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COMPLEX MEMBRANE PROTEINS
IN CELLULAR DEVELOPMENT AND DISEASE

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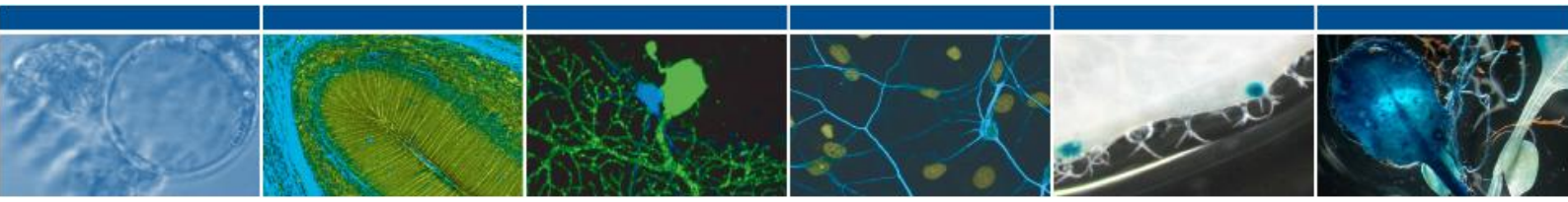

INTERNATIONAL RESEARCH TRAINING GROUP

4th Joint Symposium

Bad Dürkheim

September 14-16, 2015

DFG
TECHNISCHE UNIVERSITÄT KAISERSLAUTERN
UNIVERSITÄT DES SAARLANDES
UNIVERSITY OF ALBERTA
NSERC CRSNG

Compendium

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10.15 am</td><td>Breakfast</td><td>Breakfast</td></tr>
<tr><td>9 am</td><td>Manuel Daumann</td><td>Katherine Badior</td></tr>
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<tr><td>1.00 pm</td><td rowspan="4">Poster Session I</td><td>Laura Hofmann</td><td>Anil Kumar</td></tr>
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<tr><td>3.00 pm</td><td>Chris Cheeseman</td><td rowspan="3">Poster Session II</td><td rowspan="3">Trainee Meeting
(Good scientific Practice)

&

PI Meeting
(Renewal Proposal)</td></tr>
<tr><td>3.30 pm</td><td rowspan="4">Guided tour and wine tasting
at the awarded vineyard
“Fitz Ritter”</td></tr>
<tr><td>4.00 pm</td></tr>
<tr><td>4.30 pm</td><td rowspan="3">Meetings of Guidance
Committees</td><td>Concluding remarks</td></tr>
<tr><td>5.00 pm</td><td rowspan="4">End of Meeting
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Departure</td></tr>
<tr><td>5.30 pm</td></tr>
<tr><td>6.00 pm</td><td rowspan="4">Dinner in the
“Bad Dürkheimer
Riesenfass”</td><td>Dinner</td></tr>
<tr><td>6.30 pm</td><td rowspan="3">Visit of the “Wurstmarkt”
(Firework at 9 pm)</td></tr>
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20 min talk & 10 min discussion

Day One (Monday, September 14, 2015)

Arrival and Registration until 10 am

Session 1 (Großer Kurssaal; Chair: Anouar Belkacemi)

10:15-10:30	**Ekkehard Neuhaus**	Welcome
10:30-11:00	**Larry Fliegel**	Structural and functional analysis of genetic defects in the human Na^+/H^+ exchanger
11:00-11:30	**Ray Amith**	Na^+/H^+ exchange in breast cancer metastasis
11:30-12:00	**Kerstin Duscha**	Investigations on the necessity and function of the C-terminal tail of the sodium/proton exchanger *At*SOS1

12:00 – 13:00 Lunch

13:00 – 15:00 Poster Session I (Raum Hamburg)

Session 2 (Großer Kurssaal; Christopher Girke)

15:00-15.30	**Chris Cheeseman**	Important roles of cysteine residues in human glucose transporter 9 (HSLC2A9)

15:30 – 17:30 Guided tour and wine tasting (vineyard “Fitz Ritter”)

18:00 Dinner (Bad Dürkheimer Riesenfass)

Day Two (Tuesday, September 15, 2015)

08:00 – 09:00 Breakfast

Session 3 (Großer Kurssaal; Chair: Alican Erdogan)

09:00-09:30	**Manuel Daumann**	*A. thaliana* equilibrative nucleoside transporter 1 (*At*ENT1) - biochemical properties and topological analyses
09:30-10:00	**Hawraa Bzeih**	Maturation of synaptobrevin2-containing lytic granules (LGs) following endocytosis in primary mouse cytotoxic T lymphocytes (CTLs)
10:00-10:30	**Anna-Maria Miederer**	A STIM2 splice variant negatively regulates store-operated calcium entry

10:30 – 11:00 Coffee break

Session 4 (Großer Kurssaal; Chair: Manuel Daumann)

11:00-11:30	**Sabrina Marz**	Identification of potential interaction partners of glycine transporter 2
11:30-12:00	**Jessica Klement**	Reconstitution of the membrane enzyme protein OmpLA

12:00 – 13:00 Lunch

Session 5 (Großer Kurssaal; Chair: Qiaolin Hu)

13:00-13:30	**Laura Hofmann**	Structure-function correlations in TRPV6: A common mechanism in TRP channels?
13:30-14:00	**Qian Wang**	Critical residues involved in the channel function of TRPP3
14:00-14:30	**Sarah Haßdenteufel**	Role of accessory proteins of Sec61 in the human ER protein translocation machinery

14:30 – 15:00 Coffee break

15:00 – 16:30 Poster Session II (Raum Hamburg)

16:30 – 18:00 Meetings of Guidance Committees

	Raum Hamburg	***Raum Berlin***	***Foyer Raum Hamburg***	***Foyer Raum Berlin***	***Großer Kurssaal***
16:30 – 16:50	A. Blum M. Schmitt J. Rettig? J. Casey	H. Bzeih J. Rettig E. Cordat	K. Duscha E. Neuhaus J. Deitmer L. Fliegel	A. Erdogan V. Flockerzi	S. Haßdenteufel R. Zimmermann X.-Z. Chen
16:50 – 17:10	H. Lyrmann B. Niemeyer M. Schmitt	M. Textor S. Keller T. Möhlmann	Z. Naoshin H. Becker J. Deitmer E. Neuhaus	S. Marz E. Friauf V. Flockerzi T. Alexander	M.-C. Klein R. Zimmermann B. Niemeyer X.-Z. Chen
17:10 – 17:30	L. Forero J. Deitmer J. Casey	S. Schwartz M. Schmitt E. Cordat			A. Melnyk R. Zimmermann X.-Z. Chen
17:30 – 17:50	M. Daumann T. Möhlmann S. Keller J. Casey		H. Sarder M. Schmitt E. Neuhaus? E. Cordat		L. Hofmann V. Flockerzi X.-Z. Chen

18:00 – 19:00 Dinner

20:00 Visit of the Wurstmarkt

Day Three (Wednesday, September 16, 2015)

08:00 – 09:00 Breakfast

Session 6 (Großer Kurssaal; Chair: Gareth Amanious)

09:00-09:30	**Katherine Badior**	The many facets of SLC4 transporters; working towards a structure
09:30-10:00	**Darpan Malhotra**	Role of SLC4A11 in endothelial corneal dystrophies
10:00-10:30	**Andrea Blum**	A BRET assay for cell surface protein expression

10:30 – 11:00 Coffee break

Session 7 (Großer Kurssaal; Chair: Marie-Christine Klein)

11:00-11:30	**Cameron Smithers**	Using biophysical techniques to develop novel cancer therapeutics
11:30-12:00	**Linda Forero**	Potential role of astrocytic mono-carboxylate transporters in epilepsy

12:00 – 13:00 Lunch

Session 8 (Großer Kurssaal; Chair: Shane Wiebe)

13:00-13:30	**Anil Kumar**	Identification of novel peroxins modulating flavivirus replication
13:30-14:00	**Yannik Finger**	Managing adenine nucleotide pools across mitochondrial membranes
14:00-14:30	**Gareth Armanious**	Keeping the beat: Investigation of phospholamban variants from human clinical studies

14:30 – 15:00 Coffee break

15:00 – 15:30 Seminar on good scientific practice for Trainees
(Großer Kurssaal, afterwards Trainee Meeting **until 16:30**)

15:00-16:30 PI Meeting (Raum Hamburg)

16:30 Concluding remarks

End of Meeting

Talk abstracts in chronological order

Structural and functional analysis of genetic defects in the human Na^+/H^+ exchanger

L. Fliegel
Department of Biochemistry, Faculty of Medicine & Dentistry, University of Alberta, Edmonton, Alberta, Canada

The mammalian Na^+/H^+ exchanger isoform 1 (NHE1) is an integral membrane protein ubiquitously expressed in mammalian cells. It is made up of two domains - a 500 amino acid membrane domain that transports and removes protons, and a regulatory intracellular cytosolic domain of 315 amino acids. It plays a key role in cell growth and development and differentiation of cells and is involved in heart disease. We examined the role of NHE1 in genetic diseases in humans. Lichtenstein-Knorr syndrome is a rare autosomal recessive condition that associates sensorineural hearing loss and cerebellar ataxia. In Lichtenstein-Knorr syndrome a combination of homozygosity mapping and whole exome sequencing identified the homozygous Gly305Arg missense mutation in *SLC9A1* (NHE1) that segregates with the disease in a large consanguineous family. We demonstrate that the Gly305Arg mutation causes the near complete de-glycosylation, miss-targeting and loss of proton pumping activity of NHE1. NHE1 is identified for the first time, as being causal in this disease, evoking ataxia and hearing loss due to a complete or near complete loss of function. We also examine the effect of a genetic mutation of the Na^+/H^+ exchanger isoform 1, N266H. This was discovered in a human patient through exome sequencing. It was present in a patient that presented with spastic diplegia, seizures, autism, intellectual disability. Mutant N266H protein was expressed in AP-1 cells, which lack their endogenous Na^+/H^+ exchanger protein. Targeting of the mutant protein to the cell surface was normal and expression levels were only slightly reduced relative to the wild type protein. However, the N266H mutant protein had no detectable Na^+/H^+ exchanger activity. A histidine residue at this location may disrupt the cation binding site or the pore of the Na^+/H^+ exchanger protein. Supported by CIHR.

Na^+/H^+ exchange in breast cancer metastasis

R. Amith
Department of Biochemistry, Faculty of Medicine & Dentistry, University of Alberta, Edmonton, Canada

The leading cause of fatality in patients with breast cancer is metastasis. Triple-negative breast cancer (TNBC) is a clinical subset of breast cancer that occurs in 15-20% of patients. It is aggressively metastatic and has high recurrence rates, low responsiveness to chemotherapy, and poor prognoses for patient survival. Currently, no targeted therapies against TNBC exist. We have previously demonstrated a role for the Na^+/H^+ exchanger (NHE1) in controlling the metastatic potential of triple-negative MDA-MB-231 cells, where NHE1 activity was important in determining the rates of migration, invasion and *in vivo* xenograft tumor growth of these cells. We further investigated the effect of changes to NHE1 regulation by: $p90^{RSK}$/14-3-3 (ΔS703A), ERK1/2 (ΔS766, 770,771A or ΔSSSA), and calmodulin (ΔK641R643,645,647E or Δ1K3R4E). ΔS703A cells showed a dramatic difference in morphology, becoming smaller and reverting from a mesenchymal to an epithelial-like phenotype with a concomitant loss of expression of mesenchymal marker vimentin. Compared to cells expressing wild-type NHE1, ΔS703A cells had significantly lower rates of migration, invasion, anchorage-dependent and -independent colony formation, and spheroid growth. Migration and colony formation of ΔSSSA cells were also adversely affected by interfering with the activation of NHE1 by ERK1/2, but invasion and spheroid growth were

not. The phosphomimetic ∆1K3R4E mutant, where NHE1 auto-inhibition and its ability to bind calmodulin were negated, had much higher rates of migration, invasion and spheroid growth. Taken together, our data demonstrate, for the first time, a link between epithelial-mesenchymal transition and NHE1 regulation that is likely dependent on $p90^{RSK}$-mediated signaling downstream of ERK1/2, and the binding of 14-3-3. We suggest that Ser703 is a critical phosphorylation switch that regulates epithelial-mesenchymal transition in TNBC cells, and is thus a promising novel target for the development of new chemotherapies.

Investigations on the necessity and function of the C-terminal tail of the sodium/proton exchanger *At*SOS1

K. Duscha[1], L. Fliegel[2] & H. E. Neuhaus[1]
[1]Department of Plant Physiology, Faculty of Biology, University of Kaiserslautern, Germany
[2]Department of Biochemistry, University of Alberta, Edmonton, Canada

Soil salinity is a major environmental factor impairing plant growth and world-wide crop yield. The predominant overabundance of sodium imposes both, an osmotic and an ionic stress, thereby limiting cell metabolism and photosynthesis. In order to maintain sodium homeostasis, plants have developed three different mechanisms: (i) restriction of sodium influx, (ii) active extrusion of sodium from the cell across the plasma membrane or (iii) sodium sequestration in the vacuole. In *Arabidopsis thaliana*, the salt overly sensitive (SOS) signaling pathway is crucial for ion homeostasis and salt tolerance. It consists of three key components: SOS3, a calcium-sensor protein; SOS2, a serine/threonine protein kinase; and SOS1, a highly specific Na^+/H^+ exchanger localized to the plasma membrane.
SOS1 exhibits 12 transmembrane domains followed by a remarkably long (about 700 aa) hydrophilic C-terminal extension facing the cytosol. The necessity and function of this C-terminal tail unique to the SOS1 antiporter is still poorly understood. Physiological analyses revealed that the overexpression of the C-terminus results in a multitude of metabolic changes in the mutant plants during salt stress. The most stunning finding is that these mutants accumulate less sodium in leaves when exposed to 100 mM of salt stress over a period of 3 days, even though they only overexpress the soluble, cytosolic part of the transporter. The cellular trigger for these fascinating changes still has to be elucidated.
Furthermore, potential novel binding partners interacting with the C-terminal region have been identified by us in a pull-down assay and some of these physical interactions have been confirmed using Bimolecular Fluorescence Complementation. Thus, we have verified the interaction of SOS1 with the two 14-3-3 proteins upsilon and omega. The binding site has been found to be located at the very end of the SOS1 C-terminal tail, e.g. somewhere between the amino acids 978 to 1146. 14-3-3 proteins are regulatory proteins that act as phosphoprotein sensors, modulating the activity of a multitude of proteins after phosphorylation. The exact output of this interaction between the SOS1 and the 14-3-3 proteins still has to be elucidated.

Important roles of cysteine residues in human glucose transporter 9 (HSLC2A9)

W. Long, P.j Panwar, K. Wong, D. O'Neill & **C. Cheeseman**
Department of Physiology, University of Alberta, Edmonton, Canada.

Urate (uric acid) is a major end product from the breakdown of nutrients and cellular purines. A high level of serum urate, hyperuricemia, is associated with diseases like gout, diabetes and hypertension. The human glucose transporter 9, encoded by the hSLC2A9 gene, is a urate/hexose transporter and was found predominantly in the liver and kidney, where urate homeostasis is achieved. Many studies have been carried out to elucidate the structure function relationship between hSCL2A9 and urate transport. However, no study has been undertaken to look into the importance of cysteine residues in this transporter. Objective: To demonstrate the roles of different cysteine (C) residues in urate transport mediated by hSLC2A9 by mutating these cysteines into the corresponding residues that from hSLC2A5. Methods: Proteins were expressing in *Xenopus laevis* oocytes. Functional studies by 1. Radiolabelled ^{14}C urate flux measurements and 2. Micro-electrode Voltage Clamp. Quantitative and Qualitative protein analysis by biotinylation, Western blot and immunohistochemistry, Results and Significance: We employed the inhibitor, p-Chloromercuribenzoic acid (pCMBS) for urate transport mediated by hSLC2A9. Furthermore, we illustrated that C181 is partially responsible for the pCMBS inhibitory effect during urate transport. We also determined that C301 and C459 are two regulatory residues essential for maintaining the protein's structure required for urate, but not fructose transport. These findings advance our understanding of the structure of hSLC2A9, which will be beneficial for future development of drugs and disease treatment. Supported by CIHR; Department of Physiology Stipend Funding.

A. thaliana equilibrative nucleoside transporter 1 (AtENT1) - biochemical properties and topological analyses

M. Daumann & T. Möhlmann
Department of Plant Physiology, Faculty of Biology, University of Kaiserslautern, Germany

A lot of effort was expended to study the physiological role, the subcellular localization and biochemical properties of equilibrative nucleoside transporters (ENT´s) in mammalian, fungal and plant organisms. Whereas a lot of information on structure and topology for mammalian nucleoside transporters could be gained due to their role in treatment of cancer and viral diseases, that kind of knowledge is relatively little for plant ENT´s.

The *Arabidopsis thaliana* ENT1 (*At*ENT1) represents the sole tonoplastic ENT, exhibiting specific structural features. Via selective mutagenesis two residues were identified participating in binding of inhibitors. As a consequence, the formerly insensitive protein, reveals strong transport inhibition in the presence of NBMPR and Dilazep. Therefore, the mutated protein occupies distinct features known for mammalian ENT´s. Furthermore, Dixon blot analysis indicated that both inhibitors compete for the same binding site like *At*ENT1 substrates adenosine and uridine. To gain more structural information 3D modelling and topology analysis can open up new ways to elucidate structural properties of the membrane protein. Substituted cysteine accessibility assays represent one way to answer this protein topology questions. The methodical approach and respective results will be presented.

Maturation of synaptobrevin2-containing lytic granules (LGs) following endocytosis in primary mouse cytotoxic T lymphocytes (CTLs)

H. Bzeih, H.-F. Chang[1], V. Pattu[1] & J. Rettig[1]
Department of Physiology, Faculty of Medicine, Saarland University, Homburg, Germany

CTLs are specialized cells of the immune system that function in killing viral infected and tumor cells, releasing the content of their LGs at the cell-cell interface, the immune synapse (IS). After being exocytosed at the IS, LGs are recycled and reused for another round of killing by the CTL. We aim to elucidate the maturation of a recycling LG by tracking the endocytosis of a specific LG marker, synaptobrevin2 (syb2). We used synaptobrevin2-mRFP (syb2-mRFP) knock-in mice as a tool in our study. CTLs were incubated with target cells to induce LG exocytosis at the IS. Upon exocytosis, syb2-mRFP on the vesicle membrane moves to the plasma membrane and thus the mRFP is exposed to the extracellular medium making it accessible to anti-RFP antibodies added extra-cellularly. We used fluorescently labeled anti-RFP antibodies to either pH-insensitive Alexa Fluor dyes or pH sensitive dyes like pHrodo green. We investigated the time required for the endocytosed LGs to be acidified using an anti-RFP antibody double-labeled with Alexa-405 and pHrodo green. We could visualize the time point of LG endocytosis and acidification by this method. We found that endocytosed LGs are acidified shortly after endocytosis within an average time of one minute. Then we used different markers of the endosomal pathway such as rab5, 7, and 11 to elucidate the maturation of endocytosed LGs. We could show that endocytosed LGs colocalize with rab5-containing early endosomes to again become killing competent. Currently we are investigating the mechanism by which endocytosed LGs regain granzyme B (GzmB), how and where does this take place.
We have also investigated the interacting partners of syb2 during excocytosis. Using co-immunoprecipitation, we have found that SNAP 23 interacts with syb2 during exocytosis; other interacting partners remain to be elucidated.

A STIM2 splice variant negatively regulates store-operated calcium entry

A-M. Miederer, D. Alansary[1], G. Schwär[1], M. Jung[2] & B.-A. Niemeyer[1]
[1]Department of Biophysics, Faculty of Medicine, Saarland University, Homburg, Germany
[2]Department of Biochemistry, Faculty of Medicine, Saarland University, Homburg, Germany

Cellular homeostasis and Ca^{2+} signaling rely upon precise regulation of intracellular Ca^{2+} concentration. Alterations in basal [Ca^{2+}] can lead to various diseases and likely contribute to development of abnormal growth. Different regulators such as calmodulin and Ca^{2+} pumps limit cytosolic [Ca^{2+}] and their down-regulation by siRNA lead to an increased basal [Ca^{2+}]. Another important regulator is the stromal interaction molecule 2 (STIM2) that shows a reduction in basal [Ca^{2+}] following knock down. STIM2 (as the second isoform STIM1) is an ER resident membrane protein which senses the Ca^{2+} content of the ER via its luminal EF-hand. After partial or complete store depletion STIM-proteins multimerize and trigger store-operated calcium entry (SOCE) by directly gating Orai channels localized at the plasma membrane.
Here, we characterize a STIM2 splice variant, STIM2.1, which retains an additional exon within the region encoding the channel-activating domain. Expression of STIM2.1 is ubiquitous but its abundance relative to the more common STIM2.2 variant is dependent upon cell type and highest in naïve T cells. STIM2.1 knock-down increases SOCE in naïve CD4 + T cells, contrasting knockdown of STIM2.2 decreasing Ca^{2+} and SOCE. Conversely, overexpression of STIM2.1, but not of STIM2.2, decreases SOCE, indicating its inhibitory

role. FRET analysis showed that STIM2.1 interaction with Orai1 is impaired and prevents Orai1 activation. We are currently working on deciphering the *in vivo* relevance, endogenous protein localization and regulation of expression of STIM2.1 in T cells, natural killer cells and in neuronal cells. In addition, we aim to further clarify the Ca^{2+} dependency of STIM2.1's dominant-negative effect.

Identification of potential interaction partners of glycine transporter 2

S. Marz, C. Fecher-Trost & E. Friauf
Department of Animal Physiology, Faculty of Biology, University of Kaiserslautern, Germany

An important inhibitory neurotransmitter in the central nervous system of mammals is glycine. Glycinergic neurotransmission is terminated by glycine transporters (GlyTs). GlyT2 is localized in presynaptic membranes of neurons and takes up glycine from the synaptic cleft into presynaptic terminals, thereby maintaining glycine concentrations. GlyT2 have received growing attention as potential target for treatment of pain and hyperekplexia. Since protein-protein interactions can determine regulation of GlyT2, it is mandatory to investigate its interactome. Until today, seven interaction partners of GlyT2 have been identified: calnexin, syntaxin-1, Na^+/K^+-ATPase, syntenin-1, unc-33-like protein 6, plasma membrane Ca^{2+}-ATPase and Na^+/Ca^{2+}-exchanger. Yet a comprehensive analysis has not been performed thus far. The aim of this study is the establishment of a sophisticated proteomic approach for the identification of the interactome of GlyT2. To do so, co-immunoprecipitations (co-IPs) of GlyT2 and GST-Pull-Downs of GlyT2 C- and N-termini were optimized. As a prerequisite, a suitable GlyT2 antibody for co-IPs was launched. GlyT2 knockout animals as well as unspecific IgGs were defined as negative controls for co-IPs.
Furthermore, the expression of GST fusion proteins was established and the Pull-Down with GST itself was determined as negative control. Interactors, which bound to GlyT2, its C- or N-terminus during co-IPs or GST-Pull-Downs, were precipitated, separated on acrylamide gel electrophoresis and identified by mass spectrometry or western blot. GlyT2 co-IPs under stringent solubilization conditions revealed in a mass spectrometric analysis of three biological replicates 287 proteins were exclusively identified in the co-IPs of $GlyT2^{+/+}$ animals. 46 proteins of those were found in all biological replicates. Among these exclusive identified proteins, three already known interaction partners of GlyT2, calnexin, Na^+/K^+-ATPase and the plasma membrane Ca^{2+}-ATPase were identified. Because unc-33-like protein 6 interacts with GlyT2 only when phosphatase inhibitors are present during the co-IP its absence was expected. Together, these results provide proof of concept for a complementary proteomic approach with co-IPs and GST-Pull-Downs for the identification of novel interaction partners of GlyT2

Reconstitution of the membrane enzyme protein OmpLA

J. Klement
Department of Biophysics, Faculty of Biology, University of Kaiserslautern, Germany

Integral membrane proteins fulfil diverse physiological functions, represent nearly 30% of all proteins encoded in the human genome, may cause various diseases, and are targets of 50% of all drugs[1] However, the vast majority of currently available *in vitro* data pertains to water-soluble proteins because most membrane proteins lose their native structure and activity in aqueous environments. They are amenable to biochemical and biophysical

investigation only after reconstitution into artificial lipid membranes, which is often a trial-and-error approach [2]

To optimize the reconstitution process and correlate it with the biological activity of reconstituted proteins, we have chosen the membrane-bound enzyme outer membrane phospholipase A (OmpLA) for systematic reconstitution studies. OmpLA is a well-studied membrane protein, can be purified in high amounts and purities, catalyses a reaction that can be measured directly in liposomes with the aid of an enzyme activity assay[3,4], and has previously been used in our laboratory to dissect the influence of membrane mechanical properties on functional membrane-protein refolding[5]

The gene encoding OmpLA, *pldA*, was cloned and recombinantly expressed in *E. coli* cells before the protein was solubilised and refolded from inclusion bodies, which was followed by different chromatographic purification steps. This provided the starting material for our reconstitution experiments. So far, OmpLA could be successfully reconstituted—with a high protein/vesicle ratio—from lauryldimethylamine *N*-oxide (LDAO) micelles into 1-palmitoyl-2-oleoyl-s*n*-glycero-3-phosphocholine (POPC) liposomes preserving its biological activity. To prevent vesicle aggregation observed during the reconstitution process, further experiments with the lipophobic fluorinated detergent F_6OPC and a lipid mixture of POPC/POPG will be done. Dynamic light scattering and isothermal titration calorimetry experiments with F_6OPC have shown that F_6OPC does not solubilise POPC vesicles[6] and negatively charged POPC/POPG liposomes should repel each other; thus, both effects are expected to hamper vesicle aggregation. Moreover, first stop-flow light scattering measurements with OmpLA provided initial hints that OmpLA could contribute to water flow across the out bacterial membrane, which we will study in more detail in the future.

[1] Overington et al. 2006. How many drug targets are there? *Nat. Rev. Drug Discovery* 2006, 5: 993−996.
[2] Rigaud and Lévy 2003. Reconstitution of membrane proteins into liposomes. *Methods Enzymol*. 372: 65−86.
[3] Moon and Fleming. 2011. Side-chain hydrophobicity scale derived from transmembrane protein folding into lipid bilayers. *PNAS.* 108: 10174−10177.
[4] Snijder and Dijkstra. 2000. Bacterial phospholipase A: structure and function of an integral membrane phospholipase. *Biochim. Biophys. Acta.* 1488: 91−101.
[5] Herrmann et al. 2015. Modulating bilayer mechanical properties to promote the coupled folding and insertion of an integral membrane protein. *Eur. Biophys. J.* 2015.
[6] Frotscher et al. 2015. A fluorinated detergent for membrane–protein applications. *Angew. Chem. Int. Ed.* 2015, 54: 5069–5073

Structure-function correlations in TRPV6: A common mechanism in TRP channels?

[1]**L. Hofmann**, W. Zheng, [2]Q. Wang, S. Hussein, Q. Hu, F. Shen, T. Kong, [1]V.Flockerzi & [2]X.-Z. Chen
[1] Department of Experimental & Clinical Pharmacology & Toxicology, Faculty of Medicine, Saarland University, Homburg, Germany
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The function of mammalian TRP channels has been extensively studied for the past two decades (Montell, 1995; Philipp et al., 1996). Recently the publication of the near-atomic (≤4Å) structures of TRPV1 and TRPA1 by electron cryo-microscopy (Liao et al., 2013; Paulsen et al., 2015) has brought deeper insights into possible intra-molecular interaction and regulation. These biophysical and structural data are important to understanding TRP channel function but how the structure correlates with the function and gating has remained largely unclear. Based on sequence alignments we examined conserved amino acid residues in the N-terminal pre-S1 helix and C-terminal TRP (-like) domains of TRPP3, TRPP2, TRPV6 and other TRP members. It has been shown that these two domains are in close proximity and can interact with each other via hydrogen bonding and salt bridging in

TRPV1 and TRPA1, respectively. Within these domains we selected the conserved tryptophan (W) residue in the pre-S1 domain and W or tyrosine (Y) residue in the TRP-like domain in TRPP, -V, -M and -C subfamilies to determine their importance in channel function and gating. In this presentation I will show some functional data on TRPV6 and TRPP2. For TRPV6 and its mutants W361A, K360A (both N-terminal), and W633A (C-terminal) I employed the two-electrode voltage clamp technique after injection of cRNAs in *Xenopus laevis* oocytes. We found that both W361A and W633A exhibit substantial increases in channel function as compared with the WT channel while mutant K360A is similar to WT. Of note, the conserved residue K360 corresponds to the K in TRPV1 and –A1 that was thought to be critical in structure and function based on structural data. By Western blot and surface biotinylation analysis we confirmed that the protein expression and plasma membrane targeting of these mutants are similar to those of WT. We thus conclude that W361 and W633, but not K360, are functionally critical to TRPV6 channel function. For TRPP2 I employed Fura-2 Ca imaging to detect thapsigargin-induced increases in the cytoplasmic [Ca] of HeLa cells as a functional readout since TRPP2 is known to be mainly localized on the ER membrane. Preliminary data indicated that mutants W201A, Y684A and D511V (a known dead mutant as a control) have significantly reduced channel function as compared with WT TRPP2, suggesting that the conserved W and Y residues are functionally critical as well. Further Ca imaging experiments as well as those addressing protein expression and targeting will be performed before we could make conclusions. As TRP channels are distinctly regulated by PIP2, e.g., TRPV6 and –P2 are activated and inhibited by PIP2, respectively, it is interesting to note that the W/Y-to-A mutations in TRPV6 and –P also resulted in opposite changes in the function. Together with our more in-depth studies on TRPP3, presented by Qian Wang (oral) and Qiaolin Hu (poster), we think that, as a shared mechanism, TRP N- and C-tails bind to each other through π- π interaction mediated by these W/Y residues. We also think that this interaction may be involved in PIP2-induced regulation. Our studies on the corresponding W residues in TRPC4 and –M4 are underway.

Critical residues involved in the channel function of TRPP3

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TRPP3 belongs to the transient receptor potential polycystin (TRPP) superfamily, and is homologous to TRPP2 (also called polycystin-2) that is mutated in 15% of autosomal dominant polycystic kidney disease (ADPKD). TRPP3 was initially cloned from human retina expressed sequence tag and has been found with a wide expression pattern, especially in neurons of many organs such as brain, tongue and ear. When expressed in *Xenopus* oocytes, TRPP3 forms a Ca activated non-selective cation channel however the mechanism of activation is unknown. In order to investigate the molecular basis of TRPP3 channel gating and opening and, in particular, to identify amino acid residues critical for Ca-induced activation, we employed two-electrode voltage clamp technique in *Xenopus* oocytes together with mutagenesis. We have identified two critical residues, cysteine 38 (C38) and tryptophan 81 (W81) at the N-terminus and one critical amino acid tyrosine 564 (Y564) at the C-terminus of TRPP3. We found that 1) C38 is essential for the channel function of TRPP3, probably through mediating palmitoylation, and 2) There exists a structure and functional correlation between the N-terminal W81 and C-terminal Y564 residues. Ongoing experiments are being conducted to identify residues involved in channel gating of TRPP3 in the transmembrane

domain 6. In summary, given the recent reported structure of TRPV1 and TRPA1 which revealed a close proximity between the N-terminal pre-S1 domain and the C-terminal TRP domain, in which TRPP3 W81 and Y564 residues are respectively located, our data indicate that TRPP3 N- and C-termini may interact with each other through residues W81 and Y564 thereby controlling the opening or activation of the channel through conformational changes in response to ligand stimuli.

Role of accessory proteins of SEC61 in the human ER protein translocation machinery

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Directed and efficient protein translocation into the lumen of the human Endoplasmic Reticulum (ER) is mediated by a complex machinery of membrane embedded and associated soluble proteins. The central protein conducting channel formed by the heterotrimeric Sec61 complex has a highly dynamic structure which needs to be regulated. Thus, accessory components in close proximity influence the open-closed equilibrium of the channel to allow protein translocation across the membrane while keeping ion flux into the cytosol under control.

To resolve the role of Sec62, Sec63 and ER luminal Hsp70 chaperone BiP, we combine protein depletion in human HeLa cells by siRNA mediated gene silencing or proteolytic cleavage by bacterial Subtilase AB toxin, with *in vitro* protein transport into the ER of semi-permeabilized HeLa cells. Characterization of the posttranslational transport mechanism of two human short secretory proteins revealed a specifically involvement of Sec62, related to the translocational mode and precursor length, demonstrated by the effect of C-terminal extension by DHFR.

Other components of the translocation machinery, like Sec63 and BiP, appear to be involved independently of the utilized translolactional mode, rather in a substrate specific manner. Moreover, by using Sec63 mutants, we also shed light on different substrate specific functions of Sec63.

For further analysis of the substrate features conferring BiP dependency, we make use of signal peptide (SP) selective Sec61 inhibitor CAM741 and chimera derived of wild type Apelin or Statherin and vice versa exchanged SPs.

Though translocation in presence of CAM741 revealed different sensitivity corresponding to different properties of the Apelin and Statherin SPs, SP mutagenesis affecting CAM741 sensitivity is not influencing BiP dependency. Thus, the different dependency on BiP in translocation of Apelin compared to Statherin is most likely in accordance with the structural properties of the mature domain, confirmed by signal sequence exchange experiments.

We postulate a mature domain (Md) specific Sec61-gating via BiP, which is based on different modes of cytosolic Md engagement with components of the translocation machinery, like Sec63. As a consequence, Sec63 bound BiP may support channel opening by direct interaction with Sec61α-Loop7 on the lumenal site, as shown by the lacking rescue capacity of the Sec61-Y344H mutant defective in BiP binding.

The many facets of SLC4 transporters; working towards a structure

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The bicarbonate-transporting SLC4 membrane protein family contains ten biochemically and physiologically fascinating transporters. Members of this family include AE1, a red blood cell membrane protein with turnover rates of $5 \times 10^4\ s^{-1}$, and SLC4a11, which is responsible for a number of corneal dystrophies when mutated. For a membrane protein family with such widespread physiological significance, little is known about the structure of SLC4 transporters. With the goal of crystallizing a member of a SLC4 transporter, we have used a high throughput *Saccharomyces cerevisiae* expression system to identify highly-expressing members of the SLC4 family. Taking advantage of the genetics of *S. cerevisiae,* we use *in vivo* homologous recombination to quickly create a large number of recombinant plasmids for expression screening. With the addition of a GFP tag, 20 SLC4 proteins were screened for expression, and the full length *Arabidopsis thaliana* Bor1 (a plant orthologue of SLC4a11) was overexpressed at 1 mg/L culture. Large-scale expression and purification of AtBor1, followed by crystallization trials, is being conducted and optimized.

Role of SLC4A11 in corneal endothelial dystrophies

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Corneal Endothelial dystrophies are characterized by cloudy appearance of the cornea, loss of endothelial cells from their attachment site on the underlying Descemet's membrane (basement membrane) and subsequent loss of vision. SLC4A11, a water channel, is localized at the basolateral surface of corneal endothelial cells (CECs). Mutations of SLC4A11 result in loss of water transport across the endothelium and hence water accumulates in the stroma. This explains the clouding of the cornea. We hypothesize that SLC4A11 promotes adhesion of CECs to Descemet's membrane. Extracellular loop 3 of SLC4A11 does not play a role in the water transport function of the protein and may anchor CECs to Descemet's membrane. We coated pepsinized Descemet's membrane components on 96-well plates and studied the adhesion of HEK293 cells to these plates. Preliminary data suggests that cells expressing SLC4A11 adhere more strongly to the descemet's membrane components than do vector-transfected cells.
COL8A2, encoding Collagen type VIII, alpha 2 chain, is the major component of Descemet's membrane. Mutations of COL8A2 have also been reported to cause corneal endothelial dystrophies. Col8A2 may be the interaction partner of SLC4A11 in the Descemet's membrane.

A BRET assay for cell surface protein expression

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SLC4A11 facilitates water flux across the basolateral surface of the corneal endothelium. Point mutations of SLC4A11 usually result in misfolded and ER-retained proteins, which cause some cases of the corneal endothelial dystrophies, Congenital Hereditary Endothelial Dystrophy (CHED), Harboyan Syndrome (HS), and Fuchs Endothelial Corneal Dystrophy (FECD). Corneal transplantation is the only effective treatment option available for these

diseases, and hence it is necessary to develope alternative therapeutic strategies. The rescue of misfolded SLC4A11 to the plasma membrane by small molecular correctors could be used as a potential therapeutic strategy.
Bioluminescence resonance energy transfer (BRET) is the transfer of energy from a donor enzyme to a complementary acceptor fluorophore after substrate oxidation. This energy only excites the acceptor fluorophore if the acceptor is localized in a close proximity to the donor, for example, in the same membrane compartment. The acceptor excitation results in a light emission at a longer wavelength. In this study, we are utilizing BRET as a tool to identify compounds that restore the cell surface expression of the ER-retained mutant SLC4A11 proteins. In my part of the work, I optimized the assay for use on 96 well plates, created mutants and screened some previously known compounds to test for the folding correction of the protein.

Using biophysical techniques to develop novel cancer therapeutics

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Potential role of astrocytic and neuronal monocarboxylate transporters in epilepsy

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Monocarboxylate transporter (MCT isoform 1-4) mediate electroneutral co-transport of high-energy metabolites like lactate or ketone bodies with one proton. In the brain, MCT1 is expressed ubiquitously, MCT2 predominantly in neurons and MCT4 in astrocytes. Some studies have reported changes in the cellular distribution of MCTs in patients with temporal lobe epilepsy (Lauritzen, F. *et al*., Brain Struct Funct., 2013). One of the treatments used for refractory epilepsy is a ketogenic diet (KD), which has been shown to reduce the incidence of seizures. Additionally, KD has been shown to have an effect on the expression pattern of MCTs (Leino R. *et al.,* Neurochem Int., 2001). However, the molecular mechanisms by which ketone bodies reduce the incidence of seizures under KD remain poorly understood. In this project, we have characterized the transport of ketone bodies and lactate in primary neuron-astrocyte co-culture and acute slices from mouse cerebral cortex in normal mice and mice under ketogenic conditions. Ketosis was induced in neuron-astrocyte co-culture by incubation with β-hydroxybutyrate, and in the animal model, ketosis was induced by a four-week KD. To characterize the transport, intracellular H^+ concentration was measured using fluorescence imaging with confocal laser scanning microscopy (LSM). Epileptogenic activity was induced by exposing the slices to Mg^{2+}-free, bicuculline-containing saline (0 Mg^{2+}/BIC) and recorded with confocal laser scanning microscopy, using a calcium-sensitive dye. H^+ imaging experiments showed that ketosis leads to an increase in the transport activity of MCTs in cortical astrocytes in co-culture and acute slices, while no changes were observed in neurons. The frequency of Ca^{2+} spikes induced by (0 Mg^{2+}/BIC) was dramatically decreased in astrocytes from animals fed with the KD as compared to the control group. Inhibition of MCT1 and MCT2 with AR-C155858 showed an increase in the frequency of Ca^{2+} spikes in slices from ketogenic mice while a decrease in Ca^{2+} spiking

frequency was found in the slices from control mice. From these results we conclude that transport activity of MCT1 and MCT2 in astrocytes and neurons, respectively, in the cortex may be involved in the reduction of seizures under normal and ketogenic conditions.

Identification of novel peroxins modulating flavivirus replication

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Dengue virus and West Nile virus are two important mosquito transmitted human pathogens belonging to family *Flaviviridae*. In most cases both viruses produce a self-limiting disease with flu-like symptoms. However, a significant amount of patients develop the severe and lethal form of the diseases characterized by hemorrhage and shock syndrome for dengue and acute encephalitis in West Nile virus. Management of both diseases are presently limited to supportive care as no drugs or vaccines are yet available. Further investigation into the viral biology and identification of novel drug targets are critical in developing new therapeutics.

The present study seeks to identify and characterize novel human proteins that are essential for flavivirus infection. Based on previous studies in our laboratory, a significant drop in the number of peroxisomes was observed in flavivirus infected cells. Pex19, a peroxisomal protein was identified as interacting with flavivirus capsid and is also degraded during virus replication. In order to further analyze the role of peroxisomal proteins in flavivirus replication, I developed a high-throughput RNAi screening platform for both viruses. We have systematically screened ~40 peroxisome associated proteins and identified several proteins modulating dengue virus and West Nile virus replication. Currently we are validating these hits and investigating the possible mode of action of selected candidates.

Managing adenine nucleotide pools across mitochondrial membranes

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A fundamental challenge for living cells is meeting the energy demands to maintain essential processes. In order to achieve this, not only is it necessary to produce energy-rich molecules such as ATP, but also to transfer the energy stored in these molecules from their place of synthesis to where they are needed, i.e. where energy-demanding processes take place. To avoid the transport of ATP along large gradients by diffusion, several enzyme networks evolved which transfer energy-rich phosphoryl groups from mitochondria to other organelles. One of these networks is formed by adenylate kinases (AK), which catalyze the transfer of the γ-phosphoryl-group from ATP to AMP, resulting in the production of two ADP molecules. This reaction is fully reversible and multiple AKs can be linked to a so-called adenylate wire, in which phosphoryl transport is achieved at near-equilibrium concentrations from source to sink.

This transport chain also has to cross multiple membranes. Therefore several different isozymes of AKs, which are localized to different subcellular compartments, form phosphoryl-"bucket chains". One critical AK isozyme is AK2, which, due to its localization to the intermembrane space (IMS) of mitochondria, fulfills a relay role between mitochondrial matrix (ATP-source) and the rest of the cell (ATP-sink). The importance of AK2 is underlined by the

severity of reticular dysgenesis, a rare and invariably fatal immunodeficiency which is caused by loss-of-function mutations in the gene for AK2.
So far it remains unclear how AK2 becomes imported into the IMS, how it efficiently facilitates ATP transfer over inner and outer membrane and how it is regulated. We identified the protein as interaction partner of both the proteins Mia40 and ALR. The former protein is mainly involved in IMS import and oxidative protein folding, while functions for ALR, except for maintaining Mia40 functional, have not yet been demonstrated.
We could already demonstrate that AK2 constitutes a putative IMS-import substrate of the oxidoreductase Mia40. By a combination of cell biological and biochemical approaches we will further investigate the roles of Mia40 and ALR in AK2 import and folding, and control of AK2 function.

Keeping the beat: Investigation of phospholamban variants from human clinical studies

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Defects in the function of calcium handling and transport proteins are a hallmark of of cardiac dysfunction. Defects in calcium handling present themselves as changes in the cardiac calcium transient resulting in a reduced amplitude, and increased duration. This abnormal calcium handling results in decreased contractility and a reduced cardiac output.
SERCA achieves 70% of the calcium removal from the myoplasm of cardiomyocytes by actively transporting two calcium ions from the myoplasm into the sarcoplasmic reticulum (SR) per hydrolyzed ATP during diastole. During systole, the efflux of the stored calcium from the SR results in the activation of the contractile apparatus of the cardiomyocyte. Reversible inhibition of SERCA by the 52 amino acid SR membrane protein phospholamban (PLB) is crucial to controlling the rate of calcium sequestration, as well as the magnitude of the calcium gradient between the sarcoplasm and myoplasm. This in turn determines the rate of diastole and the force of contraction during systole. Unphosphorylated PLB decreases the apparent calcium affinity of SERCA, while β-Adrenergic-mediated phosphorylation of PLB at S16 by PKA, or at T17 by CaMKII partially restores SERCA activity.
New human mutations in PLB have been recently identified. An A15T mutation was identified in a 4 year old female DCM patient, and a P21T The effects that these variants of PLB have on the kinetics of SERCA, as well as their implications to the regulation of PLB via phosphorylation by PKA is currently under investigation.

Poster Sessions

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6	Klein, Marie-Christine	Functional characterization of a common variable immune disease-related SEC61A1 mutation in humans
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10	Rodriguez, Cristina	Investigations on the necessity and function of the C-terminal tail of the sodium/proton exchanger *At*SOS1
11	Sarder, Hasib	Intracellular trafficking and mis-trafficking of disease related plasma membrane proteins (kidney AE1) in yeast and mammalian cells.
12	Schwartz, Sara	Structural and functional analysis of yeast killer toxin K28
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Poster abstracts in alphabetical order

Identification & characterization of the TRPC6 protein

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The TRPC6 protein belongs to the transient receptor potential (TRP) protein family and shares 75% amino acids sequence identity with the closely related members TRPC3 and TRPC7. The expression of the TRPC6 cDNA in HEK293 cells or other cell lines is sufficient to yield an ion conducting TRPC6 channel, a calcium permeable non-selective cation channel. These results indicate that the TRPC6 protein is the ion conducting pore or the alpha subunit of the channel. In HEK293 cells TRPC6 can form a homomultimeric channel but it can also associate with other proteins like TRPC3 and TRPC7 to create a heteromultimeric channel. Whether such TRPC3/TRPC6/TRPC7 heteromeric channels are present *in vivo* and whether additional non-TRP proteins contribute to the TRPC6 channel complexes *in vivo* as beta or gamma subunits has yet to be shown. To identify TRPC6 proteins in human tissues we generated antibodies which allow detecting the respective protein in tissue homogenates and in Western blots. Thereby we identified the TRPC6 protein in human platelets. We then tested different detergents to solubilize efficiently the TRPC6 protein containing complexes from human platelets. Initial attempts have been made to establish an antibody-based affinity purification scheme to enrich TRPC6 protein complexes solubilized from platelets in the presence of 1% Digitonin. The bound TRPC6 protein complexes were eluted under non-denaturing conditions from the immobilized antibodies by the antigenic peptide which was originally used to generate the anti-TRPC6 antibody. As a control the same procedure was performed but using a not defined immunoglobulin fraction instead of the anti-TRPC6 antibody. The eluted proteins were run on blue native gels followed by mass spectrometry to identify the proteins associated and enriched with the TRPC6 protein. By pursuing these procedures we could identify the TRPC6 protein, which was absent in the control, but we could not detect TRPC3 and TRPC7. Instead a number of proteins appeared to be associated with the TRPC6 protein, the Inositol 1,4,5-trisphosphate receptors type 1 and type 2 (IP3RI/II) and the ARF GTPase-activating protein (Git1) proteins. By coimmunoprecipitation the interaction of IP3R1/TRPC6, of IP3R2/TRPC6 and of GIT1/TRPC6 could be confirmed. In the future work we want to map the binding site of these proteins for TRPC6 and to study their functional impact on the TRPC6 channel activity.

Designing an organelle specific calcium sensor to estimate the calcium concentration of lytic granules in murine cytotoxic T lymphocytes

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Cytotoxic T lymphocytes (CTLs) play an important role in our body's immune system. Their main effector function is to recognize and destroy viral infected, tumorigenic target or foreign cells. They contain cytotoxic proteins such as Granzymes and Perforin in specialized secretory granules termed lytic granules (LGs). LG contents are released upon target cell recognition at the highly dynamic CTL:target cell interface called the immune synapse. Ca^{2+} signaling inside the CTL is essential for its activation, effector function, tolerance of self-antigens and homeostasis. Despite it being such an important player, the calcium concentration inside LGs is unknown. We aim to estimate the Ca^{2+} concentration inside LGs and examine how this concentration regulates LG function. To this end, we have generated

organelle specific Ca^{2+} and pH sensors, which we attempt to use as a tool to calculate the absolute calcium concentration and the pH of LGs (secretory lysosomes). We selected ratiometric, FRET based troponin C calcium sensors (twitch calcium sensors), which have a K_d ranging from 150nM to 9.5μM (Oliver Griesbeck et al., 2014). The calcium sensors were fused to the C-terminus of the LG membrane protein Synaptobrevin2 (Syb2) therefore the sensor lying in the lumen of the LG. The calcium concentration inside the LG was measured by generating calibration curves at pH 7.3 with the help of Ionomycin. The pH of LGs is however acidic and therefore knowledge of the absolute pH inside LGs is essential for calibration of the Ca^{2+} sensor. To that end a ratiometric pH sensor called clopHensorN was generated by fusion at the C-terminus of GranzymeB. To confirm the localization of these sensors to lytic granules, mouse CTLs were co-transfected with Syb2-twitch or GraB-clopHensorN fusion constructs and GranzymeB-mTFP or stained with GranzymeB antibody. From the pH calibrations data, pH in LGs was measured to be 5.9±0.3. Further, we would like to calibrate the calcium sensor at pH 6 to obtain the absolute calcium concentration inside the LGs. For our future studies we aim to stimulate CTLs to induce LG fusion and then measure the calcium concentration through different stages of LG maturation.

Redox processes in complex I assembly

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Complex I of the respiratory chain of mitochondria is crucial for cellular energy production. It is also a major source of reactive oxygen species, and dysfunctions of the complex have been implicated in the pathogenesis of a variety of neurodegenerative disorders. Dysfunctions often occur as a result of an impaired assembly, but so far only little is known about the biogenesis and maintenance of Complex I in mammalian cells. Seven of its subunits are encoded in the mitochondrial genome, while the remaining 38 subunits have to be imported from the cytosol. Subunits are then sequentially assembled to give rise to the holoenzyme. We aim to characterize the function of four proteins in the assembly/maintenance of Complex I. The four uncharacterized nuclear-encoded Complex I subunits NDUFS5, NDUFB7, NDUFB10 and NDUFA8 lack typical mitochondrial import signals but instead contain conserved cysteine residues. The proteins are likely imported and trapped in the intermembrane space in a redox-dependent manner (twin-Cx_9C proteins). This process of oxidative folding is facilitated by the oxidoreductase Mia40 and is coupled to the activity of the respiratory chain. It is unknown how this redox pathway affects the assembly and maintenance of Complex I and whether the coupling to the respiratory chain provides a feedback control for respiratory chain assembly. Here, we will present our findings on the import and the function of these proteins as well as the role of Mia40 in Complex I biogenesis and maintenance on the molecular level.

Roles of C-terminal residues in TRPP3 channel function and gating

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Abstract: TRPP3, also called polycystic kidney disease 2-like-1(PKD2L1), is a member of the transient receptor potential (TRP) superfamily. TRPP3 is a Ca- andacid-activated cation channel that is involved in hedgehog signaling, intestinal development, and sour tasting. However, the mechanisms of TRPP3 channel gating and activation have remained unclear. Our lab recently identified a novel C-terminal domain K575-T622 as the first TRPP3 domain essential for both TRPP3 trimerization and channel function. In the present study, we did sequence alignments and found a highly conserved amino acid tyrosine (Y) in the C-terminal TRP-like domain. Using the two-electrode voltage clamp technique and *Xenopus laevis* oocytes expression, we found that mutants Y564A, Y564F, Y564H and Y564L are either functionally dead or show a significantly decreased function, while mutant Y564W is similar to WT TRPP3. Immunofluorescence or biotinylation studies indicate that they ll target to the surface membrane similarly as WT TRPP3. Together with data presented by Qian Wang, we think that the C- and N-termini bind with each other through π interaction between Y564 and the N-terminal W81, and that this binding is critical for channel activation or regulation by a ligand. Furthermore, based on the residues proposed to be involved in gating TRPV1 and TRPA1, we identified L557 in the TRPP3 transmembrane domain 6 to be a candidate gating residue. We then generated a series of mutants in which L557 is changed to a hydrophilic, hydrophobic or cationic residue. Our functional studies on these mutants support that L557 indeed acts as a gating residue of TRPP3. In summary, the present study identified C-terminal residues critical for TRPP3 channel activation or gating.

Lactate flux via monocarboxylate transporters is facilitated by 'proton-collecting antennae'

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The monocarboxylate transporters MCT1-4 mediate the H^+-coupled shuttling of lactate between glycolytic and oxidative cell types in different tissues like muscle and brain. We have previously shown that carbonic anhydrase isoform II (CAII) enhances transport activity of MCT1 and MCT4 by a non-catalytic mechanism (1, 2). CAII-induced augmentation of transport activity is mediated by functional interaction between MCT1/4 and CAII that requires direct binding of the enzyme to a glutamic acid cluster in the C-terminal of the transporter (3, 4). This binding of CAII to the C-terminal of MCT1/4 is mediated by CAII-His64, which is also involved in H^+-shuttling. To further investigate the augmentation of MCT activity by a putative H^+-shuttle, we inserted a cluster of histidine residues as "proton antenna" into the C-terminal of MCT4. Indeed, introduction of this His cluster led to a significant increase in MCT4 transport activity also in the absence of CAII, comparable to CAII-induced transport augmentation. This finding supports the hypothesis that it is a "proton antenna" in CAII that enhances the transport activity. We therefore tried to analyze the structure of the CAII "proton antenna" by heterologous protein expression in *Xenopus* oocytes. Our results showed that both Glu69 and Asp72 in CAII, which were proposed to be involved in intermolecular proton transfer between CAII and its surrounding area (5), are also essential for the enhancement of lactate transport via MCT1/4. Mutation of these residues

abolished the CAII-induced increase in MCT1/4 activity, although the direct interaction between enzyme and transporters seemed unaffected, as indicated by pull-down assay. However, injection of 4-methylimidazole, which acts as an exogenous H^+ donor/acceptor, can restore the ability of these mutants to enhance transport activity of MCT1/4. These results suggest that when CAII is directly bound to MCTs, identified residues of CAII can act as a "proton antenna", which removes or donates H^+ from or to the transporter pore, depending on transport direction, and distribute H^+ along the membrane to enhance H^+-driven lactate flux.

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Functional characterization of a common variable immune disease-related SEC61A1 mutation in humans

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The endoplasmic reticulum (ER) of human cells is a major storage compartment for calcium ions (Ca^{2+}) and a main site for protein synthesis. The translocation and membrane integration of newly synthesized proteins into the ER lumen and ER membrane, respectively, by the membrane resident Sec61 complex represents a crucial point in intracellular calcium homeostasis: calcium ions leak from the ER into the cytosol due to the steep ion concentration gradient between these two compartments. That's why the Sec61 channel needs to be tightly controlled by 'gate keepers' like calmodulin on the cytosolic and BiP on the ER lumenal side of the ER membrane. Their mechanism of limiting the calcium leakage from the ER has been investigated by our lab employing live cell Ca^{2+} imaging of HeLa cells using the ratiometric Ca^{2+} indicator FURA-2 in combination with siRNA mediated cellular depletion of proteins of interest or their inhibition of small molecules.
Recently, a SEC61A1 mutation which may lead to a Sec61 channel defect attracted our attention. This mutation is associated with the common variable immunodeficiency (CVID) which is the most abundant symptomatic primary immunodeficiency. Patients display a very heterogenous clinical phenotype which ranges from recurrent infections of the upper respiratory tract, an increased risk of developing autoimmune diseases to selective cancers. A hallmark of the phenotype is the complete absence of plasma cells from the peripheral blood.
According to our model, the underlying defect in B cell differentiation into plasma cells may result from an altered calcium homeostasis in affected cells. This would again emphasize the importance of preserving the Sec61 ion permeability barrier and its impact on the calcium homeostasis even with a pathophysiological background. To test this hypothesis live cell calcium imaging experiments are pursued in both a transient and a stable HeLa model cell system as well as further functional characterization.

Quantitative analysis of calcium dependent migration in human killer cells

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The killer cells of the immune system, such as cytotoxic T lymphocytes (CTL) and natural killer cells (NK cells) search the body for virus-infected and cancerous cells. After recognition of a target cell, the killer cell releases lytic granules to kill the target. To optimize the immune function, the migration machinery of the killer cells has to be tuned to maximize the probability of target cell encounter.
We studied the calcium dependence of NK and CTL migration by time-lapse microscopy on a flat substrate (2D). Our analysis revealed that the cells alternate between a mobile and a stationary state, where the probability of each state depends on the external calcium concentration. On a short time scale (seconds/minutes), the killer cells show directional persistence, whereas migration on a longer time scale (hours) is random. This mode of migration can be modelled as an intermittent, persistent random walk. Our model predicts that the direction as well as the speed of the cell is determined by the number of active motors within the cell. Through shape analysis during migration we want to test if the number of active motors predicted by the model coincides with the number of lamellipodia formed by the cell membrane. Furthermore simulations with this model reveal that, depending on the persistence time, a search time optimum can be reached in a given space for certain boundary conditions. We are currently investigating the behaviour of migrating killer cells upon contact with boundaries in 2D. We believe that modelling killer cell migration and search behaviour can help us modify and optimize the immune response in health and disease.

Sealing the human Sec61 translocation channel – Luminal chaperone BiP and the impact of the co-chaperone specificity

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Beside its relevance for protein synthesis and folding the mammalian endoplasmic reticulum (ER) constitutes also a main storage compartment for free calcium ions (Ca^{2+}). During translocation of newly synthesized proteins into the ER lumen by the membrane resident Sec61 complex calcium ions leak from the ER into the cytosol due to the ion concentration gradient between ER and cytosol. In previous work, calmodulin was shown to bind to the cytosol located IQ motif of Sec61α in a calcium dependent manner, thus leading to channel closure after calcium has started to leak out. Furthermore, the luminal Hsp70 chaperone BiP, an essential component of the translocation machinery, has been shown to counteract the loss of Ca^{2+} during translocation by binding to the ER luminal loop7 of the Sec61 channel and closing of the latter. To investigate and characterize this interaction of BiP and Sec61α we performed several binding studies such as surface plasmon resonance (SPR) spectroscopy, peptide arrays, pulldown- and photo-cross-linking experiments. Based on the findings that Kar2p, the yeast orthologue of BiP as well as a BiP mutant which does not bind the J-domain of Hsp40 co-chaperones are both unable to seal the channel, we focused on the interactome of the luminal chaperone network, to gain knowledge about the mechanism of recruiting BiP to the Sec61 channel. In combination with live-cell Ca^{2+} imaging we identified two Hsp40 co-chaperones, ERj3 and ERj6 whose depletion lead to an increased cytosolic calcium influx which is even more pronounced after simultaneously depletion of

BiP, which leads us to the model that these two ERj-proteins are co-chaperones of BiP in limiting ER Ca^{2+} leakage at the level of the Sec61 complex.

The impact of electrogenic sodium-bicarbonate cotransporter 1 (NBCe1) and sodium-potassium-chloride cotransporter 1 (NKCC1) on cytosolic sodium in mouse cortical astrocytes

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Astrocytes possess the electrogenic sodium bicarbonate co-transporter isoform 1 (NBCe1, 1 Na^+: 2 HCO_3^-) which is the major regulator of intracellular pH in astrocytes. Sodium-potassium-chloride cotransporter 1 (NKCC1, 1 Na^+:1 K^+: 2 Cl^-) regulates $[Cl^-]_i$, $[K^+]_i$ and volume in astrocytes. As both, NBCe1 and NKCC1 co-transport Na^+, activation of these transporters can lead to changes in $[Na^+]_i$ in astrocytes. To investigate their role on cytosolic Na^+ homeostasis, Na^+ changes were studied in primary cortical astrocyte cultures with the Na^+-sensitive fluorescent indicator. Our results shows that in WT astrocytes, changing from CO_2/ HCO_3^- free to CO_2/ 26 mM HCO_3^- -buffered saline mediated a Na^+ transient of ~6.4 ± 0.22 mM and removal of CO_2/ HCO_3^- led to a rapid undershoot of $[Na^+]_i$ which gradually returned to the initial baseline (recovery from sodium depletion). However, these CO_2/ HCO_3^- mediated changes in Na^+ were reduced or even reversed in NBCe1-KO astrocytes, indicating the transients were due to NBCe1 activity. In addition, application of a weak acid (40 mM butyrate) or changing $[HCO_3^-]_e$ (10 mM and 61 mM) also led to significant difference in $[Na^+]_i$ in WT and NBCe1-KO astrocytes. These observations show the involvement of NBCe1 in cytosolic Na^+ homeostasis. To investigate impact of NKCC1 on cytosolic Na^+, the activity of NKCC1 was inhibited by bumetanide and it was observed that the CO_2/ HCO_3^- mediated changes in $[Na^+]_i$ were significantly larger in bumetanide-treated WT astrocytes than in untreated astrocytes. Moreover, significant changes in the rate of recovery were observed in bumetanide-treated cells during recovery from sodium depletion, which indicated that NKCC1 carry Na^+ into cells after intracellular Na^+ depletion. These results suggest that NKCC1 is primarily a Na^+ loader while NBCe1 can operate in both directions across the membrane in cortical astrocytes and hence increase and decrease intracellular Na^+.
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Investigations on the necessity and function of the C-terminal tail of the sodium/proton exchanger *At*SOS1

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Soil salinity is a major environmental factor impairing plant growth and world-wide crop yield. The predominant overabundance of sodium imposes both, an osmotic and an ionic stress, thereby limiting cell metabolism and photosynthesis. In order to maintain sodium homeostasis, plants have developed three different mechanisms: (i) restriction of sodium influx, (ii) active extrusion of sodium from the cell across the plasma membrane or (iii) sodium sequestration in the vacuole. In *Arabidopsis thaliana*, the salt overly sensitive (SOS) signaling pathway is crucial for ion homeostasis and salt tolerance. It consists of three key components: SOS3, a calcium-sensor protein; SOS2, a serine/threonine protein kinase; and SOS1, a highly specific Na^+/H^+ exchanger localized to the plasma membrane.

SOS1 exhibits 12 transmembrane domains followed by a remarkably long (about 700 aa) hydrophilic C-terminal extension facing the cytosol. The necessity and function of this C-terminal tail unique to the SOS1 antiporter is still poorly understood. Physiological analyses revealed that the overexpression of the C-terminus results in a multitude of metabolic changes in the mutant plants during salt stress. The most stunning finding is that these mutants accumulate less sodium in leaves when exposed to 100 mM of salt stress over a period of 3 days, even though they only overexpress the soluble, cytosolic part of the transporter. The cellular trigger for these fascinating changes still has to be elucidated.
Furthermore, potential novel binding partners interacting with the C-terminal region have been identified by us in a pull-down assay and some of these physical interactions have been confirmed using Bimolecular Fluorescence Complementation. Thus, we have verified the interaction of SOS1 with the two 14-3-3 proteins upsilon and omega. The binding site has been found to be located at the very end of the SOS1 C-terminal tail, e.g. somewhere between the amino acids 978 to 1146. 14-3-3 proteins are regulatory proteins that act as phosphoprotein sensors, modulating the activity of a multitude of proteins after phosphorylation. The exact output of this interaction between the SOS1 and the 14-3-3 proteins still has to be elucidated.

Intracellular trafficking and mis-trafficking of disease related plasma membrane proteins (kidney AE1) in yeast and mammalian cells

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Kidney anion exchanger 1 (kAE1) is a bicarbonate exchanger in the basolateral membrane of α-intercalated cells of the human kidney responsible for the reabsorption of bicarbonate ions (HCO_3^-) by exchange with Cl^- ions to ensure acid excretion through urine [1]. Various genetically inherited mutations in the kAE1 encoding gene have been described which negatively affect HCO_3^-/Cl^- exchange, ultimately resulting in clinical disorders known as distal renal tubular acidosis (dRTA). Until now, autosomal dominant (AD) as well as autosomal recessive (AR) mutations have been identified which are either linked to kAE1 mislocalization or mistrafficking [2]. Since the underlying mechanisms of kAE1 mistrafficking are still poorly understood, we here use yeast as simple eukaryotic model organsim to dissect expression and intracellular targeting of wild-type kAE1 and its mutant variants in more detail. Since yeast Bor1p is proposed to be the homologue of mammalian kAE1 [3], we intend to (i) express codon-optimized versions of wild-type kAE1 and its clinically relevant mutant variants in a yeast Δbor1 knock-out mutant, (ii) analyze for functional complementation and (iii) determine intracellular kAE1 localization by confocal laser scanning microscopy (CLSM). In addition, a genetic screen of selected yeast knock-out and overexpressing mutants will be performed to identify cellular components involved in proper kAE1 trafficking and/or capable to restore kAE1 mistrafficking by redirecting mutant kAE1 back to the plasma membrane. In close collaboration with the IRTG partner lab of Emmanuelle Cordat, results from the yeast screen will be translated into the situation of mammalian cells to mechanistically understand the underlying principles of proper kAE1 targeting and clinically relevant mistrafficking in human cells.

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Structural and functional analysis of yeast killer toxin K28

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Controlled disulfide bond reduction is a key step in the intracellular trafficking of A/B protein toxins such as cholera toxin, Shiga toxin, ricin and yeast killer toxin K28. To investigate and molecularly dissect this process, we use K28 as model A/B toxin. Killer toxin K28 is produced and secreted by virus-infected *S. cerevisiae* strains and consists of two subunits (α and β) connected by a single disulfide bond. It is taken up by sensitive host cells by receptor-mediated endocytosis and travels the secretory pathway backwards via early endosomes, Golgi and ER to finally translocate into the cytosol. The molecular mechanism of its ERAD- and ubiquitin-independent retrotranslocation and the nature of the translocation channel in the ER membrane are still largely unknown. After reductive cleavage of the connecting disulfide bond in the cytosol, cytotoxic K28α is released and enters the nucleus to kill the host cell by blocking DNA synthesis. While K28 producing cells likewise take up K28, the retrotranslocated α/β heterodimer is rapidly degraded after being complexed by the cytosolic preprotoxin (pptox) precursor. To achieve this self-protection, S-S bond reduction within the K28/pptox immunity complex should be tightly controlled. In the present project we therefore investigate the properties of cysteine residues (Cys) and thiol redox control in K28 and try to solve its 3D crystal structure. Mature K28 possesses four cysteines (C56 in the α-subunit; C292, C307, C333 and C340 in the β-subunit). Mutational analysis and subsequent phenotypic and biochemical analyses of mutant K28 variants indicated that C56 and C333 are the most likely candidates which form the single inter-chain disulfide bond connecting both toxin subunits. We recently discovered that the three remaining Cys residues (C292, 307, and 340) are actively involved in the dissociation of the α/β heterodimer, while C292 seems not essential for K28 *in vivo* toxicity. In addition, we found that K28 is irreversibly inactivated at pH higher than 6 through the formation of S-S-linked toxin oligomers. Since such pH conditions are likely to occur *in vivo* during toxin passage through the Golgi (pH 6) and the ER (pH 7.2), we assume the existence of cellular factors in the ER (e.g. PDI) that control these reactions and prevent the undesired formation of toxin oligomers and α-subunit release.

Structural and funtional analysis of the human endoplasmic reticulum

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A decisive step in the biogenesis of many secretory and membrane proteins is the translocation into the endoplasmic reticulum (ER). This is mediated by a complex machinery of membrane embedded and associated proteins. The protein transport across the ER membrane can occur either co- or post-translationally through the highly conserved Sec61-complex. Immunoglobulin binding protein (BiP) is the major Hsp70 chaperone in the ER lumen. It facilitates translocation into the ER lumen because of its high affinity for hydrophobic peptides in the ADP-bound state. Therefore, each of the two peptide translocation mechanisms through the ER membrane, either co-translational or post-translational, can be inhibited in human cells by applying BiP-specific proteolytic inactivation or siRNA mediated gene silencing. After translocation into the ER lumen, the

polypeptide chains are processed, folded, and finally sent to their place of destination by vesicular transport.
Studies on the translocation machinery comprise the structural elucidation of the translocon complex as well as the functional characterization of transport mechanisms across the mammalian ER membrane.
To gain better insight into the substrate specific functions of components of the ER translocation machinery, we combine siRNA mediated gene silencing with quantitative mass spectrometry. Subsequently, candidate substrate polypeptides are evaluated in transport assays in vitro as well as in living cell. To determine the overall structure of the protein conducting channel Sec61 and additional complexes, cryoelectron tomography (CET) is used in combination with small interfering RNA-mediated gene silencing or plasmid-coded gene overexpression.
At present, sub-densities in the oligosaccharyl-transferase complex (OST) visualized by CET have to be structurally assigned to known subunits. The protein STT3A is present in the majority of OST complexes and mediates cotranslational N-glycosylation of most N-glycosylation sites on target proteins. In addition, results of cross-linking studies proved the spatial proximity of Sec61 and TRAM (translocating chain-associating membrane) protein, which regulates the exposure of nascent secretory proteins to the cytosol during translocation into the ER.
To ascertain the position of TRAM and STT3A in the ER membrane, several plasmids containing the coding sequences of both proteins were constructed by molecular cloning. Furthermore, after silencing of the respective genes in HeLa cells the resulting proteome was investigated by mass spectrometry. Based on these data, transport assays will be performed to validate the polypeptide transport into the ER after gene knock-down.

Reconstitution and membrane topology of Mistic from *Bacillus subtilis*

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Membrane proteins comprise more than half of all drug targets and, thus, are of outstanding scientific and medical importance [1]. However, membrane proteins are usually not readily amenable to *in vitro* studies because they require membrane-mimetic environments to retain their native structures and functions. To this end, detergent-solubilized membrane proteins are reconstituted into lipid bilayer membranes by lowering the detergent concentration to subsolubilizing concentrations. In contrast with other reconstitution strategies, detergent complexation with cyclodextrins [2] allows for a tight control of the reconstitution process and facilitates the rational optimization of experimental protocols because cyclodextrins sequester detergents at defined stoichiometries.
In this project, a quantitative model has been established to describe the phase transition during cyclodextrin-mediated reconstitution, which is monitored by isothermal titration calorimetry [3]. Proof-of-principle reconstitution of the α-helical membrane protein Mistic from *B. subtilis* [4] demonstrates the formation of well-defined, unilamellar, and uniformly sized proteoliposomes, as verified by dynamic light scattering and circular dichroism spectroscopy.
With the rational reconstitution protocol at hand, the membrane topology of Mistic was investigated with the aid of fluorescence quenching experiments and oriented circular dichroism. On the basis of these experiments, Mistic appears to be the first exception from the commonly observed transmembrane orientation of α-helical membrane proteins, since it

exhibits a highly unusual in-plane topology, with each of its two pairs of helices inserted into one of the two bilayer leaflets. This novel topology goes in line with coarse-grained molecular dynamics simulations of Mistic in a self-assembled DPPC bilayer [5].
Future efforts aim at elucidating how this topology is related to Mistic's function as an essential regulator of biof-ilm formation in *B. subtilis* and, in particular, how it affects Mistic's putative interaction with the potassium ion channel YugO [6].

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The role of NHE8 in renal proximal tubule calcium reabsorption

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Abnormalities in calcium homeostasis can result in osteoporosis and kidney stones. Improved therapies for these disorders can only be achieved through increased fundamental knowledge about calcium handling. In the kidneys, filtered calcium (Ca^{2+}) is reabsorbed along the nephron. To delineate the renal regulation of calcium reabsorption, we performed a micro array on mRNA from mice treated with the calcium sensing receptor agonist cinacalcet. This revealed that the sodium/hydrogen exchanger isoform 8 (NHE8) mRNA expression was decreased. These results were confirmed by quantitative PCR. Further administration of vitamin D to mice also decreased NHE8 mRNA expression. In contrast, by semi-quantitative western blot analysis renal NHE8 protein expression was increased in the same mice treated with cinacalcet or vitamin D. We therefore hypothesized that NHE8, similar to NHE3, plays a role in calcium homeostasis by facilitating the reabsorption of filtered calcium. To this end, we are validating a renal cell culture model, normal rat kidney (NRK) cells. We confirmed apical surface expression of NHE8 in NRK cells using surface biotinylation and immunofluorescence techniques imaging the Z plane of the cells. Functional studies demonstrate EIPA inhibitable sodium/hydrogen exchanger activity. We are also knocking down NHE8 and repeating these studies. To delineate the molecular role of NHE8 in calcium homeostasis, we will use this model to measure paracellular and transcellular Ca^{2+} flux across confluent monolayers in the presence and absence of functional NHE8. Future studies will explore the calcium phenotype in the NHE8 knockout mouse.

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Venue by car:
Use the highway A61, exit “Autobahnkreuz: Ludwigshafen” take the federal highway B 37, in Bad Durkheim follows signposting "Kurhaus / Casino"

Venue by train:
Mannheim main train station (ICE-connection), continue with the Rhein-Haardt-Bahn or via Neustadt main train station and then take the regional train. Exact departure times are available online: www.deutschebahn.com (from the station in Bad Dürkheim to the conference center it takes about 5 minutes by feet).