surmann, K.; *Ćudić, E.; Hammer, E., and Hunke, S. (2016). MicrobiologyOpen doi: 10.1002/mbo3.353 (*these authors contributed equally to this work.)*
*[6] Zhou, X.; Keller, R.; Volkmer, R.; Krauss, N.; Scheerer, P. and Hunke, S. (2011) J. Biol. Chem.* ***286****, p. 9805-9814.*
*[7] Tschauner, K.; Hörnschemeyer, P.; Müller, V.S.; Hunke, S. (2014) PLoS ONE* ***9(9)****. e107383. doi:10.1371/journal.pone.0107383*

**Unraveling the working mechanism of a tumor suppressor lipid**

Shashank Dadsena[1], Svenja Bockelmann[1], Manual Nuno Melo[2], John Mina[1], Patrick Niekamp[1], Helene Jahn[1], Alicia Kraffzik[1], Sergei Korneev[1], Siewert-Jan Marrink[2], Joost C. M. Holthuis[1]

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[2]*The Zernike Institute for Advanced Materials, University of Groningen, The Netherlands*

While sphingolipids are vital components of cellular membranes, their production relies on a potentially toxic metabolic intermediate: ceramide. Deregulation of this putative tumor suppressor lipid is frequently linked to induction of apoptosis, but the underlying molecular principles are not clear. We previously showed that mitochondrial translocation of ER ceramides specifically commits cells to death. Using a photo-activatable and clickable ceramide analog (pacCer), we now report identification of the voltage-dependent anion channels VDAC1 and VDAC2 as the principal ceramide-binding proteins in mitochondria. VDAC3 and TOMM40, two other protein channels in the outer mitochondrial membrane, lack affinity for ceramide. In line with our pacCer labelling studies, molecular dynamics simulations revealed that VDAC1 and VDAC2, contrary to VDAC3, possess a ceramide-binding site around the 4$^{th}$ of 19 predicted amphipathic beta-strands. The main sequence difference in the 4$^{th}$ beta-strand between VDAC1/2 and VDAC3 is a Glu73Gln substitution. Introducing this substitution in VDAC1 abolished its interaction with ceramide in molecular dynamics simulations. Treatment of mitochondria with dicyclohexylcarbodiimide (DCCD), a compound known to react with Glu73 in VDAC1/2, blocked pacCer-labelling of these proteins, confirming that this residue is critical for ceramide binding. Apart from mediating metabolic cross-talk between mitochondria and cytosol, VDAC1/2 have been implicated as critical players in the cytosolic release of mitochondrial apoptogenic proteins such as cytochrome *c*. Consequently, our current efforts are aimed at generating ceramide-binding mutants of VDAC1/2 to address their potential role as down-stream effectors in ceramide-induced mitochondrial cell death.

## Molecular analysis of the adhesion-invasion microcompartment formed during interaction of *Salmonella enterica* with polarized epithelial cells

Sonja Geißelsöder and Michael Hensel

University of Osnabrück, Division of Microbiology

*Salmonella enterica* is a pathogen with a facultative intracellular lifestyle which enters cells of the intestinal epithelium. After adhesion to the apical membrane mediated by a type I secretion system (T1SS) and its substrate adhesin SiiE, *Salmonella* injects effector proteins into the host cell by a type III secretion system (T3SS). This leads to effacement of the brush border, a remodeling of the host cell actin cytoskeleton and uptake of *Salmonella* by a process termed macropinocytosis. We propose the transient formation of an adhesion-invasion microcompartment composed of bacteria T1SS and T3SS, adhesins, host cell ligands, and host cell cytoskeleton.

The molecular composition of the microcompartment formed during adhesion and invasion is unknown. To identify the bacterial and host cell components involved in this microcompartment, proteomic analyses will be used. We deploy proximity biotinylation for identification of proteins in close proximity to a bait protein. The enzymatic tag APEX2 is fused to *Salmonella* outer membrane proteins, adhesins, or translocated effector proteins and will label nearby proteins with biotin. APEX2 is an engineered ascorbate peroxidase, which converts biotin-phenol upon addition of hydrogen peroxide into a radical. This radical is short-lived and reacts with electron-rich sidechains of amino acids in close proximity to the APEX2-tag. This makes it possible to pull down biotinylated proteins of the adhesion-invasion microcompartment using streptavidin matrix or alternatives and to analyse the proteome by mass spectrometry.

# Analyzing Membrane Ruffle Formation during Invasion of *Salmonella* Typhimurium in Polarized Epithelial Cells with Correlative Light and Electron Microscopy (CLEM)

Carina Kommnick and Michael Hensel

University of Osnabrück, Division of Microbiology

*Salmonella enterica* serovar Typhimurium is a facultative intracellular pathogen known for causing self-limiting gastroenteritis by ingesting contaminated food. To mediate forced uptake into polarized epithelial gut cells, this process requires tight adhesion by the giant adhesion SiiE (Barlag & Hensel, 2015) and remodeling of the host cell actin cytoskeleton by effector proteins of the type III secretion system (T3SS) encoded by S*almonella* pathogenicity island 1 (SPI1) (LaRock *et al.*, 2015). We observed that *Salmonella* interaction with polarized epithelial cells also results in transient effacement of the apical brush border.

Known to manipulate the reformation of the F-actin cytoskeleton in host cells, SPI1-T3SS effector protein SopE is a major factor resulting in effacement of the brush border and uptake of *Salmonella* by micropinocytosis (Felipe-López, dissertation, 2014). In a mutant background lacking SPI1-T3SS effector proteins SipA, SopA, SopB, SopE and SopE2, complementation with SopE alone was sufficient to mostly restore the invasion process in polarized cells. Due to accumulation of F-actin on the contact side of *Salmonella* invasion, the formation of membrane ruffles is a distinct morphologic feature, which needs to be elucidated. Thus, scanning electron microscopy was performed to gain ultrastructural information for membrane ruffle morphology of polarized cells infected by WT or *sopE* deletion strains 25 min post invasion.

As invasion is a fast and dynamic process, taking only 5 to 7 min from first contact of *Salmonella* to completed uptake into epithelial cells and delayed regeneration of microvilli, each event needs to get correlated individually by time. To define the morphologic stages of membrane ruffles, a correlative light and electron microscopy (CLEM) approach using confocal laser scanning and scanning electron microscopy was devised. Our first results show that this is a promising method to clarify the morphology of membrane ruffles over the time and gives the opportunity to visualize more *Salmonella* specific structures e.g. SiiE during invasion.

## Nuclear ROXY interactions with TGA transcription factors

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Glutaredoxins (GRX) are small oxidoreductases present in both, prokaryotes and eukaryotes. Participation of GRXs in many crucial processes could be shown, e.g. in oxidative stress response, regulation or modulation of metabolic enzymes, in signalling processes or in the assembly and delivery of Fe-S clusters.

In land plants, a specific group of GRXs is present, the CC-type GRXs, named ROXYs in *Arabidopsis thaliana*. Current data show an involvement of ROXYs in flower development (*ROXY1/2*) and in pathogen response (*ROXY19*) [1]. *ROXY1* is required for petal primordia initiation (*A. thaliana* wildtype plants have 4 petals, whereas the *roxy1* mutant forms on average only 2.5) and later during flower development for petal morphology. The *roxy1 roxy2* double mutant shows defects in anther development and microsporogenesis.

The TGA-transcription factor PERIANTHIA (PAN) has been identified as an interaction partner of ROXY1. Both proteins act together in the same genetic pathway in petal initiation. A nuclear localization of the ROXY1 protein and its interaction with TGA in the nucleus is pivotal for normal petal development [2]. Since Glutaredoxins are oxidoreductases and known to post-translationally modify target proteins due to a redox-signal, ROXY1 thus might modify PAN post-translationally. Recent *in vitro* studies revealed a redox-sensitive DNA- binding of PAN, which is mediated by its N-terminus comprising five cysteines. It was shown that these cysteines participate in a redox-dependent control of the PAN interaction with a conserved regulatory cis-regulatory element, emphasizing the importance of redox-modifications in the regulation of flower developmental processes [3].

The *in vivo* characterization of the subnuclear localization of ROXY1 and its interaction partner PAN will give insight in the formation of nuclear microcompartments in Arabidopsis.

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## In *situ* single cell pull-down for probing stability and stoichiometry of cytosolic protein complexes

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Investigation of composition, stoichiometry and interaction dynamics of signaling complexes is crucial for understanding the various signaling pathways. We have developed in *situ* single cell pull-down (SiCPull) for specific and efficient pull-down of protein complexes from individual cells, based on engineered, micropatterned functionalized surface architectures [1]. Cells cultured on these surfaces are lysed by mild detergents, leading to almost instantaneous in *situ* capturing of GFP-tagged protein complexes, thus enabling the determination of their life-time, by monitoring the dissociation of prey proteins interacting with GFP-tagged bait proteins. Using SiCPull, we quantitatively determined the stability of various signal transducers and activators of transcription (STAT) complexes, ranging from seconds to nearly an hour. Strikingly, complex stoichiometry could be determined on the single cell level by single molecule imaging techniques. In combination with single cell manipulation, e.g. by microfluidics, this generic methodology provides robust possibilities to monitor stability and stoichiometry of cytosolic signaling complexes at different cellular states.

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**Analysis of Mechanism of Autophagosome-Vacuole Fusion in Yeast**

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In both yeast and mammals, autophagosome formation is a complex process that begins with the assembly of the phagophore or isolation membrane. Once complete, autophagosomes mature to autolysosome by fusing with lysosomes for degradation. The mechanism of interactions between lysosomes and autophagosomes is still poorly understood. The Mon1-Ccz1 complex is the GEF of the yeast Rab7 GTPase Ypt7. The Ccz1 and Mon1 proteins are required for multiple vacuole delivery pathways including the general autophagy, Cvt pathway and endocytosis. However, it is not clear about if and how the Mon1-Ccz1 complex recognizes autophagosomes in yeast, or whether it promotes fusion with the vacuole. In my presentation, I will show that Mon1-Ccz1 and Ypt7 are recruited to autophagosomes at an early stage of the autophagic process and that this recruitment depends on Atg8. I identified two LIR (Atg8/LC3-interacting region) motifs in Ccz1. LIR mutants miscolocalized with Atg8 and caused accumulation of autophagosomes without degradation. To determine the parameters of autophagosome fusion with vacuoles, I established a novel in vitro assay with purified organelles. I show that fusion requires ATP, physiological temperature, vacuolar SNAREs and Mon1-Ccz1. My findings uncover a novel mechanism of Mon1-Ccz1 recruitment to an organelle, and provide the basis to unravel autophagosomal maturation in the test tube.

**Dissecting the behaviour of RNA binding proteins in neuronal stress granules with super-resolution microscopy**

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Maintenance of cellular polarity as well as fast response upon extracellular cues requires a fast and tightly regulated expression of proteins, especially in morphologically complex cells like neurons. Messenger ribonucleoprotein particles (mRNPs) contribute to the regulation of gene expression via posttranscriptional coordination of mRNA translation, localization and degradation. These self-assembling structures lack a limiting membrane and can be considered as dynamic microcompartments.

The Ras GTPase activating protein SH3 domain binding protein 1 (G3BP1) and the Insulin like growth factor II mRNA binding protein 1 (IMP1) are present in stress granules (SGs), i.e., mRNPs that are induced after cellular stress. According to confocal laser scanning microscopy (cLSM) these two proteins colocalize in SGs and overexpression of either of them is sufficient to induce SG formation. Furthermore, IMP1 appears to be rather immobile while G3BP1 is frequently fluctuating between granules and cytosol. Currently, two concepts exist which try to explain the behaviour of mRNPs. The scaffolding assembly or the liquid droplet model.

In order to unravel the organization of G3BP1 and IMP1 inside of stress granules and to distinguish between the 2 concepts, epitope-tagged G3BP1 and IMP1 constructs were transiently transfected and expressed in neuronally differentiated PC12 cells. Posttranslational, substoichiometric labeling of these constructs and induction of granules via sodium-arsenite treatment was followed by Total Internal Reflection Fluorescence (TIRF) microscopy and subsequent Single Molecule Tracking (SMT) or density-based cluster analysis.

Our data indicate an increased dwell time of G3BP1 and IMP1 in stress granules if both proteins are co-expressed compared to control experiments where only one of the proteins was expressed. This suggests an interaction of both proteins inside stress granules which might favour the scaffolding assembly mechanism over the liquid droplet model. Currently, we perform SMT of the movement of G3BP1 and IMP1 inside of vesicles and apply cluster analysis to determine the distribution in granules. The experiments will be complemented by 3D SMT in order to further discern G3BP1 and IMP1 interaction.

**Single vesicle recording in hippocampal 'xenapses' reveals diffusional dispersion of SV proteins after fusion**

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In order to maintain neuronal transmission after exocytosis of synaptic vesicles (SVs), the vesicular proteins have to be cleared away from the active zone. Until now it remained controversial whether SV components remain clustered during translocation from sites of exocytosis or disperse by free diffusion. To adress this question, we developed a novel purely presynaptic neuronal preparation which enables single vesicle recording by TIRFM.

Using click-chemistry we functionalized micropatterned coverglasses with protein domains of synaptic cell adhesion molecules, serving as artificial postsynapses. On these host substrates purely presynaptic boutons form 'en face' directly onto the coverslip, termed 'xenapses'. Serial section TEM as well as focused-ion-beam SEM showed that xenapses contain a few hundred SVs, many of them docked in several clusters at the bottom membrane. 4Pi and TIRF-STORM confirmed the existence of several active zones. Thus, xenapses offer the unique opportunity to record exocytosis of single vesicles by TIRFM. Using fusion constructs of the pH-sensitive pHluorin, single fusion events were visible as diffraction-limited spots on stimulation with single action potentials. We could localize fusion events synchronous to action potentials with ~20 nm precision and follow the fate of released SV proteins. We observed diffusional dispersion of vesicular proteins post fusion with diffusion constants in the range of 0.1 – 1 $\mu m^2/s$. Thus, our results point to free diffusion as mechanism for fast clearance.

**Activation of the cytoplasmic domain of the *Np*SRII/*Np*HtrII complex studied by EPR spectroscopy**

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The photoreceptor/transducer complex *Np*SRII/*Np*HtrII is responsible for the phototaxis of *Natronomonas pharaonis*. It is known that the signaling cascade upon light activation comprises changes of the conformation of the receptor *Np*SRII and of the transmembrane part of the transducer *Np*HtrII.[1] Recently, it was shown by EPR spectroscopy how the signal changes the first HAMP domain's conformation.[2]

In addition to light activation, transducer methylation, the molecular mechanism of adaptation in the *Np*SRII/*Np*HtrII complex, modulates conformation, dynamics and signaling by transducer methylation.[3] Furthermore, a thermodynamic equilibrium between a more compact and more dynamic state of the transducer's tip domain was been found. [4]

The influence of methylation states on protein dynamics can be mimicked by mutation of the respective glutamate residues to glutamine. With this mutants and methanothiosulfonato spin label on well known positions EPR spectroscopy provides information about the conformation and dynamics of the transducer domain of *Np*SRII/*Np*HtrII.

Additionally, EPR spectroscopy has been applied to investigate the structure and the conformation of the tip domain of the transducer to understand the further transmission of the signal. To investigate the activated state a *Np*SRII mutant (D75N)[5] has been used, which is thought to initiate an activated state similar to the light-activated state of wild type *Np*SRII. EPR spectra of the complexes of wt *Np*SRII and *Np*SRIID75N with *Np*HtrII spin labeled at eleven different positions have been monitored and compared. To verify the results dependences of the spectra on different parameters have been tested. The EPR spectra have been fitted and the amplitudes of different components, which differ in the spin label mobility, yield information about potential conformational changes and equilibrium shifts.

For more details about phototaxis of *Np*SRII/*Np*HtrII the set up of a laser to measure time-resolved EPR-spectra is in progress. Hence, further results about changes in structure and dynamics due the transmission of the signal are expected.

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# Poster Abstracts

## *Brucella abortus* exit mechanism from its host cell

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Brucellosis is a zoonotic disease of worldwide distribution that affects both animals and humans and inflicts economical and public health burden in endemic areas. An important feature of *Brucella* that is essential for its pathogenesis and persistence is its capacity of being a facultative extracellular intracellular pathogen. Once *Brucella* infects its mammalian host, it invades professional phagocytes. These cells become a survival/replication niche and vectors for systemic dissemination to other organs of the reticuloendothelial system. In these organs, *Brucella* survives intracellularly and replicates within macrophages/monocytes. Once the bacteria are internalized they transit within the *Brucella* Containing Vacuole (BCV) and then reach their replicative niche, the endoplasmic reticulum. Following replication the BCV acquires autophagic vesicle markers. It is not clear what mechanisms are involved in the exit pathway. To understand *Brucella* exit and propagation to new host cells we have determined by plate counts the time in which bacteria exit and analyzed by immunofluorescence infected cells at this time of infection. Our preliminary results show that, infected cells begin budding, its nuclei undergo a degradation process and at this point, we detect extracellular bacteria clumps. These bacteria clumps are probably located within vesicles since they are not accessible to antibodies in unpermeabilized samples. Finally, the, bacteria begin to exit from these vesicles. Preliminarily we proposed a model in which *Brucella* promote host cell death and surrounded by a host membrane infect new cells.

**The Two Component System BvrRS of *Brucella abortus* senses intracellular conditions and triggers signals required for replication**

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The two component system BvrR/BvrS (BvrRS) is essential for the intracellular lifestyle of Brucella abortus and controls the expression of virulence determinants such as the transcriptional regulator VjbR and the Type Four Secretion System (T4SS) VirB. To understand the role of BvrRS we have analyzed the expression and phosphorylation of the system in bacteriological cultures and in intracellularly grown bacteria in cultured mammal cells. The TCS was highly expressed at early hours of the bacterial growth curve, and steadily declined at later times. Incubation of log phase bacteria in minimal medium at pH 5.5 induced the phosphorylation of BvrR and increased the expression of VjbR and VirB. In contrast, stationary phase bacteria were unable to respond to these conditions. The BvrS was undetectable in stationary phase in all conditions. Consistently, cells infected with B. abortus at stationary phase achieved significantly lower levels of replication than those infected with bacteria at exponential phase. Bacteria extracted from infected cells demonstrated phosphorylated BvrR and increase in VjbR and VirB8 proteins. Intracellular replication of B. abortus was affected by inhibition of endosomal acidification of cells. However, the intracellular replication under these conditions was reestablished if the bacteria were extracellularly incubated in acidic minimal medium before the infection. Our results support a model where the TCS BvrRS senses the transition from an extracellular milieu to an intracellular niche triggering a transcriptional response required to guide the bacteria during the first hours of its intracellular journey.

**The role of the TMA64 (eIF2D) and TMA20 (MCTS1) proteins in translation reinitiation on uORF containing mRNAs**

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eIF2D and its homologs MCTS1 and DENR are eukaryotic translation factors with unknown function. In mammalian translation reconstitution system both eIF2D and MCTS1/DENR dimer have an activity of tRNA stabilization in the P-site of 40S ribosomal subunits. This suggests a role in translation initiation, termination or recycling played by these proteins *in vivo*. In drosophila, the orthologous proteins were reported to be necessary for translation of uORF containing mRNAs. To dissect the function of eIF2D, MCTS1 and DENR further, we turned to yeast cell-free translation systems prepared from two *S.cerevisiae* strains, *wt* and the double knockout strain lacking both *TMA64* (a *EIF2D* ortholog) and *TMA20* (an *MCTS1* orholog) genes. A set of luciferase encoding mRNAs with 5' untranslated regions bearing different uORFs was prepared and translated in these *in vitro* systems. To check specificity, we performed rescue of *Δtma64/Δtma20* lysate by addition of the recombinant human MCTS1/DENR dimer. We observed an enhanced translation of the uORF containing mRNAs in the absence of TMA64 and TMA20, in contrast to uORF-less ones. The effect required termination of the uORF translation before or shortly behind the luciferase start codon. It also depends on uORF length and a distance between uORF and the reporter coding sequence. We concluded that reinitiation rate is increased in the absence of TMA64 and TMA20. Thus, these proteins are involved in translation reinitiation. We hypothesize that they may operate to protect the post-termination ribosomes from acquiring novel initiation complex and thus inhibits unsanctioned translation reinitiation on eukaryotic mRNAs.

## Evaluation of a fluorescent analog sensor sphingolipid as a predictor of resistance or sensitivity to chemotherapy in a breast cancer model

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Cancer is a genetic disease characterized by a large variety of abnormalities leading to therapy resistance. Therefore, novel strategies are needed to target pathways downstream from those oncogenic disturbances including metabolism. For example, the sphingolipid (SL) or ceramide pathway is an attractive target since it plays important roles regulating survival and death. One important property of this pathway is that it harbors a sphingolipid rheostat, whereby the ratios of several inter convertible metabolites determines cell survival or death. The present study aims to evaluate the potential use of a fluorescent sphingolipid analogue as sensor to predict chemotherapy resistance in breast cancer cells, including single cell resolution to deconvolve cancer heterogeneity. Time-resolved images of live cells were acquired upon perturbations such as treatments with chemotherapeutic agents and known sphingolipid pathway inhibitors. In order to track individual cells, we designed an image analysis pipeline to evaluate the dynamic behavior of multiple image features including shape, texture, intensity and nucleus/cell correlation. Using these data we built mathematical models to identify whether and how the sphigolipid pathway is able to sense cell resistance or sensitivity to drugs and suggest double perturbations to the system to overcome chemotherapy resistance.

**A search for ceramide binding proteins using bifunctional lipid analogous yields the CERT-related phosphatidylcholine transfer protein StarD7**

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Cells routinely synthesize ceramides in the endoplasmic reticulum (ER) as precursors for sphingolipids to form an impermeable plasma membrane. In addition to their role as central intermediates of sphingolipid biosynthesis, ER ceramides have been implicated as signaling molecules in cellular stress responses and can trigger apoptosis when translocated to mitochondria. Consequently, cells must regulate their ceramide levels closely to meet metabolic demands without compromising their viability. The aim of this project is to unravel the molecular principles that govern ceramide trafficking between ER and mitochondria and to identify down-stream effectors responsible for executing ceramide-mediated cell death.

To this end, we developed a screen for ceramide-binding proteins using a bi-functional ceramide probe that contains a photoactivatable and clickable group: pacCer. Application of pacCer in photo-affinity labeling experiments with cytosol from mouse GM95 melanoma cells allowed us to capture the well-characterized ceramide transfer protein CERT. We used this protein as starting point to expand our screen to a more directed approach, which enabled us to probe the lipid binding specificity of CERT and map residues critical for coordinating ceramide in the protein's lipid binding pocket. With this assay in hand, we next determined the lipid binding profiles of various CERT-related proteins and found that the mitochondria-associated phosphatidycholine (PC) transfer protein StarD7 displays dual specificity for ceramide. Site-directed mutagenesis of StarD7 indicated that the binding sites for PC and ceramide overlap at least partially. While StarD7 lacks ceramide transfer activity *in vitro*, we found that its ability to transport PC is modulated by ceramides. Whether StarD7 acts as a ceramide-sensitive PC transfer protein at the ER-mitochondrial interface *in vivo* is the subject of ongoing investigations.

**Protein interaction dynamics of the plant-specific microtubule-associated protein TORTIFOLIA1 from Arabidopsis**

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Microtubule-associated proteins (MAPs) regulate cell division and the direction of cell expansion in plants. Defects in cell expansion can lead to right- or left-handed organ twisting. Plant mutants with twisting phenotypes are either defective in microtubules, in transport of the plant growth regulator auxin or in cell wall structure. This research aims at understanding better how microtubules are regulated by MAPs. Therefore TORTIFOLIA1 (TOR1), which encodes a plant-specific MAP that labels plus-end comets as well as microtubule cross-over sites, was studied in detail. *tor1* is a tropism mutant and defective in plant movements. We investigate the dynamics of TOR1 and other MAPs and their interactions using a range of techniques. We employ proteomics, yeast-2-hybrid analyses and bimolecular fluorescence complementation studies to identify TOR1 protein interaction partners. TOR1 and further MAPs are analyzed by superresolution and single-molecule microscopy. This is performed in tobacco BY-2 cells. We found that individual TOR1 molecules are relatively immobile despite the apparent translocation of TOR1-containing microcompartments on the microtubule plus-end. This indicates that TOR1 molecules specifically recognize the plus-end tip and do not move by themselves. This suggests a mechanism for plus-end recognition. In addition several interaction partners of TOR1 could be determined.

**Molecular dissection of a candidate ceramide sensor**

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We previously identified SMSr, an ER-resident ceramide phosphoethanolamine (CPE) synthase, as a critical regulator of ER ceramides and suppressor of ceramide-induced mitochondrial apoptosis. Intriguingly, SMSr-mediated ceramide homeostasis relies on both the enzyme's catalytic activity and its *N*-terminal sterile-alpha motif or SAM domain. BLAST searches revealed that SMSr-SAM is closely related to the SAM domain of diacylglycerol kinase DGKδ-SAM central regulator of lipid signaling at the plasma membrane. Native gel electrophoresis of recombinant SAM domains showed that SMSr-SAM, analogous to DGKδ-SAM, self-associates into oligomers. Co-IP and chemical cross-linking studies in HeLa cells revealed that SMSr forms homo-oligomers (trimers/hexamers) in the ER, and that SMSr oligomerization is critically dependent on its SAM domain. These results were confirmed by single molecule photobleaching analysis, which allowed us to quantitatively monitor SMSr oligomerization in the ER of intact cells. Treatment of cells with curcumin, a compound disrupting ceramide and calcium homeostasis in the ER, promoted SMSr-oligomerization and induced formation of a heterologous SMSr-protein complex. However, blocking *de novo* ceramide synthesis with myriocin or chelating intracellular calcium with BAPTA-AM did not prevent curcumin-induced SMSr-oligomerization, suggesting that this process is independent of ceramide or calcium fluctuations in the ER. Disrupting SMSr oligomerization by site-directed mutagenesis caused the enzyme to relocate to the Golgi. Our ongoing work focuses on whether oligomerization-defective SMSr mutants are able to suppress ceramide accumulation in the ER and on identification of SMSr binding partner(s).

**The I-BAR protein Ivy1 regulates vacuole biogenesis via phosphatidylinositol 3,5-biphosphate levels**

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Phosphoinositides (PIs) are well known for the regulation of trafficking process. The generation of these lipids in the endocytic pathway depends on PI kinases complexes such as Vps34, which generates phosphatidyl-inositol- 3-phosphate and Fab1, which converts PI-3-P to PI-3,5-P2. We have recently showed that the yeast equivalent to the metazoan *Missing in metastasis* (MIM) protein, Ivy1 binds PtdIns-3-P *in vitro*, binds the Rab7 GTPase Ypt7 and localizes to distinct sites on the vacuolar membrane. Here, we identify the interaction site between Ypt7 and Ivy1, and provide evidence for a role of Ivy1 in the regulation of vacuole morphology by affecting the biogenesis of phosphoinositides on the yeast vacuole.

## Resolving Interactions Inside Living Cells With Engineered Upconversion Nanoparticles

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Upconversion nanoparticles (UCNP) are efficiently excited by sequential multiphoton absorption of NIR light and emit photons in the UV/VIS regime and therefore can be detected with negligible background [1]. Moreover, lanthanide resonance energy transfer (LRET) from UCNP to molecules in immediate proximity opens exciting possibilities as spectroscopic reporters or photoactuators with very high spatial resolution [2]. However, even though strategies for improving the optical properties of UCNP emerged [3], the determinants of UCNP-based LRET as well as its application in a biological context are still poorly resolved. We have engineered biofunctional UCNP optimized for LRET as novel reporters for spatially-resolved protein interaction analysis within living cells. To this end, we implemented microscopic techniques for UCNP excitation and synthesized various nanoparticle species to systematically improve UCNP emission and energy transfer efficiency. These phenomena strongly profited from power densities far beyond commonly published values – in agreement with recent studies on this topic [3]. Strikingly, we observed further enhancement of LRET when breaking with traditional paradigms of UCNP design. In order to exploit these unique optical properties in a biological context, we established a biofunctional coating with an anti-GFP nanobody for selective targeting in the cytoplasm of living cells. With the mitochondrial TOM complex as model system we demonstrated specificity of UCNP functionalization by colocalization and further confirmed this interaction by LRET-sensitized dye emission.

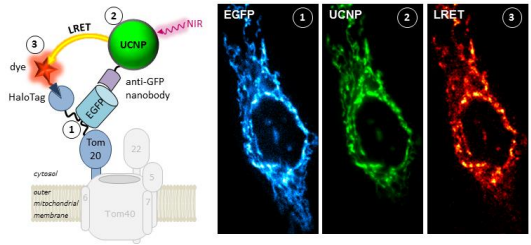


**Fig. 1.**
UCNP functionalized with anti-GFP specifically bind to mitochondrial Tom20-EGFP-HaloTag. Once excited by NIR light, UCNP luminesce and transfer energy to the acceptor dye label of the HaloTag.

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## Neprilysins control insulin signaling via cleavage of regulatory peptides

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Insulin and IGF signaling are critical to numerous developmental and physiological processes, including regulation of growth, metabolism, reproduction, stress responses, aging, and lifespan. Consequently, perturbations in these processes are pathognomonic of various diseases, with Diabetes being a most prevalent. However, while the functional roles of the respective signaling pathways have been extensively studied, the control of insulin production and release is only partially understood. By showing that proper expression of major insulin-like peptides critically depends on the catalytic activity of a *Drosophila* neprilysin, we provide novel mechanistic insight into this issue and relate neprilysin activity to the regulation of insulin signaling for the first time. Concomitant phenotypes of altered Neprilysin 4 expression included impaired food intake, reduced body size, premature lethality, and characteristic changes in the metabolite composition of respective transgenic animals. Significantly, ectopic expression of a catalytically inactive protein variant did not elicit any of the phenotypes, which demonstrates that enzymatic activity and thus regular peptide hydrolysis are critical to proper insulin expression. In a screen for novel substrates of the neprilysin, we identified numerous peptides known to be involved in regulating insulin-like peptide expression, feeding behavior, or both, thus providing a conclusive explanation for the observed phenotypes. The high functional conservation of neprilysins and their substrates renders the characterized principles applicable to numerous species, including higher eukaryotes and humans.

**TGA/NPR/ROXY: Analysis of conserved nuclear interaction dynamics**

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Glutaredoxins (GRXs) are small glutathione-dependent oxidoreductases that participate in redox regulated processes via thiol-modulation of proteins and/or are involved in the assembly and transfer of Fe-S clusters. In land plants, a unique glutaredoxin group, the CC-type GRXs (ROXYs) are known to function in flower developmental processes and stress signaling (1). In *Arabidopsis thaliana,* ROXY1 participates together with the plant-specific bZIP TGA transcription factor PERIANTHIA (PAN) in the regulation of petal initiation. Loss of *ROXY1* activity leads to a reduced petal number, whereas *pan* mutants show an opposing flower phenotype with the formation of five petals. Both proteins interact in the nucleus and a nuclear localization is a prerequisite for the formation of wildtype-like flowers (2). Another group of proteins involved in the TGA-dependent expression control is the *NON EXPRESSOR OF PR1* (*NPR*) family with its member NPR1, one of the best studied redox regulated cofactors in *Arabidopsis thaliana* (3).

All three gene families expanded strongly throughout land plant evolution likely contributing to the adaption to new challenges, like abiotic and biotic stresses or the development of more complex tissues and organs. The bryophyte *Marchantia polymorpha* is currently being established as a model organism to study gene functions in basal land plants. In Marchantia, only two ROXYs, one TGA and one NPR gene exist in contrast to 21, 10 and 6 in Arabidopsis, respectively. We therefore use Marchantia to study the earliest emerging ROXY-TGA-NPR gene functions. To understand the impact of the cellular redox state on the interaction dynamics between these proteins, we use the FLIM technique with mTURQ2-mVenus as fluorophores in transient transformed *Nicotiana benthamiana* epidermal leaf cells.

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## Gender differences in synaptic microcompartments of mice

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Biologically based differences between the sexes influence human development, behavior and disease progression in both obvious and subtle ways. Sex differences therefore have an increasing impact on biomedical research, and subsequently on healthcare delivery.

Our main goal was to explore whether gender differences are present on the level of mouse dendritic spines in different brain regions even after the reproductive phase of mice. To address this question we used a laboratory mouse strain C57/Bl6J at 24 months of age and analyzed dendritic spine parameters in both genders. Dendritic spines are highly dynamic sub-organellar functional microcompartments. They represent the postsynaptic entities, which serve as organizing platforms for molecular changes in the synaptic connection of the majority of excitatory inputs in the hippocampus and cerebral cortex.[1]

Animals were transcardially perfused with 4% formaldehyde to maintain the neuronal structure. The mouse brains were cut using a vibratome, into160 μm-thick slices and imaged with laser scanning confocal microscope.[2] High-resolution images were obtained from sensory and associative cortices and of the CA1 and CA3 subfields of dorsal hippocampus. We focused our analysis on the apical dendrites of the pyramidal cells. The micrographs were subjected to 3D blind deconvolution and algorithm-based recognition of spine density and morphology. Origin Pro was used for statistics and graphs.

Our preliminary results suggest that there is a robust difference in spine density on the cortical pyramidal neurons resulting in significantly higher spine density in males. In contrast, we observed only subtle differences in spine parameters between genders on the hippocampal pyramidal neurons, mainly regarding morphology of spines in the CA3 subfield.

Taken together, our data indicate that in the current era of translational research, it is increasingly important to take sex differences into account, even at advanced ages of the subjects, so that the potential effects of drugs and therapies can be more fully and adequately understood.

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**Diverting CERT-mediated ceramide transport to mitochondria triggers Bax-dependent apoptosis**

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A deregulation of ceramide biosynthesis in the ER is frequently linked to induction of mitochondrial apoptosis. While in vitro studies suggest that ceramides may initiate cell death by acting directly on mitochondria, their actual contribution to the apoptotic response in living cells is unclear. In here, we analyzed the consequences of targeting the biosynthetic flow of ceramides to mitochondria using a ceramide transfer protein equipped with an outer mitochondrial membrane anchor, *mito*CERT. Cells expressing *mito*CERT import ceramides into mitochondria and undergo Bax-dependent apoptosis. Apoptosis induction by *mito*CERT was abolished by: i) removal of its ceramide transfer domain; ii) disrupting its interaction with VAP receptors in the ER; iii) addition of antagonistic CERT inhibitor HPA12; iv) blocking de novo ceramide synthesis; v) targeting a bacterial ceramidase to mitochondria. Our data provide first demonstration that translocation of ER ceramides to mitochondria specifically commits cells to death and establish *mito*CERT as a valuable new tool to unravel the molecular principles underlying ceramide-mediated apoptosis.

## S/M proteins and their role in SNARE assembly and membrane fusion

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In eukaryotic cells, fusion of membranes is an important process that is required for exchanging material between different compartments within the cell, maintenance of homeostasis and cell-cell communication. In order to maintain the cells physiology, membrane fusion is a critical aspect that needs to be temporally and spatially regulated. The key players in this process are part of a conserved fusion machinery in the endomembrane system that contains small Rab GTPases, SNAREs (soluble N-ethylmaleimide-sensitive-factor attachment receptors) and Sec1/Munc18 family (S/M) proteins. When the membranes are in close proximity, SNARE proteins drive fusion by forming a stable four-helix coiled-coil complex also called trans-complex. This complex of SNAREs is highly stable and it requires the hydrolysis of ATP by the ATPase NSF (N-ethylmaleimide-sensitive-factor) for disassembly after fusion. S/M proteins contain a ~600 amino acids conserved sequence that forms a clasps-like structure. They can interact with monomeric SNAREs and with SNARE complexes to promote intracellular membrane fusion, however, mechanistic and structural insight into this process is incomplete. The aim of my research is to identify how S/M proteins interact with SNAREs to fulfill their function For this purpose we measure the affinity of the interaction of S/M proteins with monomeric SNAREs as well as with (sub)assemblies to identify the cognates substrate complex(es) as target for crystallization and structure determination. I have so far been successful in establishing a purification protocol for individual SNARE and SNARE subassembly and the crystallization of isolated S/M protein. My current focus is on the reconstitution and crystallization of the S/M-SNARE complexes for structural determination.

## Membrane Topology of AtpI in *Escherichia coli*

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AtpI, encoded by the first gene of the *atp* operon of *E. coli*, is a hydrophobic, chloroform/methanol extractable protein of 14 kDa, which is present in substoichiometric amounts in purified $F_O$ and $F_OF_1$-ATP synthase preparations, although not necessarily part of the functional enzyme complex. Whereas AtpI is essential for the oligomerization of the *c* ring in $Na^+$-translocating $F_OF_1$-ATP synthases during assembly of $F_OF_1$, it is not absolutely required for the assembly of $H^+$-translocating $F_OF_1$-ATP synthases, although in its presence, stability and activity of the membrane-bound ATPase activity is modestly increased ([1] and references therein). Nevertheless, the *atpI* gene is highly conserved in most bacterial *atp* operons, although the protein exhibits a high variability in sequence and length.

Hydropathy plots as well as the positive-inside rule suggest a membrane topology of AtpI with four transmembrane segments and a $N_{in}$-$C_{in}$ orientation. To determine its topology, intact cells as well as inverted membrane vesicles enriched in AtpI carrying single cysteine substitutions were incubated with membrane-impermeable 4-acetamido-4‘-maleimidyl- stilbene-2,2‘-disulfonic acid to block thiol groups accessible from the water phase. Subsequently, blocked and untreated samples were labelled with membrane-permeable 3- (N-maleimido-propionyl) biocytin and detected with fluorescently labelled streptavidin after electrotransfer [2].

Our analysis verifies the presence of four transmembrane helices as predicted. At the periplasmic side of the membrane only a few amino acid residues were accessible to the non-permeable thiol reagent indicating the presence of small loops only. While at the cytoplasmic side of the membrane, the accessibility of both termini as well as the cytoplasmic loop is more extended as initially expected. Furthermore, hydrophobic, membrane-spanning segments shorter than 20 amino acids in length indicate the presence of a hydrophilic cavity probably comparable to the one observed for $F_O$ subunit *a* in the $Na^+$-translocating $F_OF_1$-ATP synthase of *Propionigenium modestum* [3] or the $H^+$-translocating $F_OF_1$-ATP synthase of *Polytomella sp*. mitochondria [4]. Further investigations are necessary to describe the interaction between those membrane-spanning segments of AtpI as well as the interface between AtpI and the subunit *c* ring.

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**Switching head group selectivity in mammalian sphingolipid biosynthesis by active-site engineering of sphingomyelin synthases**

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Sphingomyelin (SM) is a fundamental component of mammalian cell membranes that contributes to mechanical stability, signaling and sorting. Its production involves the transfer of phosphocholine from phosphatidylcholine onto ceramide, a reaction catalyzed by SM synthase SMS1 in the Golgi and SMS2 at the plasma membrane. Mammalian cells also synthesize trace amounts of the SM analog ceramide phosphoethanolamine (CPE), but the physiological relevance of CPE production is unclear. Previous work revealed that SMS2 is a bifunctional enzyme producing both SM and CPE whereas a closely related enzyme, SMSr/SAMD8, acts as a monofunctional CPE synthase in the ER. Using domain swapping and site-directed mutagenesis on enzymes expressed in defined lipid environments, we here identified structural determinants that mediate head group selectivity of SMS family members. Notably, a single residue adjacent to the catalytic histidine in the third exoplasmic loop profoundly influenced enzyme specificity, with Glu permitting SMS-catalyzed CPE production and Asp confining the enzyme to produce SM. An exchange of exoplasmic residues with SMSr proved sufficient to convert SMS1 into a bulk CPE synthase. This allowed us to establish mammalian cells that produce CPE rather than SM as principal phosphosphingolipid and provide a model of the molecular interactions that impart catalytic specificity among SMS enzymes.

**Microcompartmentation of the enzyme ferredoxin: NADP(H) oxidoreductase within the chloroplast.**

Manuela Kramer and Guy Hanke

Ferredoxin:NADP(H) oxidoreductase (FNR) is the final enzyme in the photosynthetic electron transport chain. It is capable of binding to, and being released from, several membrane complexes and dedicated tethering proteins in a regulated manner. These proteins are localized to the thylakoid membrane in chloroplasts – a dynamic and complex membrane structure comprised of sheets of "stromal lamellae" membrane stretching between stacks of pancake like structures known as "grana", to create a continuous membrane system with a single lumen. The protein contents differs between these microcompartments of the thylakoid membrane, resulting in localized areas with different pathways of electron transport. There is some evidence that relocalization of FNR can also regulate electron transport, but the spatial relationship of FNR interactions remains a mystery. In the first part of the project we have sought to define the different microcompartments on the thylakoid membrane and identify the regions to which FNR binds using immunostaining of isolated chloroplasts followed by confocal microscopy and immunostaining of leaf sections followed by transmission electron microscopy.

**Functional analysis of cardiac ECM components and their relevance to proper tissue physiology**

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The *Drosophila* dorsal vessel is embedded in an elaborate and highly dynamic extracellular matrix (ECM) that consists of tissue-specific structural proteins determining the physical / biophysical properties of the matrix, which in turn affects proper functionality of the whole tissue. Collagens like Pericardin (Prc) are essential to maintaining heart integrity, mainly due to the fact that the *Drosophila* heart lacks mechanisms for tissue repair or replacement. Although ECM alterations are present in major cardiac diseases, matrix composition or assembly are not fully understood.

To study the biophysical / biomechanical relevance of individual ECM components, we measure heart parameters, such as beating frequency, luminal distances, heart wall movement velocity, and stiffness. In this respect, the beating heart is visualized by recording live animals with a high-speed video camera. Corresponding physiological parameters are deduced by using a combination of two movement detection algorithms written in Matlab (The MathWorks. Inc., Natick, MA, USA). Utilizing the UAS-Gal4 system, we aim to increase / reduce the expression of individual ECM constituents in a heart specific manner, thereby modifying the stiffness-elasticity balance of the ECM. In addition, we will also analyze the effects of modulating the expression of factors being required for proper biosynthesis of collagens, e.g. lysyl oxidases. Finally, we aim to destabilize the ECM in a time dependent manner by collagenase treatment and monitor the effects on heart physiology over time.

These approaches may allow us to characterize the contribution of individual ECM components to the general physical properties of the cardiac ECM, and thus to understand their respective roles in maintaining proper heart function.

## Comparison of Conformational Dynamics of Sensory Rhodopsin II in Nanodiscs and SMALPs

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The transmembrane signaling complex NpSRII/NpHtrII plays a key role in negative pho- totaxis of Natronomonas pharaonis archaea. Structural information in terms of X-ray crys- tallographic data exists for the transmembrane part of this protein complex as well as for the rod shaped cytoplasmic part of the transducer due to its high homologies with chemorecep- tors. Photon absorption induces conformational changes in NpSRII, which are conducted to the transducer NpHtrII[1]. The transducer in turn regulates the phosphorylation level of a bound histidine kinase in the cytoplasm which modulates the rotation of the flagellum. The aim of this study is to understand the signaling mechanism within the sensory rhodopsin – transducer complex. To trace the kinase-activating signal along the extended transducer, we use site directed spin labeling in conjunction with EPR spectroscopy and photoactivation to probe the complex for conformational and dynamical changes starting from the transmem- brane region. The preparation of homogeneous and functional samples for the spectroscopy of membrane protein conformation and dynamics, especially for EPR applications, is chal- lenging. Current methods of purification of membrane proteins are based on using detergents, which could negatively affect the stability and activity of purified proteins[2]. Currently, two alternative membrane mimetic approaches, which provide monodisperse samples containing membrane proteins, are being developed, where the protein-lipid core are surrounded and stabilized either by an amphipathic helical protein belt, so called nanodiscs, or by a styrene- meleic acid (SMA) copolymer belt, so called SMA-lipid particles (SMALPs). It was shown that such model systems are well suitable for EPR spectroscopy or other single molecule experiments [3,4]. Here, we compare the applicability of both methods to NpSRII in pres- ence and absence of NpHtrII, reconstituted in nanodiscs and SMALPs, by using a set of time-resolved optical and EPR methods.

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**Exploring functional consequences of acute perturbations in the sphingolipid metabolic network by selective and inducible proteolytic profiling**

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Ceramides are essential but potentially lethal precursors of sphingolipids. Consequently, cells must monitor their ceramide levels closely to avoid killing themselves during sphingolipid biosynthesis. How this works is largely unexplored. We previously identified sphingomyelin synthase-related protein SMSr, an ER-resident ceramide phosphoethanolamine synthase, as critical regulator of ER ceramides and suppressor of ceramide-induced mitochondrial apoptosis. Using RNAi approaches, we showed that SMSr-mediated control over ER ceramides relies on both the enzyme's catalytic activity and its *N*-terminal sterile alpha-motif or SAM domain. The importance of SMSr as negative regulator of mitochondrial apoptosis is further emphasized by our recent finding that SMSr itself is a target of the apoptotic machinery and looses its SAM domain upon proteolytic cleavage by caspases. In this project, we use CRISPR/Cas9 technology to engineer HEK293 cells expressing endogenous SMSr carrying a tabacco etch virus protease (TEVP) cleavage site down-stream of its SAM domain. By employing HEK293 cells expressing TEVP under control of a tetracycline-inducible promotor, we seek to investigate the impact of an acute and specific proteolytic release of SMSr-SAM on ER ceramide levels and cell survival. In addition, we will integrate TEVP cleavage sites in the genomic loci of other mediators of sphingolipid homeostasis, such as the ceramide transfer protein CERT. We anticipate that our approach should help assign functional consequences of specific perturbations in the sphingolipid metabolic network and shed further light on key mechanisms underlying sphingolipid homeostasis.

**Role of retromer in cargo receptor recycling at endosome and vacuole**

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To maintain the cell homeostasis, several membrane proteins and other cargo are transported by vesicular transport along different pathways along the endomembrane system of a eukaryotic cell. Along the endocytic pathway, endosomes then mature to late endosome and finally fuse with lytic lysosome. Recycling of receptors in the retromer complex that associates with endocytic pathway requires the heteropentamer the cytosolic face of endosomes and mediates retrograde transport of transmembrane cargo to the trans-Golgi network. This process is highly regulated by Ypt7, a Rab GTPase of the yeast vacuole. To recapitulate the function of Ypt7 in receptor recycling, I have started to reconstitute the recycling process by purifying two subcomplexes of retromer: trimeric cargo recognition complex and a dimer of sorting nexins. I was able to purify retromer subcomplexes and determine their effects on membrane deformation using giant unilamellar vesicles. I also show by an in vivo recycling assay that recycling of the endosomal SNARE Pep12 requires retromer. Our data suggest a coordinated action of retromer with Ypt7 in cargo sorting and tubule formation, which is necessary for subsequent fusion with the Golgi complex.

## Interfacial fluorescence sensor proves respiratory supercomplex assembly *in cellula*

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Assembly of the mitochondrial OXPHOS complexes to macromolecular superassemblies was hypothesized in the 1960ties based on electron microscopic images. A role in physiological adaptation was derived from following supercomplex studies [1-3]. However, principally all research relies on detergent dependent membrane protein extraction. Alternatively, we here probed Fluorescence Lifetime Imaging Microscopy (FLIM) to prove respiratory supercomplex (SC) interaction in living cells (Figure 1). According to our fluorescence measurements, fluorescence lifetime sensors at two CIV subunits showed significantly reduced fluorescence lifetimes compared to subunits at other loci. 3D modelling based on the Althoff model [3] revealed that these subunits are buried in the contact site of supercomplex $CI_1CIII_2CIV_1$. Suppression of supercomplex assembly factors HIGD2A or Cox7a2l was accompanied by an increase in the mean fluorescence lifetime of the sensors. Moreover, respiration was clearly decreased in these respiratory SC downregulated cells. We interpret the results as electrostatic interaction with the adjacent proteins in this specific nanoenviroment and the first *in situ* proof of dynamic respiratory SC formation.

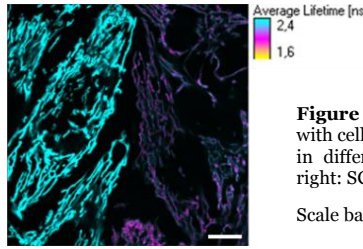


**Figure 1.** Fluorescence lifetime imaging with cell lines expressing EGFP derivative in different cell locations (left: mobile; right: SC embedded).

Scale bar: 10 µM.

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## The role of Atg9 and ist cycling system Atg2-Atg18 in the biogenesis of autophagosomes

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Autophagy is an essential degradation pathway, which not only enables the cell to adapt to nutrient starvation, but also serves as an important quality control mechanism. The hallmark of autophagy is the formation of a unique double membrane organelle – the so-called autophagosome - which engulfs portions of the cytoplasm and finally fuses with the lysosome/vacuole where the cargo degradation takes place [1].

During the formation of autophagosomes three proteins, namely Atg9 and the Atg2-Atg18 complex, form a distinct localization pattern at the edges of the emerging phagophore[2]. Although this observation gives a strong hint that Atg9 and Atg2-Atg18 must play a role during the elongation or the closure of the autophagosomal membrane, the hypothesis lacks supporting experimental data. Therefore, our aim is to use different *in vivo* and *in vitro* approaches to shed light on the structural and functional aspects of these proteins.

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## Quantitation of *Escherichia coli* phospholipids by MALDI TOF/TOF mass spectrometry

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The inner membrane of *E. coli* consists of roughly 70-75% phosphatidylethanolamine (PE), 20-25% phosphatidylglycerol (PG) and 5-10% cardiolipin (CL) with mainly three different fatty acid chains, C16:0, C16:1 and C18:1 [1]. Using MALDI TOF/TOF mass spectrometry, a semi-automatic set-up was designed that allows a quantitation of *E. coli* glycerophospholipids, extracted from intact cells, in a single step and enables a detailed analysis of the distribution of lipid classes, fatty acids as well as a comparison of the fatty acid composition of individual phospholipids.

Lipid standards of phospholipids present in *E. coli* membranes were used in defined amounts to determine the parameters essential for mass spectrometry (MALDI target plate, matrix, laser intensity, peak resolution, etc.) and to analyze the different phospholipid species. Whereas the variation in head groups has a high impact on the detection level within the MALDI TOF/TOF, the fatty acid composition of phospholipids naturally occurring in *E. coli* has no influence. To compensate the different characteristics, specific *flight factors* were defined for each lipid class. However, in dependence on the lipid mixture analyzed, individual phospholipid species reveal differences in miscibility and, therefore, different flight characteristics. To identify an internal standard that can be used independent of the composition of lipid extracts, a screening was performed for a phosphorylated compound with optimal flight properties. This internal standard now enables a quantitation of all *E. coli* phospholipid species by including so-called *miscibility factors* adapted to the corresponding lipid mixture.

To verify the method, phospholipid classes as well as fatty acid compositions of *E. coli* K-12 cells were determined at different growth phases and different growth temperatures. For the identification of the different phospholipid species a *lipid identification filter* was performed additionally to the accuracy of the molecular weight by fragmentation of the relevant molecules by post source decay. In addition, *E. coli* mutant strains carrying defects in the phospholipid head group biosynthesis were analyzed to confirm lipid identification. Interestingly, some of the mutant lipid extracts showed enrichment in precursors, which were also identified and quantified. The results obtained will be discussed in detail.

In summary, after extraction of *E. coli* phospholipids with an aqueous organic solvent mixture, all lipid species were identified and quantified by MALDI TOF/TOF mass spectrometry. Determination of phospholipid concentrations, head group *flight factors* and *miscibility factors*, if required, combined with the generation of a *lipid identification filter* now allows a rapid, detailed and comprehensive *lipidome* analysis for *E. coli*.

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## In *situ* single cell pull-down for probing stability and stoichiometry of cytosolic protein complexes

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Investigation of composition, stoichiometry and interaction dynamics of signaling complexes is crucial for understanding the various signaling pathways. We have developed in *situ* single cell pull-down (SiCPull) for specific and efficient pull-down of protein complexes from individual cells, based on engineered, micropatterned functionalized surface architectures [1]. Cells cultured on these surfaces are lysed by mild detergents, leading to almost instantaneous in *situ* capturing of GFP-tagged protein complexes, thus enabling the determination of their life-time, by monitoring the dissociation of prey proteins interacting with GFP-tagged bait proteins. Using SiCPull, we quantitatively determined the stability of various signal transducers and activators of transcription (STAT) complexes, ranging from seconds to nearly an hour. Strikingly, complex stoichiometry could be determined on the single cell level by single molecule imaging techniques. In combination with single cell manipulation, e.g. by microfluidics, this generic methodology provides robust possibilities to monitor stability and stoichiometry of cytosolic signaling complexes at different cellular states.

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**Single molecule exploration of the formation of the Wnt signalosome**

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Wnt signaling is a key regulatory mechanism for stem cell differentiation and embryonic development. A hallmark of canonical Wnt signaling is that the signaling relevant proteins assemble into multi-protein entities termed as 'signalosome'. To explore the molecular mechanisms of signalosome formation and the roles in spatiotemporal regulation of signal activation, we have orthogonally labeled the Wnt coreceptors, Fzd8 and Lrp6, in live cells. Time course single molecule imaging in the plasma membrane revealed a dynamic crosslinking of the receptors upon Wnt activation. We identified the formation of Wnt signalosome in the presence of Wnt ligands, as well as Wnt surrogates which can only form receptor hetrodimers. Our findings support a cytosolic driving mechanism for the formation of the Wnt signalosome. Further studies on the details of this mechanism and spatiotemporal manipulation of the Wnt signalosomes in live cells are ongoing.