Annual Swiss Physiology Meeting 2017

Keynote speaker: Prof. Gisou van der Goot
Laboratory of Cell and Membrane Biology
EPF Lausanne, Switzerland

"Function and dynamics of protein palmitoylation"

A Forum for Young Physiologists:

Young Investigator Award: “Oetliker Prize”
to the best oral presentation and the best poster

Program and Abstracts

5 September 2017 University of Bern
Gertrud-Woker-Str. 5, 3012 Bern, Gemeinschaftshörsaal (GmH)
1. Program

09:30 - 10:10  Registration and coffee
University of Bern I Gertrud-Woker-Str. 5 I 3012 Bern,
Gemeinschaftshörsaal

10:10 - 10:15  Welcome and introductory remarks

10:15 - 11:15  Keynote lecture: Prof. Dr. Gisou van der Goot
Laboratory of Cell and Membrane Biology, EPFL
"Function and dynamics of protein palmitoylation"

11:30 - 12:30  Young Investigator Award: poster competition
(presenters must be by their posters)

Julia Baumann; Zurich: Inhibition of Furin arrests brain
endothelial cell migration and prevents TGFβ-mediated
permeability changes at the blood-brain barrier

Joaquim Blanch Salvador; Bern: Cardiac specific IP3R
overexpression: IP3ICR contribution in Ca2+ signalling

Jessica Brunetti; Genève: Localization of the splice variant
STIM1L in Human Skeletal Muscle

Morgan Chevalier; Bern: Regulation of the cardiac sodium
channel Nav1.5 by CASK is mediated by calcineurin

Vasiliki Delitsikou; Genève: Regulation of Klotho by
proteinuria in Chronic Kidney Disease

Mannekomba Diagbouga; Genève: Does primary cilium
influence the endothelial response to aneurysmal wall
shear stress?

Julia Günter; Zurich: The role of the deubiquitinase
OTUB1 in basal kidney function and renal inflammation
Christopher Henry; Genève: Does the architecture of the endoplasmic reticulum alter the propagation of cytosolic Ca2+ signals and the efficiency of Ca2+ store refilling during store-operated calcium entry?

Ji Huang; Fribourg: Role of arginase-II in regulation of renal aquaporin-2 and water reabsorption

Anna Keppner; Fribourg: Androglobin: a newly identified globin required for male fertility in mice

Ilaria Orlando; Zurich: Regulatory DNA elements modulating oxygen-regulated erythropoietin gene expression

Amalia Ruiz-Serrano; Zurich: Haploinsufficiency of the deubiquitinase OTUB1 affects energy metabolism and oxygen physiology in vivo

Zizun Wang; Bern: Abolition of the Dominant Negative Effect of Brugada Syndrome Variants

Yuyan Xiong; Fribourg: Role of arginase-II in regulation of aging

Yi Yu; Fribourg: Non-canonical effects of arginase-II in activation of mTORC1 through myosin-1b

12:30 - 14:00  Lunch and poster viewing

14:00 - 16.00  Young Investigator Award: Oral communications (15 min. each)

Beatrice Bianchi; Bern: Altered half-life of mutant TRPM4 proteins plays a major role in cardiac conduction disorders

Sheng-Fu Huang; Zurich: A new insight into the blood-brain barrier: Glutathione crosstalk between astrocytes and endothelial cells
Olga Komarynets; Geneva: Aldosterone controls primary cilium length and Ift88 abundance via mineralocorticoid receptor in the distal segments of the kidney tubule.

Lalita Oparija; Zurich: Regulation of basolateral amino acid uniporter LAT4 by phosphorylation

Duilio Potenza; Bern: Ablation of the RyR2-Ser2030 phosphorylation site limits changes in RyR2 sensitivity during β-adrenergic stimulation

Aderonke Sofoluwe; Genève: Reducing NETosis by targeting Pannexin1 channels

Chih-Chieh Tsao; Zurich: Pericyte HIF-1 deficiency modulates stroke outcome: Implications for a therapeutic time window of HIF-1 intervention

Sabrina Vullo; Lausanne: Conformational dynamics and role of the acidic pocket in ASIC pH-dependent gating

16:00 - 16:10 Jury session
16:10 - 16:30 Business meeting of the Society
16:30 - 16:45 Award ceremony: YIA Research Prize of the Oetliker Foundation 2017
17:00 End of meeting
2. Abstracts  (In alphabetical order)

Baumann, Julia  
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**Inhibition of Furin arrests brain endothelial cell migration and prevents TGFβ-mediated permeability changes at the blood-brain barrier.**  
*Julia Baumann, Chih-Chieh Tsao, Sheng-Fu Huang and Omolara Ogunshola*

A stable well-functioning blood-brain barrier (BBB), formed by specialized endothelial cells (EC), is crucial to maintain and control cerebral homeostasis. Indeed increased barrier permeability is a common feature of numerous CNS insults and diseases. Our group has identified transforming growth factor β (TGFβ) to be involved in vessel permeability changes in vivo. TGFβ is known to affect cell migration but also exerts apoptotic effects giving various potential mechanisms to disrupt the barrier. TGFβ induces the expression of Furin, a ubiquitously expressed proprotein convertase that stimulates extracellular matrix degradation and cell movement. This study aimed to investigate the potential roles of TGFβ and Furin in hypoxic barrier disruption.

In a Lucifer yellow-based transwell setup exogenous TGFβ (0.25μM) increased permeability of primary endothelial cells, but had no effect on cell viability. Subsequent in vitro experiments were performed on rat brain microvascular ECs (RBE4) exposed to normoxia and hypoxia (1% O2) for 6h. EC migration tended to increase already within 6h of hypoxia and exposure to TGFβ significantly potentiated this effect. Hypoxic exposure in presence of exogenous TGFβ increased both Furin protein levels and activity. Blockade of the TGFβ-receptor I (ALK5) with 10μM SB431542 reduced Furin protein levels indicating that its activation relies on the TGFβ signaling pathway. Importantly, pharmacological inhibition of Furin with Naphthofluorescein (20μM) not only prevented hypoxic EC migration but also abrogated the TGFβ-induced potentiation.

Taken together our data suggests a crucial role for Furin in mediating hypoxic and TGFβ-induced BBB permeability changes after insult. Such insights present novel potential targets for future therapy during injury and disease.
Altered half-life of mutant TRPM4 proteins plays a major role in cardiac conduction disorders

Bianchi B., Ozhathil L., Medeiros-Domingo A., Gollob MH., and Abriel H.

Transient receptor potential melastatin member 4 (TRPM4), a nonselective cationic channel, has been found to mediate cell membrane depolarization in immune response, insulin secretion, neurological disorders, and cancer. Functional mutations in TRPM4 gene have been linked to several cardiac phenotypes such as complete heart block (CHB), ventricular tachycardia, and Brugada syndrome (BrS). Despite many recent findings about functional implications of TRPM4 in cardiac diseases, the molecular and cellular mechanisms leading to altered conduction are not fully understood.

In the present study, we identify and characterize four novel TRPM4 variants found in patients with CHB or tachycardia. HEK 293 cells were transiently transfected with WT-TRPM4 and TRPM4 variants and then analysed using Western Blot and patch clamp for their biochemical and electrophysiological properties.

Three of them, A101T, S1044C and a double variant A101T/P1204L, led to a decreased expression and function of the protein. On the opposite, the variant Q854R showed an increase in TRPM4 current density. Recent evidences indicate that premature or accelerated degradation of mutant proteins represents a pathogenic mechanism underlying genetic diseases. In consequence, protein turnover of WT-TRPM4 and TRPM4 variants was analyzed using cycloheximide, an inhibitor of protein biosynthesis. Upon addition of cycloheximide, WT-TRPM4 resulted to have a half-life of ~ 20 hours, while loss-of-expression variants showed a 30% increase in degradation rate, with a half-life nearby 15 hours. Together, the gain-of-expression variant showed a higher stability and a doubled half-life compared to WT-TRPM4.

In conclusion, decreased or increased protein expression of several TRPM4 variants linked to cardiac conduction disorders was found to be caused by altered TRPM4 half-life and impaired protein turnover compared to the WT form, correlating misfolded-dependent altered trafficking with clinical outcome.
Inositol-1,4,5-trisphosphate (IP3) is a second messenger produced upon agonist binding to a G-protein coupled receptor (GPCR) and subsequently triggers SR Ca2+ release through openings of IP3 receptors (IP3Rs). In cardiac muscle, IP3R type 2 (IP3R2) is expressed both in ventricle and atria tissue. Several studies have focused on the functional interaction between the ryanodine receptors (RyRs) and IP3Rs in atrial myocytes. However, it is still unclear how IP3-induced Ca2+ release (IP3ICR) may contribute to excitation-contraction coupling in ventricle. Evidence suggests that IP3ICR modulates RyR function by fine-tuning of its local Ca2+ environment. Under pathophysiological remodeling conditions (e.g. heart failure) a functional interplay of IP3R and RyR Ca2+ events may be significantly pronounced. Our aim in this study was to examine the role of IP3ICR in Ca2+ homeostasis in a cardiac specific IP3R2-overexpressing mouse model (IP3/tTA).

Our experimental approach includes: pharmacological characterization of the IP3 signaling pathway; electrophysiology under whole-cell configuration of the patch clamp technique in combination with rapid confocal Ca2+ imaging and complemented with molecular biology approaches (qRT-PCR, Western Blot, Immunostaining).

Validation of the IP3/tTA mouse model returned an increase in IP3R2 protein expression accompanied by a decrease in RyR2 expression. In similar SR-Ca2+ loads, IP3/tTA ventricular myocytes present a higher local Ca2+ event frequency compared to FVB. However, a distinctive response upon IP3 stimulation was found: as reported by others, IP3 increases spontaneous Ca2+ events in ventricular myocytes; interestingly in IP3/tTA cardiomyocytes a decrease in Ca2+ event frequency was seen. Studies on intact cardiomyocytes revealed a shift towards a more RyR-independent IP3ICR. In other words, potentially pro-arrhythmogenic acting local Ca2+ events were reduced.

We conclude that overexpression of IP3R2 in ventricular myocytes significantly modulates Ca2+ homeostasis by regulation of SR-Ca2+ content.
IP3ICR could act as a protective regulatory mechanism in the context of ventricular arrhythmogenicity under various cardiac pathologies.

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**Localization of the splice variant STIM1L in Human Skeletal Muscle**  
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STIM1 (stromal interaction molecule 1) and Orai1 were described as the two main components of the store-operated calcium entry (SOCE). STIM1 is able to sense a decrease of the calcium concentration within the endo/sarcoplasmic reticulum. Orai1, a Ca2+ channel localized at the plasma membrane is gated by STIM1 following the calcium store depletion. In 2011, a new splice variant of the well-known STIM1 was discovered and identified in skeletal muscle. This new isoform was called STIM1 Long (STIM1L) since it contains 106 additional amino acids between exon 11 and 12 of the classical isoform, that we named thus STIM1 Short (STIM1S). STIM1S is ubiquitously expressed whereas STIM1L expression is mainly found in skeletal muscle. Furthermore, the expression of STIM1L increased during muscle differentiation, with no expression in the myoblasts and an expression similar to STIM1S in myotubes.

The aim of this study was to determine the localization of STIM1L in adult skeletal muscle and to compare it with STIM1S. This represents a first step in the general goal of understanding the physiological functions of STIM1S and STIM1L both during differentiation and in adult tissue.

To study the localization of STIM1L, we used a recombinant mini-antibody raised against STIM1L that was developed by the Geneva Antibody Core Facility (Faculty of Medicine, University of Geneva). In order to test the specificity of the antibody we performed immunostaining experiments using two models: Mouse Embryonic Fibroblast (MEF) cells line derived from Stim1-/-/Stim2-/- double knock-out (DKO) mice in which we overexpressed...
STIM1S, STIM2 or STIM1L, tagged with YFP, and human primary myoblasts and myotubes. To determine the localization of STIM1L, we used cryosections of hamstring muscle sample collected during an orthopaedic surgery. The detection of STIM1S was performed using a commercially antibody that recognize both STIM1S and STIM1L. In addition, co-immunostainings with STIM1L and STIM1S and other main proteins present on skeletal muscle were performed on longitudinal and transversal cryosections.

The specificity of STIM1L antibody was tested on MEF DKO cells. Only the cells overexpressing YFP-STIM1L allowed us to detect the signal with the antibody, showing that neither STIM1S nor STIM2 are recognized by the STIM1L antibody. Furthermore, STIM1L antibody did not reveal any signal in human primary myoblasts, while a clear staining was shown using differentiated myotubes.

Next, we studied the localization of STIM1L compared to STIM1S on longitudinal section of muscle tissue. Both STIM isoforms co-localize. Moreover, we compared their localization with Ryanodine Receptor (RyR, marker of T-tubules) and -Actinin (marker of the Z-discs). -Actinin co-localize with STIM1S and STIM1L, whereas RyR does not co-localize with both isoforms.

Moreover, STIM1L co-localize with SERCA, an ATP dependant-calcium pumps expressed in longitudinal part of the SR. Finally, on transverse sections using SERCA1 or SERCA2 as markers of fast and slow fibers, respectively, we showed that STIM1L is expressed in all fiber types.

In this study, we demonstrated that the antibody developed against STIM1L specifically recognized exogenous but also endogenous STIM1L isoform. Immunostaining experiments performed on human muscle cryosections showed that STIM1L and STIM1S have a similar localization in the fibers, mainly present at the level of the longitudinal SR and not close to the T-tubules. This is a surprising result as STIM1 (short and/or long isoform was not determined) was reported to be also present close to the T-tubule in mouse skeletal muscle. Further experiments are required to understand the difference observed between mouse and human STIM1S/L localization.
Skeletal muscle contraction and differentiation is controlled by cytosolic Ca2+ signals relying partly on store-operated Ca2+ entry (SOCE), a Ca2+ homeostatic mechanism mediated by STIM and ORAI proteins that links the depletion of endoplasmic/sarcoplasmic (ER/SR) Ca2+ stores to the activation of membrane Ca2+-permeable channels. Gain of function mutations in STIM1 or ORAI1 cause tubular aggregates myopathy (TAM), a skeletal muscle disorder with muscular pain, weakness and cramping. Here, we characterize two new mutations in the ORAI1 gene associated with TAM. Mutation V107M is located near the channel selectivity filter, and mutation T184M in the third transmembrane domain.

TIRF microscopy, calcium imaging, electrophysiology, molecular dynamic simulations. Clinical features differed between patients carrying the two different mutations, prompting us to test the regulation and Ca2+ permeability of the mutated channels. When ectopically expressed in HEK cells, both mutated channels formed plasma membrane clusters, conveyed high basal Ca2+ entry and increased SOCE, indicative of constitutive activation. Electrophysiological recordings revealed that currents carried by the mutated channel were of larger amplitude. The mutation V107M altered the Ca2+ selectivity of the channel, and decreased its sensitivity to acidic pH block. Mutation T184M was neither related to a defect in ROS or pH sensitivity, nor to a loss of Ca2+ selectivity, but might impact on neighboring transmembrane domains to alter the channel permeability.

By characterizing new mutations of ORAI1, we hope to improve our understanding of the channel permeation and regulatory properties. This will allow us to identify new targets for the development of therapies against diseases with gain of function mutations in ORAI1.
The voltage-gated Na+ channel, Nav1.5, is responsible for the rapid depolarization of the cardiac action potential (AP) and thus allows for the conduction of the electrical impulse throughout the myocardium. The channel Nav1.5 is present in different membrane domains in myocyte where it interacts with specific partners such as MAGUK (membrane-associated guanylate kinase) proteins, which are key regulators of ion channels. Among this family of proteins, CASK (calcium/calmodulin-dependent serine protein kinase) has been recently shown to interact with Nav1.5 at the lateral membrane of cardiomyocytes. Here, we investigate the role of CASK on the function of Nav1.5 in cardiac myocytes.

Recently, it has also been shown that CASK interacts with the calcium-dependent serine-threonine phosphatase: calcineurin in cardiomyocytes and inhibits its activity.

To assess the functional consequences of the interaction between CASK and Nav1.5 channels, patch-clamp experiments were first performed in TSA-201 cells transfected with shRNA plasmids to silence the endogenous CASK expression.

In addition, a CASK KO mouse model has been generated and WB was performed to confirm the absence of CASK in the heart. Sodium current was also recorded in isolated cardiomyocytes.

In order to assess the role of calcineurin in the CASK dependent regulation of Nav1.5, INa were recorded in CASK silenced TSA-201 transfected cells after treatment with two calcineurin inhibitors: cyclosporin A and CN585.

Duolink stainings were also performed in order to investigate the co-localization between Nav1.5, CASK and calcineurin.

It was found that sodium current (INa) is twice higher in TSA-201 cells where CASK was silenced. In CASK KO cardiomyocytes, INa is increased by 40% without any significant modifications of the steady state activation and inactivation. In addition, AP recordings were performed. It was shown that
AP threshold is significantly lower in CASK KO cardiomyocytes while dV/dt and resting membrane potentials are not significantly modified. In presence of DMSO 0.1%, INa is doubled in CASK silenced cells while after 1-hour treatment with cyclosporin A or CN585 10, two different calcineurin inhibitors, INa remains unchanged. Moreover, Duolink staining suggest a colocalization of Nav1.5 and calcineurin in TSA-201 cells. According to these results, calcineurin appears to be a positive regulator of Nav1.5 whose activity is inhibited by an interaction with CASK.

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Regulation of Klotho by proteinuria in Chronic Kidney Disease
Vasiliki Delitsikou, Romain Dissard, Frédérique Ino, Sophie De Seigneux

Albuminuria, caused by lesions in the glomerular filtration barrier, promotes tubular inflammation, apoptosis and fibrosis in Chronic Kidney Disease (CKD). Klotho is a transmembrane protein expressed in the proximal and distal convoluted tubules where it mainly regulates phosphate homeostasis and plays important role in CKD progression and extrarenal complications. We have shown previously that albuminuria is associated with lower Klotho levels. Therefore, we are aiming to further investigate how albuminuria regulates Klotho expression.

We induce high range glomerular proteinuria in a transgenic POD-ATTAC mouse model by inducing podocyte apoptosis, and collect kidney samples at seven days post-dimerizer injection. In vitro, we use a stable HEK cell line overexpressing the human transmembrane Klotho isoform as well as HK-2 cells.

In proteinuric mice, Klotho was downregulated at the mRNA and protein levels at day seven, and already from day three. Similarly in HEK overexpressing Klotho, the total and membrane fractions of Klotho protein were decreased when exposed to albumin, while the Klotho protein half-life was also reduced. We observed no modification in cleavage by ADAM10 or ADAM17 cell-surface proteases in vitro or in vivo. On the contrary, in both systems, ER stress response genes were induced in albuminuric conditions, with predominant ATF3 and ATF4 induction. To determine whether ER stress
may be involved in Klotho regulation, we used the ER stress inhibitors 4-PBA and TUDCA which restored significantly Klotho expression in vitro and in vivo. Finally, overexpression of ATF3 in HK-2 cells induced a downregulation of both Klotho protein and mRNA expression.

In our in vivo and in vitro models, ER stress appears to mediate at least in part the observed downregulation of Klotho protein by albuminuria. Further work on the effect of ATF3 on Klotho regulation is ongoing.

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Does primary cilium influence the endothelial response to aneurysmal wall shear stress?
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Intracranial aneurysms (IA) are local outpouchings of the wall of cerebral arteries occurring in 2-3% of adults. Its risk of rupture is influenced by the characteristics of the IA itself such as its location, size or shape irregularities, but also by patient-related factors like gender, age or hypertension. The physiology of the IA wall is not well understood, but it is generally assumed that the composition of the arterial wall is influenced by biomechanical forces imposed by blood flow such as wall shear stress (WSS). WSS is detected by multiple sensors in endothelial cells (ECs), including specialized structures called primary cilia. People affected by polycystic kidney disease (PKD) have abnormal or no primary cilia and are more prone to develop IAs with a prevalence of 4-40%. Moreover, individual IAs of PKD patients also seem more prone to rupture. Here, we test the hypothesis that defective
primary cilia may alter EC behavior, which, in turn, may affect the outcome of IA disease. 
First, we performed histological analyses on human IAs domes obtained after surgical clipping at the Geneva University Hospitals. Immunostaining for α-smooth muscle actin and CD68 revealed decreased smooth muscle cell and higher macrophage content in ruptured IAs domes (N=18) compared to unruptured IAs (N=31). Masson’s Trichrome, Picosirius Red and Victoria Blue stainings showed a reduced collagen content in ruptured IAs domes and fragmented but equal level of elastin in all IAs. Furthermore, preliminary data suggest that domes obtained from PKD patients exhibit a thinner IA wall containing less collagen compared to unaffected patients. 
Next, we used arterial ECs from wild-type and Tg737orpk/orpk mice, a transgenic mouse model for PKD that lack primary cilia, and exposed them to a physiological level of WSS (30 dynes/cm2) or low aneurysmal WSS (2 dynes/cm2). The endothelial response to aneurysmal WSS was investigated by an unbiased transcriptomic analysis (RNAseq). Immunostaining for acetylated α-tubulin revealed that the percentage of wild-type ECs containing primary cilia was low and not affected by the level of WSS, indicating that our model with wild-type and Tg737orpk/orpk cells will allow us to decipher the role of primary cilia in WSS-mediated EC response. Aneurysmal WSS compared with physiological WSS resulted in 58 differentially expressed genes in wild-type ECs, whereas 296 genes were differentially expressed in Tg737orpk/orpk ECs (fold change≥3; p≤0.001). Furthermore, Gene Set Enrichment Analysis revealed various differentially expressed pathways between wild-type and Tg737orpk/orpk ECs in response to aneurysmal WSS, which include gap junction-, Wnt- and Toll like receptor pathways (cilium-dependent pathways), as well as several commonly regulated pathways that include focal adhesion and extracellular matrix pathways (cilium-independent pathways)
In conclusion, our results show that the vascular wall of human ruptured IA present wall characteristics that are associated with increased vulnerability such as lower smooth muscle cell and collagen content and a higher macrophages content. Moreover, a 5-to-6-fold increase in the number of WSS-responsive genes was detected in Tg737orpk/orpk ECs, suggesting that primary cilia may have a dampening effect on the pathological response to WSS.
Long isoform of STIM1 and canonical transient receptor potential type 1 (TRPC1) in store operated calcium entry (SOCE).
Agnieszka Dyrda1,2, Stéphane König2, Laurent Bernheim2 & Maud Frieden1
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Store-operated Ca2+ entry (SOCE) takes place after Ca2+ depletion of the endoplasmic reticulum (ER). An indispensable step in ER Ca2+ store refilling is the activation of Orai1 channels gated by stromal interaction molecule 1 (STIM1). Orai1 is a highly Ca2+ selective channel localized in the plasma membrane whereas STIM1 is an ER sensor of Ca2+ concentration. The current mediated by Orai1 is called calcium-released activated calcium (CRAC) current (ICRAC). However in several cellular systems STIM1 activates not only Orai1 but also non-selective cationic channels of the canonical transient receptor potential (TRPC) family. Participation of both Orai1 and TRPC gives rise to SOCE current (ISOCE). Both currents have been intensively studied using patch clamp technique and can be differentiated based on the characteristic features of their current/voltage (I/V) relationships. The typical signature of the ICRAC is a strong inward rectification, absence of an outward current and very positive reversal potential (Erev > +50mV).

Our laboratory is working on mechanisms controlling human skeletal muscle differentiation, and we and others, established the crucial role played by SOCE in this process. Recently we identified a new splice variant of STIM1, called STIM1L (long) that has an extra 106 aa in the C-term part and is expressed mainly in adult skeletal muscles. We documented that STIM1L is as efficient as STIM1 in eliciting SOCE, but still very little is known about the channel(s) gated by STIM1L.

To answer this question, we used the whole-cell configuration of electrophysiological patch clamp technique. Currents were recorded in HEK293T cells transiently overexpressing Orai1 and STIM1 or STIM1L proteins. The values of Erev (to monitor the channel ionic selectivity) and current amplitudes at -100 mV and +80 mV (to monitor the inward and
outward part of the I/V curves) were chosen to compare between STIM1 and STIM1L activated currents.
We have recorded statistically significant reduction of the current amplitude (by ~50%) in HEK293T cells overexpressing Orai1 and STIM1L than in those transfected with Orai1 and STIM1. However, in both cases outward currents are present which together with less positive Erev points to the participation of other channels than Orai1 in the process. Our strong candidate was TRPC1 and its involvement in ER refiling was tested using several approaches: endogenous TRPC1 down regulation (siRNA), overexpression of mutated STIM1 unable to bind to and to activate TRPC1 (STIM1_EE), and overexpression of a non-conducting TRPC1 (TRPC1_DN) protein. In all these conditions the current activated by STIM1 or STIM1L has the signature of ICRAC, highlighting the implication of TRPC1 in STIM1/1L-activated current. The role played by TRPC1 in SOCE appears to be more important in STIM1 compared to STIM1L-induced SOCE.
We conclude that both Orai1 and TRPC1 are activated by STIM1 and the splice variant STIM1L. As these molecules are also expressed in muscle tissue, it remains to be determined whether they are also involved, together with other TRPCs, in human skeletal muscle SOCE.

Funding: Swiss National Foundation (Grant 310030_166313), Foundation Marcel Levaillant
Renal inflammation and inflammation-driven fibrosis are two major contributors to progressive kidney damage. Deciphering underlying signaling pathways is essential for the development of novel therapeutics. The deubiquitinase ovarian tumor domain-containing ubiquitin aldehyde-binding protein 1 (OTUB1) has been implicated in the regulation of key players of pro-inflammatory and pro-fibrotic signaling pathways in vitro. OTUB1 was further shown to be protective in renal ischemia-reperfusion injury in mice, while it was linked to disease progression in humans in glomerulonephritis. In a bioinformatic analysis, OTUB1 was associated with the regulation of basal transport processes in the renal collecting duct. However, the exact role of OTUB1 in physiologic kidney function and renal injury remains to be elucidated.

We first confirmed OTUB1 expression in the kidney and its reduced renal expression in OTUB1 haploinsufficient mice. OTUB1 heterozygous knockout resulted in a decreased urinary excretion of a multitude of urinary solutes. Investigating pro-inflammatory signaling in renal cells, we observed that increased as well as diminished OTUB1 expression resulted in a marked reduction in TNFα-induced NF-κB activity.

Overall, our results show that OTUB1 affects basal renal function and the pro-inflammatory TNFα-dependent signaling pathway in renal cells, indicating an important role of OTUB1 for kidney homeostasis in health and disease.
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Does the architecture of the endoplasmic reticulum alter the propagation of cytosolic Ca2+ signals and the efficiency of Ca2+ store refilling during store-operated calcium entry?

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Store-operated calcium entry (SOCE) is a cell signaling mechanism mediated by endoplasmic reticulum (ER) Ca2+ sensing proteins STIM that, upon Ca2+ depletion of the ER, interact with Ca2+-permeable ORAI1 channels at the plasma membrane (PM) to operate Ca2+ entry. STIM/ORAI1 interactions take place at membrane contact sites (MCS), sites of close apposition (<20nm) between the ER and the PM. MCS are dynamic structures that increase in number and size upon ER Ca2+ depletion, with sizes ranging from a few tens to a few hundreds of nm. The distance between the cortical ER (cER) and the PM is also dynamically regulated by tethering proteins such as extended synaptotagmins and varies from 20 nm to 8 nm. Whether the proximity and dimensions of the cER structures juxtaposed to the PM determine the homeostasis of the entering Ca2+ ions is unknown. My project aims to study how the architecture of the cER impacts the propagation of cytosolic Ca2+ signals and the efficiency of ER Ca2+ refilling during SOCE.

We used total internal reflection fluorescence (TIRF) microscopy to observe events occurring in proximity (100 nm) to the PM. We measured MCS lateral dimension by imaging fluorescent STIM molecules and recorded variations of the Ca2+ concentration in the ER or at MCS with Ca2+ sensitive probes targeted to the ER lumen or grafted to ORAI1 channels. cER proximity to the PM can be modified by expressing tethering proteins MAPPER short or MAPPER long and MCS dimension by expressing different STIM isoforms or adapters proteins such as Iست2.

Cells lacking the two STIM genes were transfected with STIM1-mCherry and D1ER, a ER Ca2+ sensor. Inhibition of SERCA pumps with cyclopiazonic acid (CPA) induced the formation of STIM1-mCherry clusters at the PM, which dissociated within minutes upon CPA removal and Ca2+ readmission, allowing us to monitor MCS dynamics and changes in the ER and cytosolic
Ca2+ concentration during the refilling phase. MCS of varied sizes were defined based on STIM1-mCherry intensity and sorted in 3 groups: small, medium and large, and the ER Ca2+ refilling kinetics between the 3 groups are being analysed. This study demonstrates the feasibility of concurrent recording of ER Ca2+ concentration in MCS of different sizes by TIRF microscopy. Analysis of the ER Ca2+ refilling kinetics between MCS of different sizes will reveal whether MCS dynamics impact the efficiency of the SOCE process.

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Role of arginase-II in regulation of renal aquaporin-2 and water reabsorption
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Type-II L-arginine:ureahydrolase, arginase-II (Arg-II), is abundantly expressed in kidney. However, the function of Arg-II in kidney remains unknown. In the present study, we aim to investigate the role of Arg-II in regulation of vasopressin-regulated water channel protein aquaporin 2 (AQP2) in collecting ducts and the impact on water balance. Mouse collecting duct cell line mCCDcl1 was cultured. AQP2 and Arg-II levels were assessed by immunoblotting and/or immunofluorescence staining. Silencing and/or overexpression of Arg-II was performed to investigate a role of Arg-II in AQP2 expression and membrane translocation. Urine parameters were analyzed by performing metabolic cage experiments in wild-type (WT) and Arg-II-deficient (Arg-II/-) mice under basal condition and water deprivation (WD) to assess urine concentrating ability.
In cultured mouse collecting duct cell line mCCDcl1, desamino-d-arginine vasopressin (dDAVP), a synthetic vasopressin receptor V2-agonist, stimulated expression and membrane translocation of AQP2 as expected and upregulated Arg-II levels. Silencing Arg-II further enhanced AQP2 expression and membrane translocation in response to dDAVP. Conversely, overexpression of native or an inactive Arg-II mutant suppressed the effects of dDAVP. In agreement with these findings in vitro, total and membrane-associated AQP2 levels were significantly higher in Arg-II-deficient (Arg-II/-) than wild-type (WT) mice, suggesting a negative regulation of AQP2 by Arg-II. Furthermore, the total and membrane-associated AQP2 levels in WT mice were increased by water deprivation paralleled with elevated Arg-II level in collecting duct cells, decreased urine volume and increased urine and plasma osmolality. Arg-II/- mice showed more pronounced water preservation effect under the water deprivation condition. Arg-II in collecting duct cells influences water balance through negative regulation of AQP2 expression and membrane translocation independently of its L-arginine:ureahydrolase activity.
A new insight into the blood-brain barrier: Glutathione crosstalk between astrocytes and endothelial cells

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Glutathione (GSH) is a thiol compound present in high concentrations in cells of all organs, it has many physiological functions including drug detoxification and defense against cell stress. Notably, in aging human brain and many neurological disorders and diseases it has been shown that GSH is below normal levels suggesting GSH is essential to support brain function. In the brain, astrocytes (AC) are the major GSH producers and the cellular link between neurons and the blood-brain barrier (BBB). AC secrete GSH to support neuronal survival but the contributions of GSH to BBB integrity are still unknown. The BBB is formed by endothelial cells (EC) that strictly control exchange between blood and brain compartment using tight junctions, maintaining brain homeostasis.

Primary brain microvessel EC and AC were isolated from rat to perform this study. For hypoxic treatments cells were cultured in 1% O2 in an InVivO2 400 hypoxia workstation (Ruskinn Technology, Pencoed, UK). Ischemia was mimicked in vitro by exposure of cells to O2 deprivation in glucose-free media. Immunostaining for ZO-1, claudin-5 and occludin was performed to observe tight junction formation. Paracellular permeability was assessed using Lucifer yellow to be a fluorescent marker. The GSH levels in cell lysates and conditioned media were assessed by sulfhydryl reagent 5,5′-dithio-bis (2-nitrobenzoic acid) (DTNB) to form the yellow derivative 5′-thio-2-nitrobenzoic acid (TNB), measurable at 412 nm. The mRNA levels were measured by quantitative PCR.

Our recent metabolomics data showed that GSH levels are higher in AC than EC during hypoxia/ischemia stimulating us to investigate GSH crosstalk between AC and EC under stress. Our results show that exogenous GSH improves EC tight junction localization and maintains EC morphology during 48h of normoxia and hypoxia/ischemia. Furthermore, exogenous GSH prevented hypoxia/ischemia-induced EC barrier impairment suggesting it benefits barrier stability. Interestingly, AC rapidly secrete GSH during 16h of hypoxia and ischemia. In correlation, mRNA levels of the GSH transporters
MRP2 and MRP4) significantly increase in AC between 6h and 24h of hypoxia/ischemia. Moreover, the γ-glutamyl transferase mRNA, an extracellular GSH stabilizer, was upregulated after 6h hypoxia/ischemia. This evidence indicates that AC increase the secretion and stability of GSH during hypoxia/ischemia. We are currently tracking isotope labeled endogenous GSH movement in our AC-EC co-culture system to confirm GSH shuttling from AC to endothelial cells. Such knowledge will provide potential candidates to develop novel strategies to reduce BBB impairment and brain injury.

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Androglobin: a newly identified globin required for male fertility in mice
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Globins are small globular metallo-proteins, involved in different cellular functions via their reversible binding capacity to gaseous ligands (O2, CO and NO) and their storage, transport and detoxification. Besides the well-known hemoglobin (Hb) and myoglobin (Mb), various new globin types have been discovered in vertebrates, including cytoglobin, neuroglobin, and more recently also androglobin (Adgb). Adgb is a chimeric protein, consisting of a calpain-like domain and a globin-like domain, and is mainly expressed in testis tissue. In this study, we aimed to analyse the in vivo function of Adgb, using transgenic and knockout mice. A gene-trap construct was inserted into the mouse Adgb gene, comprising a lacZ reporter sequence, and three loxP sites, thereby generating the Adgbtm1a line. By crossing these mice with CMV-promoter driven Cre-recombinase-expressing mice, Adgbtm1b mice were obtained, in which exons 13 and 14 of the Adgb gene were removed, with remaining lacZ gene expression. Finally, by crossing Adgbtm1a mice with both Flp-recombinase and subsequent Cre-recombinase-expressing mice, full knockout Adgbtm1d
mice were obtained, lacking the complete gene-trap insert and exons 13 and 14. All three mouse lines display male infertility at the homozygous state, indicating spermatogenesis-related defects. Our preliminary data suggest that Adgb plays a role in elongating spermatids as revealed by FACS analysis, and X-gal staining on microdissected seminiferous tubules shows predominant expression in the midpiece of elongating spermatids and mature spermatozoa. Interestingly, in humans, ADGB expression is strongly reduced in infertile men as compared to fertile individuals. Since the cell proliferation rate during spermatogenesis is extremely high, and considering the high oxygen gradient present in testis, the discovery of a globin domain-containing protein is of high interest and opens a new route towards the understanding of oxygen-dependent mechanisms involved in sperm formation.
Duchenne Muscular Dystrophy (DMD) results from the lack of expression of a functional dystrophin protein. In the absence of dystrophin the plasma membrane of skeletal muscles is weakened and more vulnerable to mechanical stress resulting in an increased calcium entry. To date however the underlying mechanisms of the disease remain unclear. But recently, it has been hypothesized that a family of proteins known as phospholipases A2 (PLA2) could be involved in the DMD pathology and exacerbate the inflammation process in damaged muscles.

We tested the role of secreted PLA2s (sPLA2s) during the differentiation of myoblasts from a non-affected newborn or from a patient with a Delta 48-50 in the dystrophin gene (human immortalized cell lines provided by the Genethon). As already shown, a hypotonic shock induced an abnormal calcium entry only in DMD myotubes. Interestingly, we found that down-regulation of pla2g5 in myotubes, which is over-expressed in DMD myotubes, decreased the abnormal calcium entry in response to a hypotonic shock. On the other hand overexpression of pla2g5 conferred sensitivity to hypotonic shock in control myotubes. We also observed that pla2g5 down-regulation led to myotubes hypertrophy exclusively in the DMD cell culture model.

Taking together, our results suggest that the overexpression of pla2g5 might induce intracellular calcium deregulation and alters myotube formation, two processes potentially implicated in DMD pathology.
Aldosterone controls primary cilium length and Ift88 abundance via mineralocorticoid receptor in the distal segments of the kidney tubule.

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The kidneys are extracting and eliminating toxic metabolites and electrolytes in order to control body fluid homeostasis. At the cellular level, the apical single non-motile primary cilium is a signalling hub and may function as a urinary chemosensor. The physiological processes and signalling pathways that control and are under regulation of the primary cilium remain poorly understood. We hypothesize that aldosterone, a mineralocorticoid hormone that stimulates sodium reabsorption in the distal nephron, modulates cilium length and thereby its functional properties.

Experiments were performed in cultured mCCDcl1 cells, a model of collecting duct cells and in transgenic mice with kidney tubule-specific knockout (KO) of the mineralocorticoid receptor (MR). Primary cilia were detected by indirect immunofluorescence using anti-acetylated a-tubulin antibodies and confocal microscopy. Cultured mCCDcl1 cells were exposed to aldosterone for 24h and challenged with bafilomycin A1 (100 nM) for 1h, afterwards transepithelial current was measured. mRNA and protein levels of mTORC1 and autophagy pathways were analysed by RT-PCR and Western-blotting assay.

In mCCDcl1 cells, a model of aldosterone-sensitive collecting duct principal cells, we observed that aldosterone-stimulated Na+ transport is correlated with lengthening of primary cilia. In inducible mineralocorticoid receptor (MR) knockout (KO) mice, displaying decreased sodium reabsorption along the aldosterone-sensitive distal nephron, primary cilia along distal convoluted tubules and collecting ducts were shorter compared to wild-type (WT) mice. We also analysed female mice with randomized deletion of the MR in renal tubule cells (MR/X mice). The primary cilium length was reduced in MR KO cells in comparison to MR WT cells, indicating that MR-dependent cilium growth relies on cell autonomous mechanisms.
In mCCDcl1 cells, the primary cilium length increase in response to aldosterone was associated with increased Ift88 abundance, a subunit of the IFT-B complex working with kinesin to deliver proteins from the base to the tip of ciliary microtubule bundles. This effect relies on decreased Ift88 degradation. We observed that aldosterone treatment lowered LC3b abundance, an indication of autophagy inhibition. This effect was associated with activation of the mTORC1 signaling pathway assessed by increased p70-Rsk phosphorylation levels. We also investigated the role of mTORC1 activation in the aldosterone-induced inhibition of autophagy. Rapamycin treatment strongly decreased p70-Rsk phosphorylation but did not prevent the inhibition of autophagy by aldosterone, indicating that MR controls autophagy via mTORC1-independent pathway. To reveal the role of autophagy in the cilium length control, we treated cells with bafilomycin which prevent the degradation of autophagosomes. Bafilomycin exposure mimicked the aldosterone effect on both autophagy and primary cilium length. Moreover, the inhibitory effect of aldosterone and bafilomycin, a vacuolar proton pump inhibitor, were not additive on both on LC3b abundance and cilium length.

Thus, we have shown that aldosterone controls primary cilium length by MR in a cell autonomous manner. This process may rely on increased Ift88 abundance via inhibition of autophagy.
System L amino acid transporter LAT4 (SLC43A2) is a sodium-independent uniporter that transports branched chain- and few other essential amino acids. Deletion of LAT4 leads to a lethal phenotype in mice, showing negative impact on transepithelial amino acid transport, as well as on intrauterine and postnatal growth. The function of LAT4 is most likely regulated to meet specific metabolic needs, however the regulatory mechanism is so far unknown. Proteomic studies indicate several possible phosphorylation sites on LAT4 (serines S274, S278, S297), therefore we hypothesized that LAT4 surface expression and/or function might be regulated by phosphorylation. To test this hypothesis, we characterized multiple (non-)phosphorylation mimicking hLAT4 mutants, expressed in X. laevis oocytes. We also investigated and visualized the overall phosphorylation state and specific phosphorylation sites of mLAT4 ex vivo. Functional measurements of phenylalanine uptake in X.laevis oocytes demonstrated that the non-phosphorylation mimicking mutant S274A exhibited an increase in transport function; the double mutant S274A+S278A displayed the most similar kinetics to the wild type hLAT4, whereas the S297A mutation led to severely reduced transport function. Immunofluorescence experiments showed changes also in subcellular localization. The alanine mutants S274A and S274A+S278A localized mostly near or at the oocyte surface; however the corresponding glutamate mutants, S278A mutant and wild-type hLAT4 appeared to localize more intracellular. Based on these results we suggest that S274 might play a role in LAT4 kinetics and localization in the cell, whereas S297 could be vital for overall LAT4 function.

Using high phosphate affinity SDS-PAGE gel system, we revealed that LAT4 is indeed phosphorylated in the kidney and small intestine of wild type (WT) mice. Phosphospecific antibodies confirmed phosphorylation on S274 and S297. With immunofluorescence tests we assessed pS274 patterns in the small intestine of WT mice, that had been challenged with time-restricted
feeding (8 hours) and sacrificed either 4 (ZT 0) or 16 (ZT 12) hours after the removal of food. In mice sacrificed at ZT 12, pS274 was detected at the tips and/or at the base of the villi along duodenum and jejunum; no phosphorylation was found in ileum. Mice sacrificed at ZT 0 showed higher pS274 levels, more even pS274 distribution along the villi and phosphorylation also in ileum. No correlation between LAT4 total protein expression level and pS274 patterns was found.
Therefore we conclude that (de-)phosphorylation of S274 might regulate LAT4 function in response to the local amino acid concentrations and availability.
Overall our results suggest that the surface localization and transport kinetics of LAT4 could be regulated by phosphorylation; showing such regulatory mechanism of a basolateral amino acid uniporter for the first time.
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Regulatory DNA elements modulating oxygen-regulated erythropoietin gene expression  
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Erythropoietin (Epo), the main hormone regulating red blood cells homeostasis, is produced by the fetal liver and adult kidney. Its physiological role becomes crucial if the delivery of oxygen to our tissues/organs is compromised e.g. at high altitude, following blood loss or due to chronic kidney disease. In response to anemia or inspiratory hypoxia, renal Epo is produced by peritubular interstitial cells, called renal erythropoietin producing (REP) cells, located at the juxtamedullary cortex. While Epo transcriptional regulation has been widely studied in the liver due to the availability of human hepatoma cell lines, the transcriptional regulatory elements of the kidney still remain to be clarified due to the lack of a REP cell culture system.

To investigate the modulation of oxygen-regulated EPO expression, Hep3B and HepG2 as well as the neuronal cell line Kelly have been employed.

We previously identified a novel 5' hypoxia response element (5'-HRE) within the putative kidney inducible region upstream of the EPO gene and compared its function with the well-known downstream 3'-HRE within the liver inducible region. Using Hep3B cells, we demonstrated that both the 5' and 3'-HREs are bound and trans-activated by hypoxia-inducible factor (HIF)-2. In order to further dissect the contribution of the 5' and 3'-HREs to endogenous EPO gene expression, we used CRISPR/Cas technology to disrupt the two loci in hepatoma and neuroblastoma cells.

While the 3'-HRE but not the 5'-HRE is required for hypoxic Epo induction in hepatic cells, both the 5’ and 3’ HREs appear to contribute to neuronal Epo induction by hypoxia. These data suggest that Epo regulation in REP cells may have more in common with neuronal cells than with hepatic cells, a finding that is supported by the unexpected finding of neuronal markers on REP cells in vivo.
Ablation of the RyR2-Ser2030 phosphorylation site limits changes in RyR2 sensitivity during ß-adrenergic stimulation

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During physical exercise or stress, the sympathetic system stimulates cardiac contractility via ß-adrenergic receptor (AR) activation, resulting in protein kinase A (PKA)-mediated phosphorylation of the cardiac ryanodine receptor, RyR2. Hyperphosphorylation by PKA at the RyR2-Ser2808 site has been proposed as a key mechanism responsible for cardiac dysfunction in heart failure (HF). However, the sites of PKA phosphorylation in RyR2 and their phosphorylation status in HF are not well defined. Recently, a new PKA phosphorylation site has been identified and proposed as a mediator of the adrenergic response, Ser2030. Our hypothesis is that phosphorylation of RyR2 at Ser2030 site may be an important event in modulating RyR2 activity, and dysregulation in its phosphorylation level may be associated with altered calcium handling.

We examined the contribution of RyR2-Ser2030 to the excitation contraction (EC)-coupling mechanism using experimental approaches on cellular and subcellular levels and a transgenic mouse with ablated RyR2-S2030 phosphorylation site (RyR2-S2030A). EC-coupling gain was assessed with the whole-cell patch-clamp technique and confocal Ca2+ imaging while the ß-ARs were stimulated with Isoprotenerol (Iso). Recent studies have suggested that refractoriness of Ca2+ spark triggering depends on the sensitivity of the ryanodine receptors. Moreover, it was demonstrated that adrenergic stimulation shortens RyRs refractoriness. We measured Ca2+ spark restitution using the “low-dose ryanodine method” in which repetitive sparks originating from the same cluster of RyRs are recorded. To confirm phosphorylation at S2030 during Iso application we performed western blot (WB) experiments using phospho and de-phospho specific antibodies. In the end we also measured the latency of the first spontaneous Ca2+ wave (SCW) after a train of stimulation, an indicator of arrhythmogenicity in intact cells.
At matched Ca2+ loading of the sarcoplasmic reticulum (SR), the EC-coupling gain in Iso was diminished in mutant compared to WT cardiomyocytes. In addition, Ca2+ waves elicited by SR Ca2+ overloading showed no acceleration during Iso treatment, unlike WT cells. Mutant cells showed also decreased frequency of spontaneous Ca2+ release during Iso application (lower Ca2+ spark frequency). Using "ryanodine method”, we found that mutant RyRs sensitivity was not enhanced by Iso application, contrary to WT cells. In the end, we also found lower occurrence of Ca2+ waves, and longer latency of the first wave after electrical stimulation. Together, our results suggest that ablation of the RyR2-S2030 site may result in a blunted increase of RyR2 Ca2+ sensitivity upon β-adrenergic stimulation, and that the site represents a link between the adrenergic pathway and modulation of RyR2 channel activity.

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Haploinsufficiency of the deubiquitinase OTUB1 affects energy metabolism and oxygen physiology in vivo
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Deubiquitinating enzymes are key regulators of ubiquitin signaling, which plays a major role in virtually all cellular signaling pathways and, hence, in cell and tissue homeostasis. Ovarian tumor domain containing ubiquitin aldehyde binding protein 1 (OTUB1) is a unique deubiquitinase, catalysing the hydrolysis of Lys48 ubiquitin chains and, as non-canonical activity, inhibits the formation of Lys63 and Lys48 Ub chains. We recently discovered that OTUB1 is a substrate of factor inhibiting HIF (FIH). FIH is a cellular oxygen sensor and hydroxylates asparagine 22 of OTUB1. Mutation of N22 to a non-hydroxylatable amino acid affected the interaction of OTUB1 with important
regulatory proteins of cellular energy metabolism. OTUB1 has further been linked to the regulation of apoptosis, DNA repair, pro-inflammatory and pro-fibrotic signalling pathways and estrogen signalling. However, its physiological role in vivo is unknown. Investigating the phenotype of Otub1 haploinsufficient mice, we observed an increased fat/lean mass, body weight and plasma insulin level together with a decreased maximal exercise capacity. Furthermore, these mice demonstrated an altered respiratory response to hypoxia. Therefore, we hypothesize that OTUB1 is a novel regulator of energy metabolism in vivo and plays an important role in oxygen physiology.

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A high throughput screen identifies forskolin and a B-RAF inhibitor as podocyte-protective molecules
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Urgently needed therapies for kidney disease have been lacking for several decades. Recent discoveries revealed that damage and loss of glomerular podocytes contribute to progressive kidney diseases. Metabolic disorder-related chronic kidney diseases have become the major cause for kidney failure. Metabolic disorders such as type 2 diabetes have been linked to disturbed endoplasmic reticulum (ER) homeostasis and signs of ER stress have been found in glomeruli of patients with established diabetic nephropathy. The objective of this study is to establish and validate a high throughput screen assay to identify and subsequently characterize small molecules preventing podocytes from ER stress. Conditionally immortalized podocytes were differentiated for at least 11 days. Cell death was assessed by quantifying relative ATP-levels (high throughput screens) and annexin V positivity. shRNA-based gene silencing was performed by using a lentiviral system. mRNA and protein expression, and protein localization were analyzed by qPCR, Western blotting and IF staining, respectively. We developed, validated and applied an ATP–based high throughput podocyte viability assay to screen small molecule libraries and to identify
podocyte death–preventing compounds. A proof of concept screen of a library of clinically used and preclinical bioactive molecules identified forskolin, an adenylate cyclase agonist, and the B-RAF inhibitor GDC-0879 as inhibitors of ER stress-mediated podocyte death. cAMP (forskolin) and GDC-0879 did not alter the ER stress response and the effects were independent of their well characterized targets PKA and Epac, and BRAF, respectively. These findings led to the identification that forskolin and GDC-0879 protected podocytes from a variety of cell death-inducers. Interestingly, gene – and protein profiles pointed to a mild but effective support of the cells’ adaptive responses such as protein biosynthesis.

The development and successful implementation of a high throughput assay to identify podocyte-protective compounds revealed that forskolin and the BRAF inhibitor GDC-0879 prevent podocytes from ER stress-dependent and – independent cell death and our data indicate that their effects are linked to a beneficial fine-tuning of the cells’ adaptive responses to stress. Future studies will aim to identify these targets and their mechanism, which ultimately may contribute to a better understanding of podocyte death and provide strategies to prevent from it.
Neutrophils are the first responders during inflammation resulting from tissue injury or infection. One of the defense mechanisms employed is the Neutrophil Extracellular Trap (NET) formation (referred to as NETosis) which leads to the expulsion of nuclear contents into the extracellular environment as a trap for pathogens and foreign particles. Prolonged recruitment of neutrophils and delayed clearance of NETosis leads to tissue damage, which makes this process deleterious in inflammatory and autoimmune diseases. Pannexin1 (Panx1) is a member of the three transmembrane protein family called Pannexins. Panx1 functions as a hexameric transmembrane channel connecting the intracellular and extracellular space, which allows the exchange of ions and small molecules (up to 1 kDa). These channels are implicated in the initiation of immune response and inflammation through the regulation of extracellular ATP release. Specifically, neutrophil chemotaxis, which involves the autocrine purinergic activation of surface receptors, is also linked to ATP release by Panx1 channels. This study aims at unravelling a potential role for Panx1 in NETosis.

Here, we examined the contribution of Panx1 channels to NETosis by using bone marrow-derived neutrophils (BMDNs) obtained from wild type (WT) and Panx1-/- mice. Panx1 expression was analysed at the mRNA and protein level by RT-qPCR and immunofluorescence, respectively. In addition, Panx1 channel function in neutrophils was confirmed using YO-PRO-1 dye uptake assay, in the presence or absence of a Panx1 inhibitor. As NETosis is a dynamic...
process, we developed a fluorescent assay to measure extracellular DNA release over time using the cell impermeable Sytox green dye. NETs were induced with calcium ionophore A23187 (1μM), phorbol 12-myristate 13-acetate (10nM) or Staphylococcus aureus strains (MOI 10), to evaluate the different mechanisms of NETosis activation. Significant Panx1 mRNA expression was confirmed in the BMDNs. Protein analysis using immunofluorescence revealed specific membrane-localized expression in these cells. Measurements of extracellular DNA release over time generated sigmoidal curves, which were analyzed using various parameters. Slope analysis was performed on the quantifications and results indicate the reduction of NETosis in Panx1-/- BMDNs compared to WT BMDNs (49.34 AU ± 10.07, 80.36 AU ± 8.765 respectively, p<0.05, Student t test). Our results also indicates that Panx1 modulates kinetics during NETosis induction and this is dependent on the inducing agent.

In conclusion, our work shows for the first time that, Panx1 is expressed in mouse BMDNs and that blocking Panx1 channel function decreases NETosis. Ongoing studies should provide detailed insights into the specific role of Panx1 channels in the signaling pathway leading to NETosis. Presently, we are investigating the contribution of ATP release, via Panx1 channels, in the induction of NETosis. Understanding Panx1 channel function might point to a new therapeutic target for inflammatory diseases in which NETosis contributes to the pathophysiology.
Activation of hypoxia inducible factor-1 (HIF-1) is crucial for cells to adapt to hypoxic/ischemic stresses. For decades, HIF-1 stroke research has mainly been limited to neurons with stabilization of HIF-1 suggested as a potential neuroprotective strategy. However, successful employment of such strategy means the impact of HIF-1 induction on non-neuronal cells needs to be thoroughly understood during cerebral ischemia. Pericytes play a key regulatory and structural role in the ischemic blood-brain barrier (BBB). However, the consequences of altered pericyte HIF-1 signaling during stroke remains unknown.

Recently, we have generated a mouse line with pericyte-targeted HIF-1α knockout (KO) under the control of the inducible Cre/Lox system. The mouse line enables us to specifically explore effects of pericyte-mediated HIF-1 signaling on stroke outcome and BBB modulation after ischemic insult. We subjected pericyte HIF-1 KO and WT mice to transient middle cerebral artery occlusion (tMCAo) for 45 mins using intraluminal filament method followed by reperfusion up to 14 days. Ischemic damage, BBB permeability, neurological scores were assessed.

Intriguingly, our data suggested stabilization of HIF-1 in pericytes differentially modulates barrier integrity and stroke outcome during stroke progression. Firstly, at 72h post-tMCAo HIF-1 deletion reduced IgG extravasation and Evans blue leakage. Although overall infarct volume was unaffected, reduced neuronal degeneration, less cell apoptosis and more astrocyte activation were observed in HIF-1 KO peri-infarct cortex by immunostaining. Therefore, HIF-1 deletion preserves barrier integrity and improves stroke outcome at the subacute stage. Surprisingly however, at 14 days reperfusion, reversal of positive effects of HIF-1 deletion was seen. HIF-1 KO mice exhibited higher blood vessel density compared to WT controls in
correlation with increased permeability. Furthermore, enhanced brain shrinkage and Flurojade C-positive processes were observed in KO mice. Taken together, our results highlight a time-dependent effect of pericyte HIF-1 stabilization on BBB regulation after insult. The switch from maintenance of vascular homeostasis to negative outcome suggests a therapeutic time window should be considered when targeting HIF-1 for stroke therapy.

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The Acid-Sensing Ion Channels (ASICs) are pH sensors in the nervous system. Under normal physiological conditions, the intra- and extracellular pH is maintained between 7.3-7.0. High neuronal activity or pathological states such as inflammation or cerebral ischemia alter the pH and modulate the function of various receptors and ion channels. ASICs are Na+ channels that are activated in response to lowering of the extracellular pH. There is strong evidence for important roles of ASICs in several physiological and pathological conditions such as fear behavior, neurodegeneration after ischemic stroke, learning and memory processes, and pain sensation. ASICs exist in three different functional states: closed, open and desensitized. The crystal structure of the chicken ASIC1, which shares 90% sequence homology with the human ASIC1a, has been determined in the open and desensitized states; the conformation of the closed state is however unknown. The crystal structure reveals a channel composed of three subunits arranged along a central pore. Each subunit contains two hydrophobic transmembrane domains, short N- and C- termini, and a large extracellular loop. A close view of the ASIC structure reveals a unique region characterized by a cluster of several acidic amino acids within a small space, the so-called «acidic pocket». The presence of many negative charges in close proximity promoted this region as a promising candidate domain for proton sensing. Our study aims at investigating the role of the acidic pocket in ASIC pH-sensing and at elucidating the possible conformational changes occurring in this region during channel activity.

All experiments were carried out on Xenopus laevis oocytes expressing hASIC1a. To characterize the biophysical properties of the mutants in the acidic pocket, we employed the Two-electrode voltage-clamp technique. To obtain information on possible conformational changes occurring in this region, we used the Voltage-clamp fluorometry technique (VCF), which employs simultaneous measurement of ionic currents and of fluorescence...
intensity of fluorophores placed at specific sites of the channel. The emission of fluorophores is sensitive to the local environment (i.e. presence of a hydrophobic or hydrophilic environment) but also to the proximity of neighboring quenching groups (as Tryptophan). In order to detect changes in fluorescence (DF), we generated Cysteine-Tryptophan pairs in the acidic pocket; the engineered Cys was used as docking site for the fluorophore and the Trp as a quencher of the fluorescence. In this setting, changes in fluorescence intensity reflect changes in distance between the Cys and Trp residues, providing clues about the structural rearrangements occurring in this domain, such as the timing and the directions of these movements.

For kinetic measurements, we used a specially designed recording chamber in which the solution flows under the oocyte; this allows us to measure the pH6-induced DF and current from approximately the same oocyte surface.

In the present study, we ask whether acid sensing in the acidic pocket is required for ASIC activation and whether the timing of conformational changes in the acidic pocket is compatible with a role in activation. We show here that combination of neutralization mutations of a large number of protonable acidic pocket residues does not prevent the opening of the channel in response to an acidic stimulation, but rather fine-tunes its pH-dependence, indicating that acid sensing in the acidic pocket is not essential for ASIC function.

Protons bind to multiple ASIC domains and trigger conformational changes which contribute to the functional transitions of the channel. The second part of the project aims at elucidating the structural rearrangements within the acidic pocket occurring during channel activity. Cys (docking site for the fluorophore)/Trp (fluorescence quencher) double mutants of the acidic pocket produce transient acid-induced currents, and various types of DF signals. To associate DF signals with functional channel transitions, we compare the kinetics of the DF and the current signals, measured as rise time (time to pass from 10 to 90% of the full amplitude). Comparison of the kinetics of current and fluorescence signals indicates that some observed movements are specifically related to the channel opening and others prepare the channel desensitization. Based on the observed fluorescence signals, we propose a model of the conformational changes occurring likely in this region during channel activation and desensitization.

Many physiological processes are regulated by pH. The mechanism by which acidic pH activates ASICs is still poorly understood. We show in this study that the “acidic pocket”, the binding site of several toxins, is not essential for
channel function but has, rather, a modulatory role. Voltage-clamp Fluorometry analysis indicates the occurrence of several conformational changes in the acidic pocket, compatible with a role in both activation and desensitization, and allows us to propose a model of conformational changes in this domain. Together, our findings improve the understanding of the molecular mechanisms involved in ASIC pH gating and regulation in the acidic pocket and provide new insights in the conformational changes occurring in this region during channel activity.

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Abolition of the Dominant Negative Effect of Brugada Syndrome Variants
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The voltage-gated sodium channel Nav1.5, encoded by the SCN5A gene, is responsible for the inward depolarizing cardiac Na+ current. Loss-of-function mutations in SCN5A result in alterations in cardiac action potentials with diverse arrhythmia phenotypes, such as Brugada Syndrome. Nonetheless, as Nav1.5 is a monomeric channel, the mechanisms of the dominant-negative (DN) effect, already reported, are still unclear. The calmodulin model serves as an adaptor between different Na+ channels. Here we present findings of a new Nav1.5 variant in a patient with Brugada syndrome, p.Y87C, which leads to a DN effect when co-expressed with wild-type Nav1.5 channel. We use TsA-201 cells and patch-clamp technique to investigate the sodium current, co-immunoprecipitation (Co-IP) to study the interaction of the Nav1.5 alpha subunits. We use pull-down experiments and calmodulin-beads to demonstrate the interaction of Nter of Nav1.5 and calmodulin.
By using transient transfected TsA-201 cells, we observed a strong reduction in current density in the presence of the p.Y87C variant. Interestingly, co-transfection of the N-terminal domain (Nter) of Nav1.5 with wild-type Nav1.5 induced a significant increase in sodium current. In addition, the decrease observed with the variant p.Y87C was abolished by co-transflecting Nter, suggesting a role of the Nter in the DN effect. Moreover, the DN effect was abolished after the deletion of the 25-Nterminal residue calmodulin binding site.

The N-terminal fragment of Nav1.5, which is not functional, increases the trafficking of the full channel. Whereas a variant with the N-terminal fragments, in which the variant is able to induce a dominant-negative effect, are not able to induce the same effect. It seems there is an interaction between the full channel and the N-terminal part. Besides, these findings are consistent with the calmodulin model, which suggests that the multimerization of Nav1.5 channels depends on calmodulin.
Aging is the progressive functional decline of various organs, leading to increased risk of many aging-related diseases such as cancer, neurological degeneration, cardiovascular diseases, and glucose intolerance. Arginase-II (Arg-II) isoenzyme that metabolizes L-arginine to L-ornithine and urea has shown to promote atherosclerosis, vascular cell senescence, and obesity-associated type 2 diabetes. Here we further investigate whether targeting Arg-II would extend lifespan in mice.

Wild type (WT) and Arg-II/-/- offspring from hetero/hetero cross were interbred to obtain WT and Arg-II/-/- mice, respectively. Mouse lifespan was analysed by using Kaplan-Meier survival curves log-rank test. Protein expression and gene mRNA levels of young mice (3 to 6 months) and old mice (18-24 months) in both WT and Arg-II/-/- were analysed by western blotting and quantitative real time PCR.

A significant lifespan extension was observed in Arg-II/-/- as compared to WT mice in females, but not in males. Combined data of both gender groups demonstrate a significant improved survival at early life stage. In female mice, the median life of Arg-II/-/- mice was increased by 93 days from 666 to 759 days. Maximum life span (mean life span of the oldest 10% within a cohort) in females was also increased (WT: 782 ± 2 days and Arg-II/-/- 848 ± 4 days). However, ablation of Arg-II gene in males had no significant effect on median and maximum life span. To further investigate the potential mechanisms of prolonged life span in female Arg-II/-/- mouse, we examined the ageing related protein p66shc and the mammalian target of rapamycin (mTOR) signaling in heart and liver. Dampened p66shc and mTOR signaling pathways in heart not liver tissue of old female Arg-II/-/- mice were observed. p16Ink4a (encoded by the INK4a/Arf locus) characterized as an important cellular senescence marker, was decreased in the heart and skin of aged
female (but not male) Arg-II/- mice as compared to age-matched WT animals. Our results demonstrate that disruption of Arg-II gene is capable of extending lifespan particularly in female mice related to suppression of p66shc expression, mTOR signaling and p16INK4a in certain tissues/organs such as heart and skin. Taken together, targeting Arg-II protects against age-related cardiovascular and metabolic diseases and extends lifespan in mice.

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Non-canonical effects of arginase-II in activation of mTORC1 through myosin-1b
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The type-II mitochondrial L-arginine:ureahydrolase, arginase-II (Arg-II), has been shown to activate mechanistic target of rapamycin complex 1 (mTORC1) pathway and contributes to cell senescence and apoptosis. However, the underlying mechanisms remain unknown. In an attempt to elucidate the underlying mechanism of mTORC1/S6K1 activation by Arg-II, here we used a proteomic approach in conjunction with molecular approaches and identified a molecular motor protein of unconventional myosin of class I, i.e., myosin 1b (myo1b) as a novel mediator of the non-canonical effect of Arg-II in activation of mTORC1-S6K1 signaling. By using a hepatocyte cell line which does not express endogenous Arg-II, we demonstrate that overexpression of wild type Arg-II or myo1b induces re-distribution of lysosome from perinuclear to cell periphery, which is associated with the activation of mTORC1/S6K1 pathway. Immunofluorescence staining reveals that overexpression of Arg-II causes dissociation of the mTORC1 inhibitor TSC from lysosome without disrupting association of mTOR with lysosomes. Silencing myo1b prevents all these alterations induced by Arg-II. By analysis of myo1b mutant deficient in motor activity or with impaired membrane-binding ability and myo1b proportion in lysosome fraction, we displayed that Arg-II promotes association of myo1b with lysosomes, which triggers lysosomal redistribution, dissociation of TSC and then activation of mTOCR1. Finally, we further showed that in senescent
vascular smooth muscle cells which has increased Arg-II expression, silencing myo1b prevented Arg-II-mediated mTORC1 activation and cell apoptosis. The results demonstrate that Arg-II exerts a non-canonical effect on mTORC1 activation through myo1b and plays an important role in cell apoptosis. In vasculature, aging-associated increase in Arg-II promotes association of myo1b with lysosome, which causes peripheral lysosomal positioning resulting in dissociation of TSC from lysosome. The relief of inhibitory effect of TSC on mTOR upstream activator Rheb ultimately leads to the activation of mTORC1-S6K1 signaling, contributing to aging-associated vascular dysfunction including impaired autophagy, mitochondrial dysfunction, cell apoptosis/ senescence. This represents another novel mechanism regulating mTORC1-S6K1 pathway and is implicated in cardiovascular aging.
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