16th International Conference on Na,K-ATPase and Related Transport ATPases

P-Type ATPases in Health and Disease

September 6-11, 2022
The Banff Centre for Arts and Creativity
Banff, Alberta Canada

We acknowledge, with deep respect and gratitude, our home on the side of Sacred Buffalo Guardian Mountain. In the spirit of respect and truth, we honour and acknowledge the Banff area, known as "Minhrpa" (translated in Stoney Nakoda as "the waterfalls") and the Treaty 7 territory and oral practices of the Îyârhe Nakoda (Stoney Nakoda) – comprised of the Bearspaw, Chiniki, and Wesley First Nations – as well as the Tsuut'ina First Nation and the Blackfoot Confederacy comprised of the Siksika, Piikani, Kainai. We acknowledge that this territory is home to the Shuswap Nations, Ktunaxa Nations, and Métis Nation of Alberta, Region 3. We acknowledge all Nations who live, work, and play here, help us steward this land, and honour and celebrate this place.

In Memoriam …

Jens Christian Skou
Aarhus University
(1918 – 2018)

Peter Jorgensen
University of Copenhagen
(2018)

David Gadsby
Rockefeller University
(1947 – 2019)

George Sachs
UCLA
(1935 - 2019)

Jerry Lingrel
University of Cincinnati
College of Medicine
(1935 – 2020)

David MacLennan
University of Toronto
(1937 – 2020)

Zijian Xie
Marshall University
(1960 – 2020)

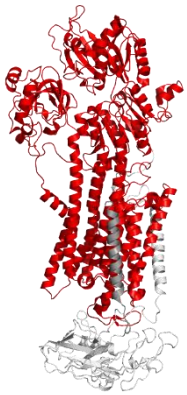
Amir Askari
University of Toledo
(1930 – 2020)

Robert Post
Vanderbilt University
(1920 – 2021)

Joseph F. Hoffman
Yale School of Medicine
(1925 – 2022)

Torben Clausen
Aarhus University
(2022)

16th International Conference on Na,K-ATPase and Related Transport ATPases



September 6-11, 2022 | Banff Centre | Banff, Alberta, Canada

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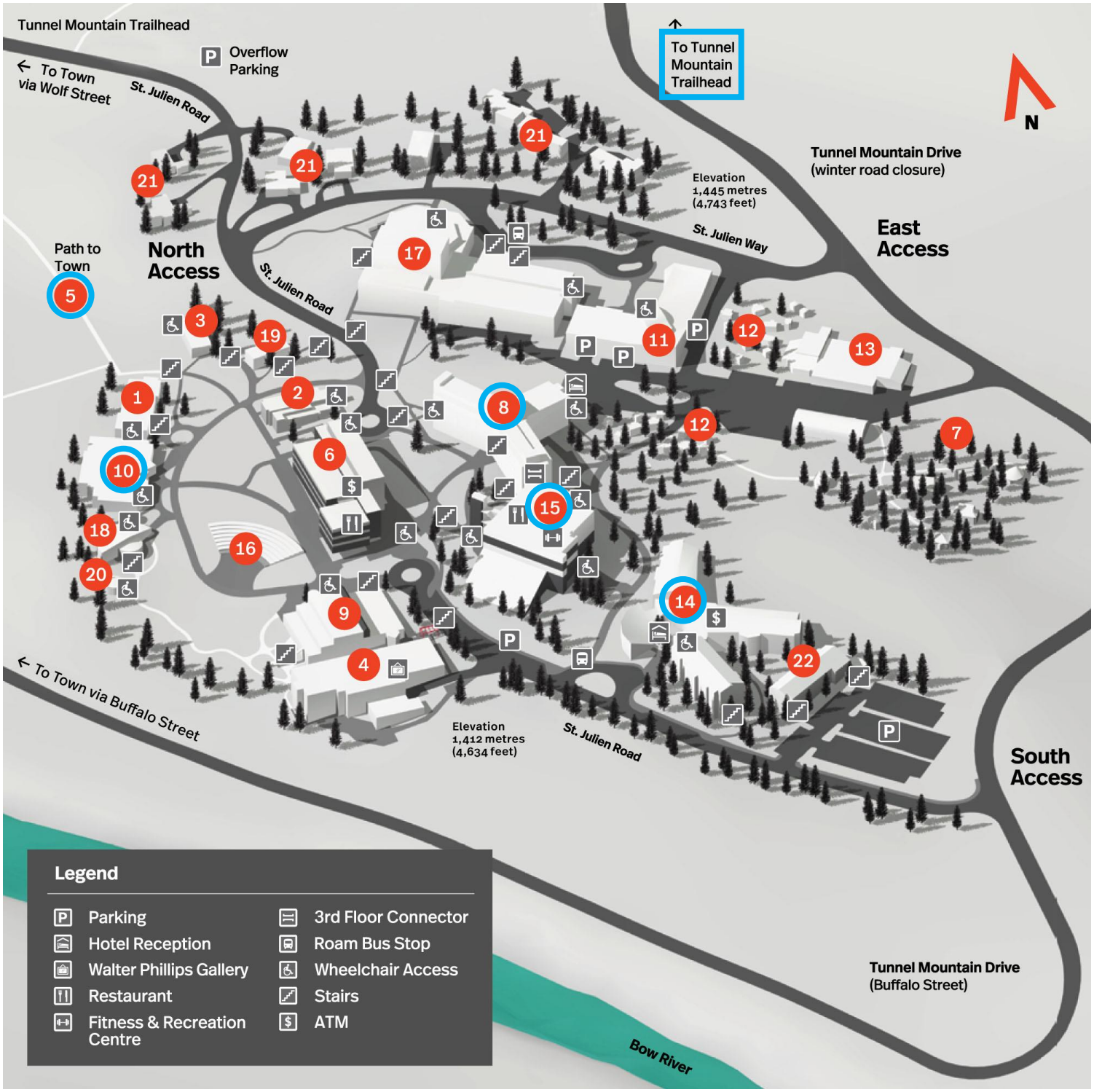
The Banff Centre Campus



Key points of interest are indicated in blue.
Map legend is on the next page.

The Banff Centre Campus

1. **Corbett Hall**
2. **Donald Cameron Centre**
   Administration Offices
3. **Farrally Hall**
4. **Glyde Hall**
   Walter Phillips Gallery
5. **Ken Madsen Path to Town**
6. **Kinnear Centre for Creativity & Innovation**
   Maclab Bistro
   Meeting Rooms & Banquets
   Paul D. Fleck Library & Archives
7. **Leighton Artists Studios**
8. **Lloyd Hall**
   Hotel Reception
9. **Jeanne & Peter Lougheed Building**
10. **Max Bell Building**
11. **Music Building**
    Bentley Chamber Music Studio
    Rolston Recital Hall
12. **Music Huts**
13. **Physical Facilities Building**
    Print Shop
    Shipping & Receiving
14. **Professional Development Centre**
    Hotel Reception
15. **Sally Borden Building**
    Fitness & Recreation Centre
    Participant Resources
    Three Ravens Restaurant
    Vistas Dining Room
16. **Shaw Amphitheatre**
17. **Theatre Complex**
    Box Office
    Jenny Belzberg Theatre
    Laszlo Funtek Teaching Wing
    Margaret Greenham Theatre
    The Club
18. **TransCanada PipeLines Pavilion**
    Banff International Research Station
19. **Vinci Hall**
20. **Yurt**
21. **Staff Housing**
22. **Staff Housing**
    Becker Hall

Map legend: Key points of interest are indicated in blue.

Meeting at a Glance

Tuesday September 6	Wednesday September 7	Thursday September 8	Friday September 9	Saturday September 10	Sunday September 11
	7 - 8:30am Breakfast	7 - 8:30am Breakfast	7 - 8:30am Breakfast	7 - 8:30am Breakfast	7 - 9am Breakfast
Arrival	8:30am – 12pm Keynote session	8:30am – 12pm Symposium 2	8:30am – 12pm Symposium 3	8:30am – 12pm Symposium 5	Departure
	12 – 1:30pm Lunch	12 – 1:30pm Lunch	12 – 1:30pm Lunch	12 – 1:30pm Lunch	
3 – 8pm Registration Opens	1:30 – 4pm Symposium 1	Free afternoon	Free afternoon	1:30 – 4:30pm Symposium 6	
	4 – 6pm Poster session Cash bar	4 – 6pm Speed talks Group Photo	4 – 6pm Poster session Cash bar	6:30 – 12pm Banquet dinner & dance	
7:30 – 11pm Opening Reception Cash bar	6 – 7:30pm Dinner	6 – 7:30pm Dinner	6 – 7:30pm Dinner		
	7:30 – 8:30pm Keynote Session	7:30 – 9pm Keynote Session	7:30 – 9:30pm Symposium 4		

Breakfast, Lunch, & Dinner will be served in the Vistas Dining Room.
All sessions will be held in the Max Bell Building.

Welcome message

The organizing committee wishes to welcome you to the 16th International Conference on Na,K-ATPase and Related Transport ATPases. The conference is being held at the Banff Centre located in the beautiful Canadian Rocky Mountains in the picturesque town of Banff, Alberta, Canada. The Banff Centre was founded in 1933 as a retreat and learning center for Arts and Creativity. It started with a single course in drama, and it stands now as a world renowned leader in arts, culture, and creativity across dozens of disciplines. Nestled in the stunning Canadian Rocky Mountains, the Banff Centre for Arts and Creativity hosts numerous conferences with the goal of inspiring attendees to unleash their creative potential.

The conference will be focused on P-Type ATPases in all aspects of life, with sessions featuring the structure, function, regulation, and physiology of members of the superfamily of P-type ATPases. The original conference on this topic occurred in 1973 at the New York Academy of Sciences, and the conference has been held (almost) every 3 years since 1978. The original meeting in this P-type ATPase-themed series dates back almost 50 years! During the intervening decades, P-type ATPase researchers such as yourselves have asked ever-changing and increasingly-complex questions about how these proteins work and their importance in so many physiological processes and disease states. We hope this meeting will provide new knowledge and creative inspiration in the ongoing studies of our membrane-bound friends.

We are honored to host the first triennial P-type ATPase Conference to be held in Canada!

We thank our guest speakers who will share exciting results from their laboratories, the organizing committee who assembled such an exciting cast of speakers, and all the people behind the scenes who have donated their time to make this meeting a success. As you can see from the exciting program, there will be Keynote Lectures, Trainee Talks, and Poster Sessions with many of the presentations given by graduate students and postdoctoral fellows. Awards will be given for the best poster presentations.

Welcome to Banff! Enjoy the mountains, enjoy the science, and take the opportunity to network with like-minded colleagues!

Howard S. Young (University of Alberta), Chair of the Organizing Committee

Organizing Committee Members

Pablo Artigas (Texas Tech University Health Sciences Center)
Jens Preben Morth (Technical University of Denmark)
Rajini Rao (Johns Hopkins University)
Seth Robia (Loyola University Chicago)
Peter Vangheluwe (Katholieke Universiteit Leuven)

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Anatrace and Molecular Dimensions are two companies that support structural biologists in their search for 3D structures of important macromolecules. We take proteins through every step from a cloned gene to a structure solved by X-ray crystallography, Cryo-EM, or NMR.

We are happy to bring you stuff that we are famous for – ultrapure surfactants (including our growing nanodisc panel), structural biology supplies, crystallization screens, as well as recent additions such as bioprocessing columns, purification resins and columns, electrophoresis supplies and more.

Thank You to Our Sponsors!

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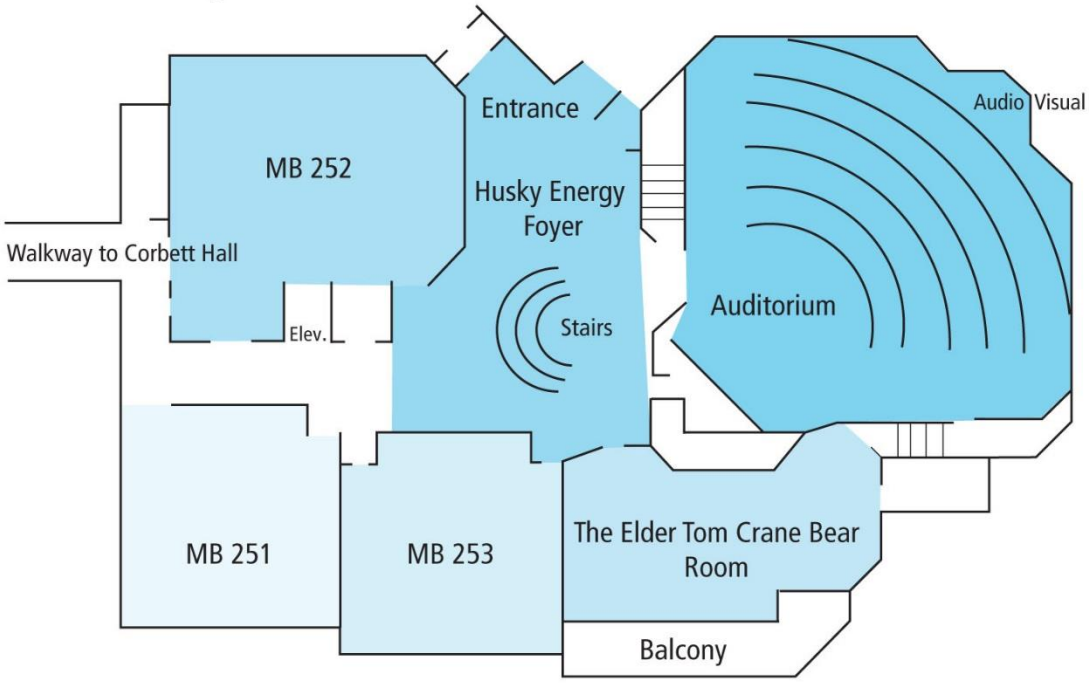
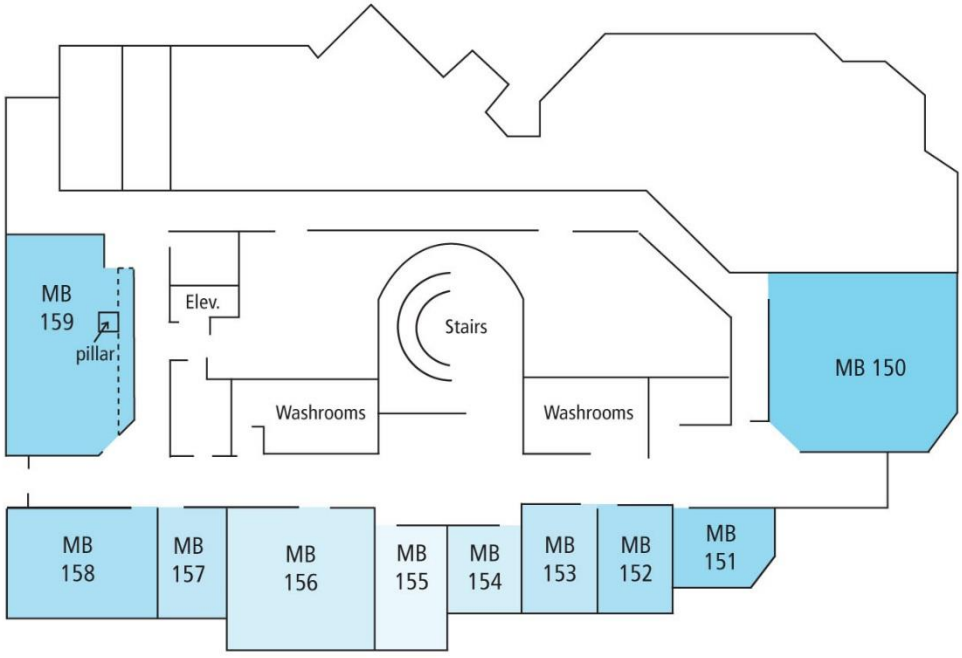
Max Bell Building

All session will be held in this building unless otherwise indicated.

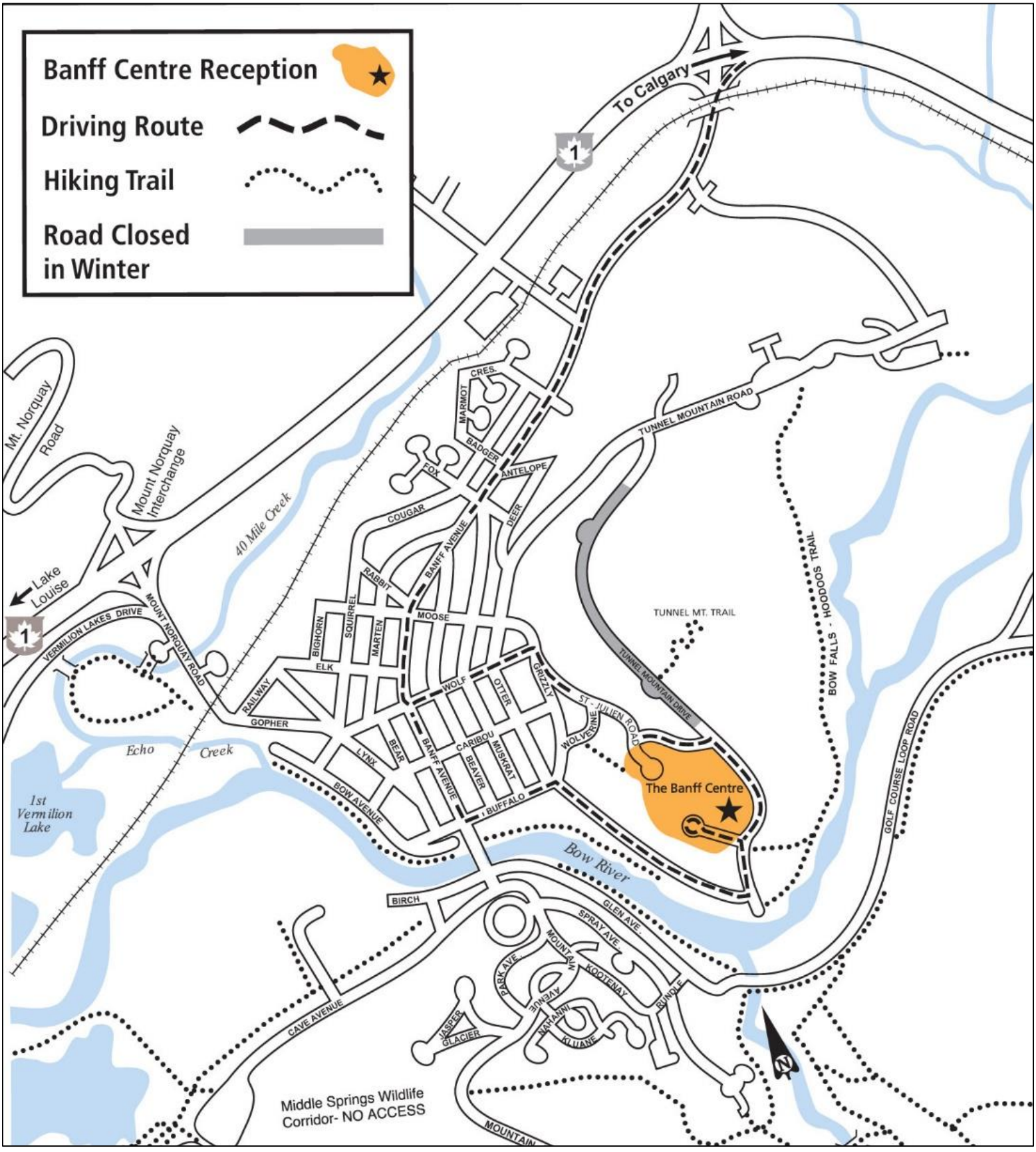
Max Bell Building Main Floor



Max Bell Building Lower Floor



Banff Town Map



Things to do in Banff

Walking and Hiking in Banff

(https://www.banfflakelouise.com/hiking) The hiking in Banff and Lake Louise is among the most scenic and inspiring in the world. Ranging from walks and easy hikes to multi-day backcountry epics, hiking is an accessible way to immerse yourself in the spectacular landscape of Banff National Park. There are numerous hikes to lakes and waterfalls throughout the park. From the Banff Centre, you can easily access the Tunnel Mountain trail, the Bow Falls & Hoodoos trail, and scenic walks along the Bow River.

World Famous Banff Hot Springs

(https://www.hotsprings.ca) Originally discovered in 1883, the Banff Hot Springs are a favorite amongst visitors and locals. It's magical to sit in the pools amidst the beautiful mountain scenery, as the warm water contrasts with the cool mountain air.

Gondolas in Banff and Lake Louis

(https://www.banfflakelouise.com/gondolas) If you want the unforgettable mountain-top views of Banff and Lake Louise without the hard work of a strenuous hike, then take a seat and ride in a gondola. There are four sightseeing gondolas and chairlifts in Banff and Lake Louise. A favorite is the **Banff Sightseeing Gondola** – Floating its way up Sulphur Mountain, the Banff Sightseeing Gondola is a five minutes drive from the town of Banff. The Gondola seats up to 4 people and takes guests to a lofty altitude of 2,281 meters (7,486 feet). Once at the top, enjoy the restaurants, cafés, short hiking trails, or an adventurous walk along the ridge line of Sulphur Mountain.

Breweries & Distilleries in Banff

Are you a big fan of fantastic local breweries or distilleries? If so, you're in luck – the Canadian Rockies are home to some of the best craft breweries and distilleries in Canada. Banff Ave. Brewing Co., Three Bears Brewery, and Park Distillery are all within the Banff town site. They offer locally brewed beer and distilled spirits, tasty eats, and mountain views. What better place to indulge than in the Canadian Rockies! We personally recommend the Alpine Dry Gin (Park Distillery), Head Smashed IPA (Banff Ave. Brewing), and Pinery Pilsner (Three Bears Brewery).

Code of Conduct

It is the policy of the Organizing Committee that all participants, including attendees, vendors, staff, volunteers, and all other stakeholders at the meeting will conduct themselves in a professional manner that is welcoming to all participants and free from any form of discrimination, harassment, or retaliation. Participants will treat each other with respect and consideration to create a collegial, inclusive, and professional environment at the P-Type ATPase Conference. Creating a supportive environment to enable scientific discourse at the meeting is the responsibility of all participants.

Participants will avoid any inappropriate actions or statements based on individual characteristics such as age, race, ethnicity, sexual orientation, gender identity, gender expression, marital status, nationality, political affiliation, ability status, educational background, or any other characteristic protected by law. Disruptive or harassing behavior of any kind will not be tolerated. Harassment includes but is not limited to inappropriate or intimidating behavior and language, unwelcome jokes or comments, unwanted touching or attention, offensive images, photography without permission, and stalking.

Violations of this code of conduct policy should be immediately reported to meeting organizers or Banff Centre staff. Sanctions may range from verbal warning, to ejection from the meeting without refund, to notifying appropriate authorities. Retaliation for complaints of inappropriate conduct will not be tolerated. If a participant observes inappropriate comments or actions and personal intervention seems appropriate and safe, they should be considerate of all parties before intervening.

P-Type ATPases in Health and Disease

Tuesday, September 6th to Sunday, September 11th, 2022

The Banff Centre
Banff, Alberta, Canada

Tuesday September 6, 2022

3:00 – 8:00 PM **Registration Opens – Max Bell Building, Husky Energy Foyer**

7:30 – 11:00 PM **Opening Mixer – Light snacks & cash bar**

Wednesday September 7, 2022

Meeting Begins

P-Type ATPases in Health and Disease

All sessions will be held in the Max Bell Building Auditorium, unless otherwise indicated.
All coffee breaks will be held in the Husky Energy Foyer.
Registration Opens in Max Bell Building, Husky Energy Foyer (8:00 AM to 8:00 PM)

7:00 – 8:30 AM **Breakfast, Vistas Dining Room**

Symposium – The Sodium Pump in Disease
KEYNOTE SESSION

Session Chair: Howard Young

8:30 – 8:40 AM **Opening Remarks – Howard Young, Chair of the Organizing Committee**

8:40 – 9:30 AM **Keynote Speaker – Kathleen Sweadner** (Massachusetts General Hospital) The Fallout of Genetic Variation in Na,K-ATPase: Structure, Biosynthesis, Function, and Disease.

9:30 – 10:00 AM **Bente Vilsen** (Aarhus University) Na,K-ATPase Structure-Function Relationship in Health and Disease– New Aspects of Rescue by Mutation.

10:00 – 10:30 AM **Coffee Break & Meet the Speakers**

10:30 – 11:00 AM **Allison Brashear** (University at Buffalo) ATP1A3 Variable Phenotypes: A Continuum of Disease or Discrete Entities?

11:00 – 11:30 AM **Michael Shattock** (King's College London) Of Mice and Men: Role of the Na/K ATPase and Phospholemman in Blood Pressure Control.

11:30 – 12 Noon **Memorial Session –** Remembering colleagues we have recently lost.

12:00 – 1:30 PM **Lunch, Vistas Dining Room**

Symposium – Function & Physiology of P-Type ATPases I

Session Chair: Mary Hernando (University of Alberta)

1:30 – 2:00 PM **Rajini Rao** (Johns Hopkins University) Unconventional Functions of the Golgi Calcium Pump SPCA2.

2:00 – 2:30 PM **Svetlana Lutsenko** (Johns Hopkins University) Copper ATPases at the Intersection of Cell Metabolism and Differentiation.

2:30 – 3:00 PM **Coffee Break & Meet the Speakers**

3:00 – 3:30 PM **Adriana Katz** (Weizmann institute of Science) Specific Na,K-ATPase-Lipid Interactions. A Role in Neurological Disease?

3:30 – 4:00 PM **Liora Shoshani** (CINVESTAV-IPN) The β_2 -Subunit (AMOG) of Human Na, K-ATPase is a Homophilic Adhesion Molecule.

4:00 – 6:00 PM **Poster Session One – Cash Bar**

6:00 – 7:30 PM **Dinner, Vistas Dining Room**

KEYNOTE SESSION

7:30 – 7:40 PM **Introduction – Peter Vangheluwe** (KU Leuven)

7:40 – 8:30 PM **Keynote Speaker – Michael Palmgren, University of Copenhagen**
Evolution of the Plasma Membrane H^+-ATPase Could Have Been Key for Plants to Conquer the Land.

Thursday September 8, 2022

7:00 – 8:30 AM **Breakfast, Vistas Dining Room**

Anatrace and Calibre Scientific Symposium – Structure and Mechanism of P-Type ATPases

Session Chair: Sean Cleary (Loyola University)

8:30 – 9:00 AM **Francesco Tadini-Buoninsegni** (University of Florence) Recording the Transport Activity of P-type ATPases on Solid Supported Membranes.

9:00 – 9:30 AM **Joseph Lyons** (Aarhus University) Structural Studies of Yeast and Mammalian Lipid Flippases.

9:30 – 10:00 AM **Kazuhiro Abe** (Nagoya University) Structural Physiology of the Gastric Proton Pump and the Lipid Flippase.

10:00 – 10:30 AM **Coffee Break & Meet the Speakers**

10:30 – 11:00 AM **Eunyong Park** (University of California, Berkeley) Understanding the Functions and Mechanisms of P5-ATPases by Cryo-Electron Microscopy.

11:00 – 11:30 AM **Benoit Roux** (University of Chicago) P-Type Ion Pumps: Simulations and Theory

11:30 – 12:00 noon **David Stokes** (New York University) A Match Made in Heaven: The Potassium Transport System KdpABC.

12:00 – 1:30 PM **Lunch, Vistas Dining Room**

Free Afternoon

Hike the Tunnel Mountain Trail, Stroll Along the Bow River, or Visit the Town of Banff!

4:00 – 4:10 PM **Franz Kerek** (SiNatur, Munich) Spherical Oligo-Silicic-Acid (SOSA) as an Endogenous Regulator of P-type ATPases.

4:10 – 5:40 PM **5 Minute Speed Talks**

5:40 – 6:00 PM **Group Photo**

6:00 – 7:30 PM **Dinner, Vistas Dining Room**

KEYNOTE SESSION

7:30 – 7:40 PM **Introduction – Preben Morth** (Technical University of Denmark)

7:40 – 8:30 PM **Keynote Speaker – Poul Nissen** (Aarhus University) Autoinhibition and Activation of P-Type ATPases.

8:30 – 9:00 PM **Sigrid Langhans** (Nemours Children's Health) Ataxia and Failure to Thrive in Mice with Genetic Deletion of Na,K-ATPase β1 Subunit in Cerebellar Granule Cells.

Friday September 9, 2022

7:00 – 8:30 AM **Breakfast, Vistas Dining Room**

Symposium – Flipping out over P-type ATPases

Session Chair: Suravi Chakrabarty (KU Leuven)

8:30 – 9:00 AM **Jens Peter Andersen** (Aarhus University) Mechanism and Transport Pathway of the P4-ATPase ATP8A2 by Mutagenesis.

9:00 – 9:30 AM **Rosa López-Marqués** (University of Copenhagen) Lipid Flippases as Key Players in Plant Stress Adaptation.

9:30 – 10:00 AM **Todd Graham** (Vanderbilt University) Evolution of P4-ATPase Structure and Function.

10:00 – 10:30 AM **Coffee Break & Meet the Speakers**

10:30 – 11:00 AM **Robert Molday** (University of British Columbia) The Flip Side of P4-ATPases: Molecular Characterization of Disease-Associated Variants.

11:00 – 11:30 AM **Osamu Nureki** (University of Tokyo) Cryo-EM Structures Elucidate the Lipid Transport Cycle of the P4-ATPase Flippase.

11:30 – 11:45 AM **Line Marie Christiansen** (Aarhus University) Structural and Functional Clues on a P4B-ATPases Suggest Autoinhibition.

11:45 – 12:00 Noon **Himanshu Kandelia** (University of Southern Denmark) Novel Mechanisms for Voltage Sensing.

12:00 – 1:30 PM **Lunch, Vistas Dining Room**

Free Afternoon

Hike the Tunnel Mountain Trail, Stroll Along the Bow River, or Visit the Town of Banff!

4:00 – 6:00 PM **Poster Session Two – Cash Bar**

6:00 – 7:30 PM **Dinner, Vistas Dining Room**

Symposium – Novel Pump Mechanisms

Session Chair: Nish Rathod (University of Alberta)

7:30 – 8:00 PM **Peter Vangheluwe** (KU Leuven) P5B-ATPases: Endo-Lysosomal Polyamine Transporters Implicated in Disease.

8:00 – 8:30 PM **Kenneth Lee** (Pennsylvania State University) Mechanism of Substrate Selection and Translocation of the P5B Polyamine Transporter ATP13A2.

8:30 – 9:00 PM **Maike Bublitz** (University of Oxford) What the Cryo-EM Structure of the Fungal Proton Pump Tells Us About its Mechanism.

9:00 – 9:15 PM **Ronald Clarke** (University of Sydney) Involvement of the Alpha-subunit N-Termini in the Mechanism and Regulation of the Na^+,K^+- and H^+,K^+-ATPases.

9:15 – 9:30 PM **Thibaud Dieudonné** (Aarhus University) Activation and Substrate Recognition Mechanism of ATP8B1/CDC50A Revealed by Cryo-EM.

Saturday September 10, 2022

7:00 – 8:30 AM **Breakfast, Vistas Dining Room**

Symposium – New & Emerging Techniques

Session Chair: Kerri Spontarelli (TTUHSC)

8:30 – 9:00 AM **Aleksey Zima** (Loyola University) A Novel Approach for Quantitation of Endoplasmic Reticulum Ca^{2+} Transport.

9:00 – 9:30 AM **Michel Espinoza-Fonseca** (University of Michigan) Deep Learning Design and Identification of Small-Molecule Effectors Targeting Ion-Motive Pumps in the Heart.

9:30 – 10:00 AM **Razvan Cornea** (University of Minnesota) High-Throughput Screens for Novel, Drug-Like Modulators of SERCA.

10:00 – 10:30 AM **Coffee Break & Meet the Speakers**

10:30 – 11:00 AM **Pablo Artigas** (Texas Tech University) How to Make a Sodium Pump.

11:00 – 11:30 AM **Magnus Andersson** (Umea University) Tracking P-type ATPase Native Structural Dynamics in Real Time.

11:30 – 11:45 AM **Gustavo Blanco** (University of Kansas Medical Center) Na,K-ATPase α4 is Involved in Sperm Energetics.

11:45 – 12:00 noon **Adriana Norris** (Vanderbilt University) ATP10A has a Protective and Sex-Specific Role in Lipid Metabolism.

12:00 – 1:30 PM **Lunch, Vistas Dining Room**

Symposium – Function & Physiology of P-Type ATPases II

Session Chair: John Yap (Loyola University)

1:30 – 2:00 PM **Hugo Adamo** (Universidad de Buenos Aires) Hydrolytic Functions of the Yeast Spf1p P5A-ATPase.

2:00 – 2:30 PM **Miguel Holmgren** (National Institutes of Health) The Interrelationships of the Structure and Dynamics of Ion Transport in Disease-Causing Mutations of the Brain-Specific Na/K-ATPase.

2:30 – 3:00 PM **Jaroslava Seflova** (Loyola University) Dimerization of the Sodium Pump in Health and Disease.

3:30 – 4:00 PM **Maria Rosario Sepulveda** (University of Granada) Contribution of Intracellular Ca^{2+}-ATPases to the Functional Modulation of Microglia: Implication in Neuropathologies.

4:00 – 4:30 PM **Jakob Silberberg** (Goethe University) Post-Translational Regulation of the K^+ Pump KdpFABC.

Banquet Dinner & Dance 6:30 – 12:00 Midnight

Sunday September 11, 2022 – End of Meeting

7:00 – 9:00 AM **Breakfast, Vistas Dining Room**

11:00 AM **Checkout and Departure.**

Speaker Abstracts

Morning session - Wednesday, September 7th

The Fallout of Genetic Variation in Na,K-ATPase: Structure, Biosynthesis, Function, and Disease.

Kathleen J. Sweadner, Elena Arystarkhova

Massachusetts General Hospital and Harvard Medical School.

Mutations in many P-ATPases can produce monogenic disease. Most of these are known for one major phenotype: Brody disease, Darier, Hailey-Hailey, Menkes, Wilson, hepatic cholestasis, and Parkinsons. DNA sequencing of undiagnosable patients, however, has uncovered new phenotypes in many genes, including Na,K-ATPase α1, α2, and α3. Mutations in ATP1A3, found in neurons, produce an extraordinarily broad range of neurological phenotypes, from fetal to adult. Specific amino acid substitutions group into at least six syndromes with distinctive characteristics (RDP, AHC, CAPOS, RECA, and two pre- and perinatal syndromes, PMG and EIEE). What are the mechanistic differences between mutations that produce microcephaly, dystonia, or episodes of alternating hemiplegia? Mutations of course might reduce activity by inactivating the ATPase, by changing its kinetics, or by preventing it from getting to the surface. Changes of Na,K- ATPase activity unquestionably play an important role, but research is showing that the severity of a syndrome does not correlate with how much activity is lost. Instead, in isogenic cells expressing different mutations, we have found so far that protein misfolding and retention in endoplasmic reticulum (in infantile epilepsy), disruption of Golgi (in alternating hemiplegia), and febrile temperature-sensitive lability (in R756H,C,L) correlate better with human syndromes. Some mutations also cause a leak. Such perturbations change cell biological homeostasis and are a "gain of toxic function". The question is why do some mutations provoke cellular responses. Crystal structures of Na,K-ATPase have been crucial to obtain insight into disease mutations, but we can also gain perspective by considering protein folding and stability. We will take a walk along the nascent polypeptide chain to investigate what the ribosome sees, and to pinpoint weak points that are likely to be common to all P-ATPases.

Na,K-ATPase Structure-Function Relationship in Health and Disease – New Aspects of Rescue by Mutation.

Bente Vilsen, Rikke Holm, Hang N. Nielsen

Aarhus University, Aarhus, Denmark

Neurological disease mutations of the Na,K-ATPase may lead to disturbance of function and/or protein misfolding and reduced expression of active enzyme. Some of those disease mutations that primarily disturb Na,K-ATPase function act by affecting the affinity for Na+. We previously found that the second-site mutation E314D rescues the reduced Na+ affinity and activity of the D928N

α1-mutant, where Na+ site III is disturbed. A successful strategy for pharmacological intervention in neurological disease might be to design a pharmaceutical that interferes mechanistically with the Na,K-ATPase in the same way as the rescue mutation by binding in the enzyme domain where the rescue mutation is located. To gain understanding of the molecular mechanism underlying the rescue effect and its applicability to relieve neurological disease, we have carried out a series of experiments exploring the rescue effects on α3-disease mutants and mutants with disturbance of Na+ sites I and II. Our findings have led to a hypothesis explaining the rescue effect, and during the course of these experiments we have discovered new rescue mutations that are more efficient than the original one.

The Story of RDP: Bench to Bedside and the Impact on Neuroscience.

Allison Brashear[1], Kathleen Sweadner[2], Ihtsham Haq[3], Beverly Snively[4], Laurie Ozelius[2]

[1]Jacobs School of Medicine and Biomedical Science, Buffalo, NY, USA
[2]Massachusetts General Hospital, Charlestown, MA, USA
[3]University of Maimi Miller School of Medicine, Maimi, FL, USA
[4]Wake Forest School of Medicine, Winston-Salem, NC, USA

First reported in 2004 as the cause of Rapid-Onset Dystonia-Parkinsonism (RDP), mutations in ATP1A3 now also cause Alternating Hemiplegia of Childhood (AHC), Cerebellar ataxia, Areflexia, Pes Cavus, Optic Atrophy, Sensorineural deafness (CAPOS) syndrome, Early Infancy Epileptic Encephalopathy, (EIEE), Relapsing Encephalopathy with Cerebellar Ataxia (RECA), Fever Induced Paroxysmal Weakness and Encephalopathy (FIPWE), Childhood Onset Schizophrenia (COS) and more recently D-DEMO (facial dysmorphism, ataxia, developmental delay without hemiplegia spells).

Years before the original 1991 description of RDP, patients presented with acute dystonia and psychiatric symptoms, only to be labeled psychogenic or cerebral palsy. RDP was proposed as a new entity after detailed clinical histories and neurologic exams of a large family revealed the interaction of physiological stress and sudden onset of neurologic disease leading to our 2004 discovery of the gene. Our subsequent work continues to be the largest and best characterized cohort of patients with RDP in the world.

The phenotypes with ATP1A3 mutations vary, but share features of dystonia, cognitive impairment, psychiatric disease, neuron loss, ataxia, and seizures. Except for prenatal brain malformations, most all the ATP1A3 phenotypes have been associated with triggers such as stress, fever, exercise, alcohol and cold. This clinical history supports the cellular mechanism ATP1A3, responsible for clearing elevated levels of intraneuronal sodium resulting from a high energy demanding task. Decades of work in RDP demonstrates how the clinician and basic science partnership can set a new course in research.

ATP1A3 related diseases is an ideal framework to discuss the interaction of external triggers, genetics, and their impact on neurologic disease. Teamwork is essential to this success and is a model for the future.

Of Mice and Men: Role of the Na/K ATPase and Phospholemman in Blood Pressure Control.

Michael Shattock

King's College London

The importance of the Na/K ATPase (NKA), and in particular the a2 subunit, in blood pressure control has long been recognized. Ouabain and its analogues raise blood pressure and agents that stimulate NKA are hypotensive. Despite a wealth of evidence implicating NKA in blood pressure control, prior to the studies described here, surprisingly little was known about the role of the muscle specific regulator of NKA activity phospholemman (PLM/FXYD1) in blood pressure control. In these studies, we describe how phosphorylation of PLM profoundly influences vascular tone and blood pressure regulation in mice, and how hypo-phosphorylation of PLM is associated with aging-induced essential hypertension. In humans, a single nucleotide polymorphism was identified in PLM exon 10 that results in an R70C mutation in PLM that leads to PLM hypo-phosphorylation in vitro and, in two human cohorts, is associated with a significant elevation of blood pressure in middle-aged men. The association between PLM de-phosphorylation and hypertension has yet to be demonstrated in man. However, it is likely that interventions that prevent PLM de-phosphorylation and Na/K ATPase down-regulation may provide a new therapeutic approach to the treatment of essential hypertension. Additionally, existing interventions that prevent downregulation of signaling pathways that terminate in PLM phosphorylation (for example, β-blockers) may in part exert their beneficial anti-hypertensive effect through this mechanism.

Afternoon session - Wednesday, September 7th

Unconventional Functions of the Golgi Calcium Pump SPCA2.

Rajini Rao

Johns Hopkins University, Baltimore, MD, USA

Secretory Pathway Calcium ATPases (SPCA) localize to the Golgi and secretory vesicles of lower eukaryotes, such as yeast, and metazoans. They differ from other Ca 2+ -ATPase subtypes in their inhibitor sensitivity, being mostly resistant to thapsigargin, and ion selectivity that extends to Mn^{2+} ions. As their name indicates, their role in regulating the ionic milieu within the lumen of the secretory pathway is important for protein sorting, glycosylation and post-translational processing. In vertebrates, there are two isoforms: SPCA1 is ubiquitously expressed and essential for embryonic development, whereas expression of SPCA2 is regulated in tissues with secretory function, such as the lactating mammary gland. However, since both SPCA isoforms have essentially similar catalytic function, the raison d'etre of SPCA2 has been of interest. We show that SPCA2 moonlights as a protein chaperone and calcium channel activator, and that these roles appear to be independent of its ion pumping activity. We will illustrate these unconventional functions of SPCA2 in the context of mammary epithelial cells with two partner proteins, E-

cadherin and Orai1. We show how these pump-independent roles of SPCA2 have far-reaching effects on cell functions, including cell adhesion and mitochondrial respiration.

Copper ATPases at the Intersection of Cell Metabolism and Differentiation.

Haojun Young, **Svetlana Lutsenko**

Johns Hopkins University School of Medicine, Baltimore, MD, USA

Copper-transporting ATPases ATP7A and ATP7B have two major functions: (i) to maintain cytosolic copper levels by sequestering excess copper in vesicles for further export and (ii) to deliver cytosolic copper to copper-dependent enzymes within the secretory pathway. Which of these two functions dominates depends on the cells' metabolic or differentiation state. In adipocytes, ATP7A is the major regulator of copper metabolism. During early adipogenesis, ATP7A relocates from the trans-Golgi network (TGN); it returns to the TGN when differentiation is completed, indicative of changes in copper distribution during cell differentiation. The early ATP7A-dependent copper sequestration is necessary for termination of the beta-catenin signaling and activation of PPAR-gamma. In differentiated cells, ATP7A activates aminooxidase AOC3, which in turn modulates the uptake of fat and glucose by adipocytes. Complete inactivation of ATP7A increases cellular copper content, alters redox balance, and blocks adipocytes differentiation, whereas partial inactivation and loss of AOC3 activity cause adipocytes hypertrophy.

Specific Na,K-ATPase-lipid interactions. A role in neurological disease?

Adriana Katz, Steven Karlish

Weizmann institute of Science, Rehovot, Israel

Function and structure of membrane protein can be affected by the lipid bilayer physical properties as well as by specific lipid–protein interactions. Crystal structures of Na,K-ATPase show several lipids bound within the transmembrane domain without a clear indications of possible functional roles. We have developed an expression system for human Na,K-ATPase isoforms in *Pichia pastoris* and purification in non-ionic detergents of α(1-3) β(1-3)/ FXYD1complexes which allows detection of specific lipid interactions, uncomplicated by general bilayer effects. Diverse biochemical and biophysical techniques complement crystal structures and reveal three specific lipid binding sites. Site A, located between trans-membrane segments (TM) αTM 8, 9 and TM FXYD, binds optimally 18:0/18:1 phosphatidylserine plus cholesterol, which stabilize the protein. Site B, located between α TM 2, 4, 6 and 9, binds optimally 18:0/20:4 or 18:0/22:6 phosphatidylethanolamine, which stimulate Na,K-ATPase activity, with similar effects in α1β1FXYD1, α2β1FXYD1, and α3β1FXYD1. Site C, yet to be located, binds optimally 18:0/18:0 phosphatidylcholine or sphingomyelin and cholesterol, which inhibit Na,K-ATPase activity.

Observations on specific lipid-protein interactions of the Na,K-ATPase may be relevant to other membrane proteins.

Specific lipid-Na,K-ATPase interactions may play a role in neurological diseases. For example, most mutations of α3 (ATP1A3) that cause alternating hemiplegia of childhood (AHC) are located within or near trans-membrane segments and inactivate the Na,K-ATPase activity. Whereas the mechanism of inactivation in many mutants is accounted for (e.g. by interference with ion binding), mutated residues facing the lipid bilayer have no obvious mechanism of inactivation, and might be interfering with specific lipid-protein interactions. An initial study with an AHC-causing mutant Q140L, located in lipid site B, appears consistent with this hypothesis. As a possible therapeutic implication, lipid composition of the membrane might be altered, by diet or dietary supplement, to alter Na,K-ATPase activity or expression.

The β_2-Subunit (AMOG) of Human Na, K-ATPase is a Homophilic Adhesion Molecule.

Maria Luisa Roldan[1], Marlet Martinez-Archundia[2], Gema Lizbeth Ramirez-Salinas[2], and **Liora Shoshani[1]**

[1]CINVESTAV-IPN, [2]Laboratorio de Modelado Molecular, Bioinformática y diseño de fármacos. Departamento de Posgrado Escuela Superior de Medicina del Instituto Politécnico Nacional

The β2 subunit of Na^+, K^+-ATPase was identified originally as the adhesion molecule on glia (AMOG) that mediates the adhesion of astrocytes to neurons in the central nervous system and that is implicated in the regulation of neurite outgrowth and neuronal migration. While β1 isoform have been shown to trans-interact in a species-specific mode with the β1 subunit on the epithelial neighboring cell, β2-subunit have been shown to act as a recognition molecule on glia. Nevertheless, none of the works have identified the binding partner of β2 or describing its adhesion mechanism. Until now, the interactions pronounced for β2/AMOG are heterophilic cis-interactions. In the present report we designed experiments that would clarify if β2 is a cell-cell homophilic adhesion molecule. For this purpose, we performed protein docking analysis, cell-cell aggregation, and protein-protein interaction assays. We observed that the glycosylated extracellular domain of β2/AMOG, can make an energetically stable trans-interacting dimer. We show that CHO (Chinese Hamster Ovary) fibroblasts transfected with the human β2-subunit become more adhesive and make large aggregates. The treatment with Tunicamycin in vivo reduced cell aggregation, suggesting the participation of N-glycans in that process. Protein-protein interaction assay in vivo with MDCK (Madin-Darby canine kidney) or CHO cells expressing a recombinant β2-subunit, show that the β2 subunits on the cell surface of the transfected cell lines interact with each other. Altogether our results suggest that the human β2 subunit can form trans-dimers between neighboring cells, when expressed in non-astrocytic cells, such as fibroblasts (CHO) and epithelial cells (MDCK).

Evening Keynote session - Wednesday, September 7th

Evolution of the Plasma Membrane H^+-ATPase Could Have Been Key for Plants to Conquer the Land.

Michael Palmgren, Anett Stéger, Cuiwei Wang

University of Copenhagen, Copenhagen, Denmark

Plasma membrane (PM) H^+-ATPases are the electrogenic proton pumps that export H+ from plant and fungal cells to acidify the surroundings and generate a membrane potential that can exceed -200 mV. Related pumps are found scattered in the prokaryote domains and in particular in extremophiles such as acidophiles, which can survive at pH below pH 2. Prokaryotic putative PM H^+-ATPases are completely dependent on alkali cations for function and likely are neutral cation/H^+ exchangers. We hypothesize that the transition to electrogenic H^+ pumps may have occurred in the last common eukaryotic ancestor, which made possible the formation of PM electrochemical gradients strong enough for secondary active nutrient uptake in aquatic and solid growth media. Plant PM H^+-ATPases are equipped with a C-terminal autoinhibitory regulatory (R) domain of about 100 amino acid residues, which cannot be identified in the PM H^+-ATPases of water-dwelling green algae but appear fully developed in the immediate streptophyte predecessors of land plants. To explore the physiological significance of this domain, we used CRISPR/Cas9 mutagenesis to create in vivo C-terminal truncations of autoinhibited PM H^+-ATPase2 (AHA2), one of the two major isoforms in Arabidopsis thaliana. As more residues were deleted, the mutant plants became progressively more efficient in proton extrusion, concomitant with increased expansion growth and root biomass. However, as the hyperactivated AHA2 also contributed to stomatal pore opening, which makes uptake of CO_2 possible, but also provides an exit pathway for water and an entrance pathway for pests, the mutant plants were more susceptible to biotic and abiotic stresses, pathogen invasion and water loss, respectively. Taken together, our results demonstrate that pump regulation through the R domain is crucial for plant fitness and might have been necessary for the successful water-to-land transition of plants.

Morning session - Thursday, September 8th

Recording the Transport Activity of P-Type ATPases on Solid Supported Membranes.

Francesco Tadini-Buoninsegni

University of Florence, Florence, Italy

P-type ATPases use the energy provided by ATP hydrolysis to transport various ions or phospholipids across biological membranes, thereby generating and maintaining essential electrochemical potential gradients. An electrophysiological method based on a solid supported membrane (SSM) has been used for the functional characterization of P-type ATPases [1]. The SSM consists of a hybrid alkanethiol/phospholipid bilayer supported by a gold electrode. Membrane vesicles/fragments, and proteoliposomes containing the ATPase of interest are adsorbed on the SSM surface. The adsorbed membrane sample is then subjected to a rapid substrate concentration jump. If the substrate concentration jump induces charge displacement across the membrane by the ATPase, an electrical current is measured due to capacitive coupling between the membrane sample and the SSM [2]. The charge transport mechanism of various P-type ATPases was investigated using SSM-based electrophysiology, such as in the case of sarcoplasmic

reticulum Ca^{2+}-ATPase (SERCA). Electrical signals generated by SERCA were measured following substrate, i.e. Ca^{2+} and ATP, concentration jumps on sarcoplasmic reticulum vesicles adsorbed on the SSM [1,2]. Charge movements related to electrogenic reaction steps in the SERCA transport cycle were detected. Recently, the SSM method was used to investigate electrogenicity of phospholipid translocation by the lipid flippase ATP8A2 [3]. We observed the generation of an electrical current by ATP8A2 in the presence of ATP and the lipid substrate phosphatidylserine. It was concluded that the electrogenicity is associated with a step in the ATPase reaction cycle directly involved in translocation of the lipid.

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Structural Studies of Yeast and Mammalian Lipid Flippases.

Joseph Lyons

Aarhus University, Aarhus, Denmark

P4-ATPases are members of the P-type ATPase superfamily that drive the inward directed translocation (flipping) of lipids within the membrane. These lipid flippase largely function as binary complexes with an auxiliary protein from the CDC50 family. Recent studies on mammalian and yeast lipid flippases have revealed structural insights into their regulatory and transport mechanisms (1,2), however the intricate details relating to substrate recognition, specificity and the role of disease associated mutations remain elusive. In this talk, I will discuss recent progress in cryo-EM derived structural studies of 1) the yeast Drs2p-Cdc50p including various lipid substrate complexes (3) and 2) WT and mutant forms of bovine ATP8A2 (4), in particular the central isoleucine of the TM4 PISL motif which has been reported as being critical in lipid flippase turnover. Comparison of WT and mutant structures of ATP8A2, in particular I336M, a mutation associated with the neurological disorder cerebellar ataxia, mental retardation, and disequilibrium (CAMRQ) syndrome, have provided a rationale for the disease mutation that supports reported biochemical characterization.

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Structural Physiology of the Gastric Proton Pump and the Lipid Flippase.

Kazuhiro Abe

Nagoya University, Nagoya, Japan

The gastric proton pump, H^+,K^+-ATPase, is responsible for the gastric acidification. A series of crystal structures has elucidated some of the key molecular mechanisms, including how H^+ is extruded to the acidic solution (pH 1) of the stomach (Abe et al., 2018, Nature), how many cations are exchanged (1 H^+/1 K^+ per ATP hydrolysis) in a single turnover (Yamamoto et al., 2019, eLife). These results revealed that H^+ is extruded by the positively-charged, invariantly conserved Lys791 (Ser in Na,K- and Ca-ATPase), and at the same time, this Lys791 physically occupies one of the cation-binding sites (site I) and allows only a single K^+ to bind, in contrast to the two K^+ ions bound to the Na^+,K^+-ATPase.

We next asked, if Lys791 prevents two K^+-binding, would simply removing this residue allow the proton pump to bind two K^+? Based on the structural analysis of a series of sodium pump mimetic mutants, we finally succeeded to generate a proton pump mutant that bonds two K^+ ions, which is visualized by cryo-EM. The results showed that the removal of Lys791 is insufficient, five mutations are required for two K^+ binding to the gastric proton pump, providing new insights into how two closely-related pumps specify the number of K^+ accommodated at their cation-binding site (Abe et al., 2021, Nat Commun).

We also determined crystal and cryo-EM structures of ATP11C, a plasma membrane flippase that is related to the apoptotic “eat-me” signal phosphatidylserine exposure, providing a molecular basis of how phospholipid traverses the membrane coupled with ATP-hydrolysis (Nakanishi et al., 2020, Cell Rep, this topic will be presented at poster session).

Understanding the Functions and Mechanisms of P5-ATPases by Cryo-Electron Microscopy.

Eunyong Park

University of California, Berkeley, California, USA

P5A- and P5B- ATPases, or collectively P5-ATPases, are eukaryotic-specific P-type ATPases that are important for functions of the endoplasmic reticulum (ER) and endo-/lysosomes, but their biological functions and substrate specificities were unknown for many years. Recent cryo-EM and biochemical studies of P5-ATPases have revealed that the P5A-ATPase extracts mistargeted or mis-inserted transmembrane helices from the ER membrane for protein quality control, whereas P5B-ATPases transport polyamines from endo-/lysosomes into the cytosol. In my talk, I will discuss the mechanisms of substrate recognition and transport by P5-ATPases and how structural

diversification of the transmembrane domain enable the P5-ATPases to adapt for transport of atypical substrates for P-type ATPases.

P-Type Ion Pump: Simulations and Theory

Jose Luis Guerra, Huan Rui, and **Benoit Roux**

University of Chicago, Chicago, IL, USA

Although the broad features of the pumping cycle of P-type ATPases are known, many aspects of the transition mechanism between the conformational states are not understood. In a first step, molecular dynamics simulations are used to provide a detailed atomic view of the pump function. Using the available 3D structures of the ATP-driven transport cycle of the calcium pump sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) from X-ray crystallography, atomic models are generated for the key states along the pumping cycle and the conformational transition pathways between the key states along the pumping cycle is determined using molecular dynamics simulations with the string method. The computations show that the large conformational change that opens the gate on the luminal side of the membrane allowing the release of the ions triggered by the phosphorylation of the pump proceeds through a two-step mechanism, with an initial rearrangement of the three cytoplasmic domains of the pump responsible for ATP binding and hydrolysis followed by the opening of the gate toward the luminal side in the transmembrane region, thereby confirming the existence of an elusive intermediate state. In a second step, we seek to develop a global view of the mechanism of P-type ATPases. For this purpose, a complete kinetic model of the Na^+/K^+ pumping cycle is constructed and its parameters are optimized with a novel Monte Carlo procedure. The energy of ATP hydrolysis and the net transported charge serve to constrain the transition rates of the cycle. In the light of the optimized model, general considerations about pump efficiency and energy landscape are examined.

A Match Made in Heaven: The Potassium Transport System KdpABC.

David Stokes

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The kdp operon is used by bacteria to combat osmotic stress, such as the loss of K^+ in the media or exposure to fresh water in the environment. Like all organisms across the kingdoms of life, elevated K^+ in the cytosol is essential for maintaining the membrane potential, driving a variety of transport processes, and in bacteria, for maintenance of pH, cell growth and division. KdpD and KdpE comprise a constitutively expressed two-component system for detecting osmotic stress and inducing the expression of the K^+ transport system KdpFABC. This system represents an unusual partnership between a K^+ channel (KdpA) and a P-type ATPase (KdpB). Recent work has produced a series of structures in a variety of different states corresponding to major intermediates in the Post-Albers transport cycle that is characteristic of the P-type ATPase superfamily. Based on this work, a detailed reaction mechanism has been proposed in which KdpA selects K^+ from

the periplasm, a unique intramembrane tunnel delivers the K^+ to canonical ion binding sites in KdpB where ATP-driven conformational changes deliver the ion to the cytosol. This mechanism highlights fascinating evolutionary adaptations of a K^+ channel, which appears to be static and no longer provides a conduit across the membrane, and a P-type ATPase, which does not directly bind ions from the periplasm and may not have innate ion selectivity. Current questions include the nature of the intramembrane tunnel, the release site of K^+ from KdpB and the energetics of the transport process.

Evening Keynote session - Thursday, September 8th

Autoinhibition and Activation of P-Type ATPases.

Poul Nissen

Aarhus University, Aarhus, Denmark

Transport in and out of cells is critical to life and tightly regulated by e.g. substrate/product gradients, autoinhibition, protein-protein interactions, post-translational modifications and lipids. Regulation is key to how transporters are integrated in molecular networks of the cell as has been highlighted recently e.g. by studies of P4-ATPase lipid flippases (1). Important to note, regulatory mechanisms are excellent entry points for development of new strategies for clinical intervention either by small molecules or antibodies.

A classic example on regulatory mechanisms of P-type ATPases is the activation of plasma-membrane Ca^{2+}-ATPase (PMCA) by Ca^{2+}-calmodulin and negatively charged lipids. We have investigated this further, also prompted by a previously reported activation of the sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) by oligomeric alpha-synuclein (αSN) (2). To our surprise we find that αSN in its native, monomeric form is a potent activator of PMCA together with negatively charged lipids, and we link this to important mechanisms for calcium homeostasis and signaling at presynaptic compartments of neurons (3). We find that the mechanism is associated with PMCA specific features, including a large, intrinsically disordered loop linking the A-domain to TM3.

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Ataxia and Failure to Thrive in Mice with Genetic Dletion of Na,K-ATPase β1 Subunit in Cerebellar Granule Cells.

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The catalytic function of Na,K-ATPase is critical to maintain intracellular ion homeostasis and cell volume, to drive secondary active transport and to fulfill more tissue-specific functions in excitable cells including neurons. Na,K-ATPase also has pump-independent roles in cell signaling and functions as a signaling scaffold; the β_1 and β_2-subunit (β_1 and β_2) have cell adhesion function. Mutations of the catalytic α_2 and α_3-subunit isoforms are associated with several neurological disorders. However, little is known about isoform-specific functions of β-subunits in the brain. Therefore, we generated a knockout mouse with targeted deletion of β_1 in cerebellar granule cells (CGCs). CGCs are one of the very few cell types expressing both β_1 and β_2 where one isoform can substitute for the other to sustain the catalytic functions of the enzyme. Thus, CGCs present an ideal isogenic system to study pump-independent, isoform-specific roles of β_1 and β_2 in neuronal cells. Heterozygous β_1 knockout mice had no apparent phenotype, had a normal life span, and were indistinguishable from control mice. In contrast, homozygous knockout mice presented with symptoms consistent with cerebellar ataxia such as wobbly gait, asynergic movements, dysmetria, and tremor that progressed with age. Homozygous β_1 knockout mice also had a shortened life span when compared to their control littermates, possibly due to a general failure to thrive. This phenotype appeared earlier and was profoundly distinct from that reported for mice with deletion of β_2 ($AMOG^{0/0}$), which exhibit motor incoordination at postnatal day 15 and die at 17-18 days of age. Our previously published studies in CGCs demonstrated isoform-specific regulation of β_1 and β_2 expression and showed that both isoforms are associated with distinct developmental signaling pathways. Together, these data suggest that the β_1-subunit of Na,K-ATPase plays an isoform-specific unique role in cerebellar granule cell function and cerebellar development.

Morning session - Friday, September 9th

Mechanism and Transport Pathway of the P4-ATPase ATP8A2 by Mutagenesis.

Jens Peter Andersen

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ATP8A2 is a phosphatidylserine- and phosphatidylethanolamine-translocating flippase highly expressed in retina and brain. To define the transport pathway for the phospholipid, we have performed mutagenesis screening of all residues in TM1-6 of bovine ATP8A2 by analyzing the ATPase activity of mutants and its dependence on the concentration of phospholipid substrate (1). Charge transfer in relation to phosphatidylserine translocation was measured for selected mutants using solid supported membranes methodology (2). These approaches have led to identification of a relatively small number of residues of critical functional importance. Recent structural work shows that several of these residues are ligands of the phospholipid head group in its occluded state in the entry site. The isoleucine of the conserved PISL motif in TM4 is notable for its involvement both as ligand of the phospholipid head group and in hydrophobic interactions with

other residues ("hydrophobic gate control"). Cryo-EM of a neurological disease mutant with methionine substitution of this isoleucine shows that the mutant is unable to occlude the phospholipid head group (3). Central to the further understanding of the transport mechanism is the question which path the phospholipid head group takes to escape from the occluded state and become inserted in the cytoplasmic bilayer leaflet. Our mutagenesis studies pinpoint specific residues that may be involved in this process.

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Lipid Flippases as Key Players in Plant Stress Adaptation.

Rosa López-Marqués

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P-type ATPases of the P4 subfamily translocate phospholipids towards the cytosolic side of the membranes at the expense of ATP. In arabidopsis, 12 P4 ATPases have been identified, named ALA1-ALA12 [1]. Our work focuses on understanding the mechanism, regulation and physiological role of ALA proteins, and we have previously provided evidence of the relevance of plant flippases in vital membrane-related cellular processes, such as lipid signaling during light sensing, adaptation to temperature changes, nutrient uptake and fertilization (for a review, see [2]). Despite the importance of these proteins for plant survival, their transport mechanism is unclear, and their regulation is unknown. Recently, a number of structures for yeast and human P4 ATPases were resolved, including several with a bound lipid. However, many of the amino acid residues suggested to be involved in lipid recognition are not conserved in plant flippases. In addition, several yeast flippases contain regulatory domains at the N- or C-terminal ends, but the presence of such a domain in plant flippases has never been demonstrated. We are currently characterizing a clade of very closely related P4 ATPases, ALA9-ALA12. While ALA10 and ALA11 are expressed mainly in roots, ALA9 and ALA12 are almost totally restricted to inflorescences [2]. The only characterized member of the family is ALA10 that is involved in lipid signaling during phosphate starvation responses and stomatal opening [3], and maintenance of the PC homeostasis in the chloroplast. To study the regulation of these proteins, we have generated a yeast mutant lacking flippase-specific kinases and provided proof-of-concept that ALA proteins are kinase-regulated. Currently, we are working in the optimization of a screening system to identify putative protein kinases from a cDNA library.

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Evolution of P4-ATPase Structure and Function.

Todd Graham

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P4-ATPases are lipid flippases that play a crucial role in the organization and function of eukaryotic cell membranes. These lipid flippases likely evolved from ancestral cation transporters and a major question we are addressing is how these P-type ATPases acquired the ability to transport bulky lipid molecules. In addition, most eukaryotic organisms express many P4-ATPases, including monomeric and heterodimeric forms, and they exhibit substantial diversity in their transport substrates. Through mutagenesis studies, we have identified residues in the membrane domains of several P4-ATPases that determine substrate specificity. These studies also helped delineate the substrate translocation pathway along the transmembrane segments from an entry gate near the exoplasmic leaflet to an exit gate near the cytosolic leaflet. We have also determined the cryo-EM structure of a monomeric P4-ATPase (Neo1) and two heterodimeric P4-ATPases, (Dnf1-Lem3, Dnf2-Lem3) from Saccharomyces cerevisiae in collaboration with Huilin Li's laboratory at the Van Andel Institute. The Neo1 structure appears to represent an intermediate in the evolution of the more prevalent heterodimeric P4-ATPases. The structures of Dnf1-Lem3 and Dnf2-Lem3 revealed transport substrate bound to the exit gate. Surprisingly, the transport lipid is drawn 10 angstroms out of the plane of the cytosolic leaflet to dock in an exit gate formed from residues in the cytosolic domains of both the alpha and beta subunits. Even though Neo1 lacks a beta subunit, the cytosolically exposed exit gate and substrate translocation pathway appear to be conserved. Insight into how substrate docking in the exit gate may be coupled to dephosphorylation of the transporter will also be discussed.

The Flip Side of P4-ATPases: Molecular Characterization of Disease-Associated Variants.

Robert Molday

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P4-ATPases comprise a subfamily of P-type ATPases that use the energy from ATP hydrolysis to transport phospholipids from the exoplasmic to the cytoplasmic leaflet of cellular membranes. This generates and maintains membrane asymmetry, a property critical for many biological processes such as vesicle transport, apoptosis and phagocytosis, blood coagulation, cell migration, synaptic pruning, cell signaling, and membrane curvature. The importance of P4-ATPases is evident in the finding that the loss in function and/or regulation of many P4-ATPases play a direct role in both monogenic and complex diseases including neurological and developmental disorders, sensory defects, cholestasis, anemia and other blood disorders, cancer, Alzheimer's disease, obesity, and diabetes. Recent clinical studies have shown that individuals homozygous or compound heterozygous for point mutations in the gene encoding ATP8A2, a P4-ATPase highly expressed in the brain, retina and testes, cause a severe neurological and developmental disorder known as Cerebellar Ataxia, Mental Retardation and Dysequilibrium Syndrome 4 (CAMRQ4). To begin to understand the molecular mechanisms responsible for CAMRQ4, we have expressed various disease-associated variants in culture cells and determined their expression level and functional activity. Our results reveal that many disease-causing variants with mutations in highly conserved motifs express at very low levels and are mislocalized to the endoplasmic reticulum of cells indicative of global protein misfolding and rapidly degradation by the proteasome of the cell. Some ATP8A2 variants, however, express at normal levels, localize like wild-type ATP8A2 to the Golgi-recycling endosomes as well as the plasma membrane of cells, but lack functional activity. The recent structures of P4-ATPases in various states together with functional studies now provide a means for understanding the molecular mechanisms underlying P4-ATPase-associated diseases and serve as a framework for developing novel therapeutic treatments for these disorders.

Cryo-EM Structures Elucidate the Lipid Transport Cycle of the P4-ATPase Flippase.

Masataka Hiraizumi[1], Tomohiro Nishizawa[2], and **Osamu Nureki[1]**

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Eukaryotic P4-ATPase translocates phosphatidyl-serine (eat-me signal) from the outer to the inner leaflet of plasma membrane to maintain lipid asymmetry, which is crucial for escaping from apoptotic phagocytosis. Here, we report the cryo-electron microscopy structures of six distinct intermediates of the human ATP8A1-CDC50a heterocomplex at resolutions of 2.6 to 3.3 Å, elucidating the lipid translocation cycle of this P4-ATPase. ATP-dependent phosphorylation of Asp409 in the phosphorylation domain induces a large rotational movement of the actuator domain around the phosphorylation site, accompanied by lateral shifts of the first and second transmembrane helices, thereby allowing phosphatidylserine binding. The phospholipid head group is transported through the hydrophilic cleft, while the acyl chain is exposed toward the lipid

environment and follows the head group movement. These findings advance our understanding of the flippase of P4-ATPases. Furthermore, we succeeded in capturing the physiological transport cycle triggered by adding excess ATP at resolutions of 2.5 to 3.1 Å, which first visualized the phosphorylated Asp409 density and first identified modulatory ATP bound to E1P state, which accelerates E2P dephosphorylation, combined with CDC50a regulatory tail sequestering ATP-binding domain.

Structural and Functional Clues on a P4B-ATPases Suggest Autoinhibition.

Line Marie Christiansen[1, 2], Thibaud Dieudonne[1], Joseph Lyons[1, 3], Poul Nissen[1, 2]

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The lipid composition of eukaryotic cellular membranes is tightly controlled by three groups of membrane proteins: flippases, floppases and scramblases. These ensure a correct lipid distribution for cell-to-cell signaling, vesicle formation and apoptosis throughout different cellular membranes. The majority of lipids flippases are P4-ATPases and are further divided into two main groups: those who function in a complex with a CDC50-family protein and those that do not. An overall understanding of the first group has emerged within recent years, whereas our understanding of the latter group (P4B-ATPases) is still in the initial stages. The group includes *Saccharomyces cerevisiae* Neo1p and mammalian ATP9A and ATP9B, whereof ATP9A dysfunction has recently been linked to neurodevelopmental phenotypes.

By a combined biochemical and structural approach, Neo1p has been utilized as a model for P4B-ATPases. Biochemical studies reveal a stabile E2P phosphoenzyme in absence of substrate, insensitive to addition of suggested phospholipid substrates. Single particle Cryo-EM lead us to determine two states: an E2P and an E1P state. The aluminium flouride inhibited 2.7 Å E1P-like state reveals a new orientation of the A-domain and supports the existing evidence of P4-ATPases adopting an E1P-like state in absence of substrate. This regardless of addition of inhibitors at the point of solubilization.

Recently, a 2.2Å structure in presence of beryllium flouride adopted an E2P state, with an only partially open substrate binding pocket. The position of transmembrane helix two and conserved P4B-ATPase residues are seen to block the proposed substrate binding site, suggesting an autoinhibited E2P state. Unlike other autoinhibited P4-ATPases (Timcenko 2018, Dieudonne 2022) no structural clues suggesting inhibition by termini were evident in the data.

Together the data suggests that the P4B-ATPase Neo1p accumulates in an autoinhibited E2P form with a yet unknown activation mechanism.

Novel Mechanisms for Voltage Sensing.

Konark Bisht, **Himanshu Khandelia**

University of Southern Denmark

Ion channels and pumps regulate electrical signaling in cells, essential for many biological processes. Regulation is imparted by the ability of channels and pumps to sense transmembrane voltage differences (dVTM). Current theories postulate that charged voltage-sensing molecular motifs move in direct response to changes in dVTM to control ion conduction. We propose that channels can also sense dVTM by utilising the reorientation of electrical dipoles in their transmembrane helices due to an external electric field. The idea draws inspiration from the concept of "flexoelectricity" (FE) in liquid crystal physics, which describes electromechanical coupling in systems with orientational order. This concept allows an ion channel to harness the electric-field induced dipole reorientations of its transmembrane helices to generate torque and sense dVTM.

We consider model peptides having electrical dipole moments in a lipid bilayer and perform molecular dynamics simulations to study the effect of variation of TM voltage on their tilt angles and ascertain the optimal parameters for designing a sensitive membrane voltage sensor.

Evening Session – Friday, September 9th

P5B-ATPases: Endo-Lysosomal Polyamine Transporters Implicated in Disease.

Peter Vangheluwe

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The human P5B-type ATPase isoforms ATP13A2-4 are genetically linked to various neurological and cardiovascular diseases. Our lab has established the P5B-type ATPases as polyamine transporters in the endosomal system. We compare the biochemical properties of P5B isoforms and study the effect of disease mutations. We also provide insights into their cellular and physiological function, and focus on the role of ATP13A2 in Parkinson's disease. Our work establishes the P5B-type ATPases as important members of the previously uncharacterized mammalian polyamine transport system, and offers new therapeutic perspectives for Parkinson's disease and beyond.

Mechanisms of Substrate Selection and Translocation by the P5B Polyamine Transporter ATP13A2.

Kenneth Lee

Pennsylvania State University, Hershey, PA, USA

Mutations in ATP13A2, also known as PARK9, cause a rare monogenic form of juvenile-onset Parkinson's disease named Kufor-Rakeb syndrome and other neurodegenerative diseases. ATP13A2 encodes a neuroprotective P5B P-type ATPase highly enriched in the brain that mediates selective import of spermine ions from lysosomes into the cytosol via an unknown mechanism. Here we present three structures of human ATP13A2 bound to an ATP analog or to

spermine in the presence of phosphomimetics determined by cryoelectron microscopy. ATP13A2 autophosphorylation opens a lysosome luminal gate to reveal a narrow lumen access channel that holds a spermine ion in its entrance. ATP13A2's architecture suggests physical principles underlying selective polyamine transport and anticipates a ''pump-channel'' intermediate that could function as a counter-cation conduit to facilitate lysosome acidification. Our findings establish a firm foundation to understand ATP13A2 mutations associated with disease and bring us closer to realizing ATP13A2's potential in neuroprotective therapy.

What the Cryo-EM Structure of the Fungal Proton Pump Tells Us About its Mechanism.

Maike Bublitz[1], Sabine Heit[1], Maxwell M. G. Geurts[1], Bonnie J. Murphy[2], Margaret Young[1], Robin A. Corey[1], Deryck J. Mills[2], Werner Kühlbrandt[2]

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The fungal plasma membrane H^+-ATPase Pma1 generates a vital proton-motive force that maintains the intracellular pH and drives the import of essential nutrients. Auto-inhibited Pma1 hexamers in the plasma membrane of starving fungi are activated by glucose signaling and subsequent phosphorylation of a C-terminal autoinhibitory domain. As related P-type ATPases are not known to form hexamers, the physiological relevance of hexameric Pma1 remained unknown. We have determined the structure of hexameric Pma1 from Neurospora crassa by cryo-EM at 3.3 Å resolution, elucidating the molecular basis for hexamer formation and auto-inhibition, and providing a starting point for structure-based drug development. Coarse-grained molecular dynamics simulations in a lipid bilayer suggest lipid-mediated contacts between monomers and a substantial protein-induced membrane deformation that could act as a proton-attracting funnel. There is also evidence of an ordered arrangement of lipids within the outer leaflet inside hexamer core, the function and identity of which we are aiming to elucidate.

Reference:
Heit S, Geurts MMG, Murphy BJ, Corey RA, Mills DJ, Kühlbrandt W, Bublitz M. Structure of the hexameric fungal plasma membrane proton pump in its autoinhibited state. 2021 Sci Adv. 12;7(46):eabj5255. doi: 10.1126/sciadv.abj5255.

Involvement of Alpha-Subunit N-Termini in the Mechanism and Regulation of the Na^+,K^+- and H^+,K^+-ATPases.

Ronald Clarke

University of Sydney, Sydney, Australia

The cytoplasmic lysine-rich N-termini of the Na^+,K^+- and H^+,K^+-ATPases have been designated as R domains because of their possible role in regulation of ion pumping activity. Prior to the

publication of any crystal structures of either protein, it was suggested by the Blostein group (1-3), based on mutagenesis studies, that regulation of the Na^+,K^+-ATPase could involve the formation and breakage of a salt bridge between Lys30 of the N-terminus and Glu233 in the first M2-M3 cytoplasmic loop of the alpha subunit. In X-ray crystallographic studies of the Na^+,K^+-ATPase, the N-terminus has never been able to be resolved, probably because it is too mobile on the time scale of the structure determination. Nevertheless, after theoretically predicting the N-terminus conformation, and adding it to the crystal structure, the distance between residues Lys30 and Glu233 makes it doubtful that a direct long-lived interaction between them is involved in pump regulation.

As an alternative hypothesis, we propose that regulation could occur via an interaction of the positively charged lysine residues of the N-terminus with negatively charged lipid headgroups (notably phosphatidylserine) on the cytoplasmic surface of the surrounding membrane, and that such an interaction could potentially be regulated by an electrostatic switch mechanism, whereby serine and tyrosine residues of the N-terminus are phosphorylated by protein kinases. Phosphorylation of these residues would decrease the positive charge of the N-terminus, allowing its release from the membrane. Experimental and theoretical data will be presented which support this hypothesis.

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Activation and Substrate Recognition Mechanism of ATP8B1/CDC50A Revealed by Cryo-EM.

Thibaud Dieudonné[1], Michelle Juknaviciute Laursen[1], Charlott Stock[1], Joseph A. Lyons[2], Poul Nissen[1]

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Lipid flippases (P4-ATPases) mediate active transport of phospholipids from the exoplasmic leaflet to the cytoplasmic one, in order to establish and maintain phospholipid asymmetry in eukaryotic membranes. This asymmetry is tightly regulated, and important for numerous cellular processes such as membrane trafficking, signaling, apoptosis, cytokinesis and cell fusion. The human genome encodes 14 different P4-ATPases, a subset of which are linked to severe diseases including cognitive disorders e.g. Alzheimer's and Parkinson's disease, diabetes, cancer or liver disorders. For example, mutations of the human lipid flippase ATP8B1, are responsible for inherited intrahepatic cholestasis, a rare liver disease and KO of *Atp8b1* in beta cells inhibits glucose-stimulated insulin release. Little is known about the exact involvement of ATP8B1 in these diseases, mainly because of the lack of consensus regarding its transported substrate and on how its activity is regulated. Previously, we have demonstrated that ATP8B1 is autoinhibited by

an evolutionary conserved mechanism involving its N- and C-terminal tails and that the activity of ATP8B1 is tightly regulated by phosphoinositide [1].

Here, we present our most recent results on the structural and functional characterization of the ATP8B1/CDC50A transport cycle. Our single-particle cryo-EM analysis reveals the structural changes happening upon ATP binding, autophosphorylation, relief of the autoinhibition, substrate binding and substrate occlusion. Interestingly, our functional data suggest that ATP8B1 has a much broader substrate specificity than initially anticipated and that surprisingly the substrate specificity is not driven by the lipid head group.

1. Dieudonné et al., 2020, eLife

Morning Session – Saturday, September 10th

A Novel Approach for Quantification of Endoplasmic Reticulum Ca^{2+} Transport.

Elisa Bovo, Roman Nikolaienko, **Aleksey Zima**

Loyola University Chicago, Maywood, IL, USA

The type 2a sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA2a) plays a key role in Ca^{2+} regulation in the heart. However, available techniques to study SERCA function are either cell destructive or lack sensitivity. The goal of this study was to develop an approach to selectively measure SERCA2a function in the cellular environment. The genetically encoded Ca^{2+} sensor R-CEPIA1er was used to measure the concentration of Ca^{2+} in the lumen of the endoplasmic reticulum (ER) ($[Ca^{2+}]ER$) in HEK293 cells expressing human SERCA2a. Coexpression of the ER Ca^{2+} release channel ryanodine receptor (RyR2) created a Ca^{2+} release/reuptake system that mimicked aspects of cardiac myocyte Ca^{2+}-handling. SERCA2a function was quantified from the rate of $[Ca^{2+}]ER$ refilling after ER Ca^{2+} depletion, then ER Ca2+ leak was measured after SERCA inhibition. ER Ca2+ uptake and leak were analyzed as a function of [Ca2+]ER to determine maximum ER Ca2+ uptake rate and maximum ER Ca2+ load. The sensitivity of this assay was validated by analyzing effects of SERCA inhibitors, [ATP]/[ADP], oxidative stress, phospholamban and a loss-of-function SERCA2a mutation. In addition, the feasibility of using R-CEPIA1er to study SERCA2a in a native system was evaluated by using in vivo gene delivery to express R-CEPIA1er in mouse hearts. After ventricular myocyte isolation, the same methodology used in HEK293 cells was applied to study endogenous SERCA2a. In conclusion, this new approach can be used as a sensitive screening tool to study effect of different drugs, post-translational modifications and mutations on SERCA function.

Deep Learning Design and Identification of Small-Molecule Effectors Targeting Ion-Motive Pumps in the Heart.

L. Michel Espinoza-Fonseca

University of Michigan, Ann Arbor, MI, USA

We have developed a platform centered on deep learning to design and discover small-molecule effectors targeting ion-motive pumps in the heart. We focus on two ion-motive pumps as proof-of-principle pharmacological targets: the calcium pump (sarcoplasmic reticulum Ca^{2+}-ATPase, SERCA) and the sodium pump (Na^{+}-K^{+}-ATPase, NKA) SERCA and NKA are essential membrane-bound transport ATPases establishing vital Ca^{2+} and Na^{+}/K^{+} gradients across the sarcoplasmic reticulum and plasma membrane, respectively. We chose these targets because they play a major role in the excitation-contraction-relaxation cycle in normal and pathological cardiac muscle, and because they are important pharmacological targets in the heart. We developed a library of lead-like small molecules that permeate across the lipid bilayer, activate cardiac SERCA in situ, stimulate Ca^{2+} transport in iPSC-derived cardiomyocyte models recapitulating healthy and disease phenotypes. In parallel, we discovered new molecules that inhibit the ATPase activity of SERCA and NKA in the micromolar range; these molecules include both FDA-approved and investigational drugs. Interestingly, a partial inhibitor of both SERCA and NKA has no apparent cardiotoxic effects in healthy cardiac cells, but it suffices to worsen the disease phenotype of genetic iPSC-CM models of cardiomyopathy. These studies support the application of deep learning for advancing drug discovery and identification of off-target effects focused on cardiac ion-motive pumps.

50,000-Compound High-Throughput Screens for Novel, Drug-Like Modulators of SERCA

Razvan Cornea

University of Minnesota, Minneapolis, MN, USA

To discover small-molecule effectors of SERCA pump activity, we have developed platforms compatible with high-throughput screening (HTS) of large chemical libraries of drug-like compounds. These platforms are based on FRET and functional assays of SERCA. Increasing SERCA expression or specific activity can alleviate pathological states fueled by chronically elevated [Ca^{2+}], most notably in the heart. We seek to develop small-molecule drug candidates that activate Ca^{2+}-pumping by SERCA. One approach we have used to this end was to adapt an NADH-coupled assay – measuring Ca-dependent ATPase activity of SERCA – to HTS format. We used this assay with isolated sarcoplasmic reticulum (SR) membranes to screen a 50,000-compound library of diverse chemical scaffolds. The other approach we have used for HTS relies on FRET constructs of SERCA ± phospholamban recombinantly expressed in HEK cells. For screening, we rely on live cells expressing these FRET biosensors, applied to 1536-well plates that are rapidly read in a fluorescence lifetime plate-reader (FLT-PR). These live-cell FRET platforms consistently enable HTS campaigns of excellent quality. Our screens have yielded numerous hits that reproducibly alter SERCA Ca-ATPase activity in cardiac and skeletal SR. The top activating hits were further tested for both Ca-ATPase and Ca^{2+} transport effects. Many hits increased Ca^{2+} uptake in both skeletal and cardiac SR, with some showing isoform specificity. Furthermore, dual analysis of both activities has identified compounds with a range of effects on Ca^{2+}-uptake and -ATPase, which fit into distinct classifications. Further studies will be needed to identify which classes are

best suited for therapeutic use, working with medicinal chemists to optimize potency and isoform specificity. These outcomes highlight the need for robust secondary assays and criteria for selection of lead compounds that address specific clinical goals.

How to Make a Sodium Pump.

Victoria C. Young[1], Hanayo Nakanishi[2], Dylan J. Meyer[1], Tomohiro Nishizawa[3], Atsunori Oshima[2,4,5], **Pablo Artigas**[1], Kazuhiro Abe[2,4]

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Ion-transport mechanisms evolve by changing ion-selectivity, such as switching from Na^+ to H^+ selectivity in secondary-active transporters or P-type-ATPases. Here we study primary-active transport via P-type ATPases using functional and structural analyses to demonstrate that four simultaneous residue substitutions transform the non-gastric H^+/K^+ pump, a strict H^+-dependent electroneutral P-type ATPase, into a bona fide Na^+-dependent electrogenic $Na+/K+$ pump. Conversion of a H^+-dependent primary-active transporter into a Na^+-dependent one provides a prototype for similar studies of ion-transport proteins. Moreover, we solve the structures of the wild-type non-gastric H^+/K^+ pump, a suitable drug target to treat cystic fibrosis, and of its Na^+/K^+ pump-mimicking mutant in two major conformations, providing insight on how Na^+ binding drives a concerted mechanism leading to Na^+/K^+ pump phosphorylation.

Tracking P-type ATPase Native Structural Dynamics in Real Time.

Magnus Andersson

Umeå University, Umeå, Sweden

P-type ATPase transport is carried out in a complex lipid environment and is regulated by both external and internal factors. We have developed a methodology to track P-type ATPase domain rearrangements in real time triggered by laser-induced release of ATP and subsequent monitoring by synchrotron X-ray pulses. This time-resolved X-ray solution scattering (TR-XSS) technique was used to capture the dynamics of an equilibrium state of the sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA1a), and transient states at 1.5 and 13 milliseconds. The 13-millisecond state showed a previously unresolved actuator (A) domain arrangement that exposed the ADP-binding site after phosphorylation. We have now identified subtle differences in SERCA1a dynamics that depend on Ca^{2+} concentration. In addition, we have characterized regulation dynamics in the isoform SERCA2b. The TR-XSS technique presents a unique possibility to simultaneously determine kinetics and structural rearrangements in P-type ATPases to better understand the transport reactions and their regulation.

Na,K-ATPase α4 is Involved in Sperm Energetics.

September Numata, Jeffrey McDermott, **Gustavo Blanco**

University of Kansas Medical Center Department of Cell Biology and Physiology

The Na,K-ATPase alpha 4 isoform (NKAα4) is expressed specifically in the male germ cells of the testes and is particularly abundant in mature spermatozoa. Genetic ablation of NKAα4 in mice (NKAα4 KO mice) results in complete infertility of male mice, due to severe impairments in sperm motility and capacitation. Here, we show that deletion of NKAα4 also leads to major defects in sperm metabolism and energetics. Sperm from NKAα4 KO mice displayed a significant reduction in the extracellular acidification rate (ECAR) compared to wild-type sperm, indicative of impaired glycolytic flux. Accordingly, glucose uptake was reduced in sperm lacking NKAα4. In addition, mitochondrial function was disrupted in NKAα4 KO sperm, as demonstrated by a decrease in the mitochondrial membrane potential and lower oxygen consumption rate (OCR). Moreover, the ratio of NAD/NADH was increased in NKAα4 KO sperm, indicating a shift in the cellular redox state of the cells. Concomitant with these metabolic changes, we found a decrease in ATP production, an increase in the production of reactive oxygen species, and higher lipid peroxidation in NKAα4 KO sperm. Altogether, these findings reveal a novel link between NKAα4 activity and sperm metabolism and establish a new mechanism for the control of sperm function and male fertility by NKAα4.

ATP10A has a Protective and Sex-Specific Role in Lipid Metabolism.

Adriana Norris[1, 2], Eugenia Yazlovitskaya[1, 2], Lin Zhu[3, 4], Bailey Rose[1, 5], Sophia Yu[3, 4], John McLean[1, 5], John Stafford[3, 4], Todd Graham[1, 2]

[1]Vanderbilt University, [2]Biological Sciences Department, [3]Vanderbilt University Medical Center, [4]Department of Medicine, [5]Department of Chemistry

Genetic predisposition and environment play substantial roles in obesity, type 2 diabetes and cardiovascular disease (CVD). Genetic association studies have linked *ATP10A*, encoding a type IV P-type ATPase (P4-ATPase), to human metabolic disease. ATP10A is a lipid flippase that catalyzes the membrane translocation of phosphatidylcholine and glucosylceramide. These lipids and their respective metabolites have been independently implicated in metabolic dysfunction. To explore the role of this flippase in metabolism, we created a novel *Atp10a* knockout (KO) mouse model. *Atp10a* KO mice display a female-specific weight gain during high-fat diet feeding and this is attributable to increased adiposity. Female *Atp10a* KO mice also exhibit elevated plasma free fatty acids, cholesterol, and triglycerides, as well as a depletion in eicosanoid species compared to the wild type (WT) littermates. Additionally, female *Atp10a* KO mice exhibit elevated fasting blood glucose levels without compensatory elevation of insulin. We also found that the liver of female *Atp10a* KO mice displays larger lipid droplets, which was associated with increased diacylglycerol acyltransferase-2 (DGAT2) expression and an attenuation of the insulin signaling pathway compared to the WT littermates. Thus far, our studies have shown that knocking out *Atp10a* in mice on a high fat diet results in sex-specific perturbations to body composition, plasma lipid levels, glucose homeostasis, and liver metabolism. We have recently found that ATP10A is specifically expressed in the endothelial cells of multiple tissues. We are now exploring these

metabolic phenotypes through the lens of endothelial cell dysfunction. These studies suggest mechanisms by which this flippase contributes to the development of CVD with obesity.

Afternoon Session – Saturday, September 10th

Hydrolytic Functions of the Yeast Spf1p P5A-ATPase.

Hugo Adamo

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The recent finding that the P5A-ATPases can extract proteins from the ER membrane has turned a long standing mystery into fascination (1). While in light of this "dislocase" activity, the ATP hydrolysis by the P5A-ATPAses can be finally rationalized, the details of the coupling between these two activities remain unsolved. Lipid/detergent micellar preparations of the recombinant purified yeast P5A- ATPase Spf1p have an ATPase activity ~ 0.2-0.8 µmol Pi/mg/min (0.4-0.8 s^{-1}) at 28 °C in a reaction medium containing 3 mM ATP, a high concentration of free Mg^{2+} and without adding any putative substrate. We have compared the hydrolytic activity of the wild type and mutant Spf1p towards different phosphorylated compounds, the effect of inhibitors and formation of EP. The hydrolysis kinetics observed under these conditions is similar to those of other P-ATPases under a normal coupled transport activity. This opens the possibility that the micellar preparation of Spf1p can hydrolyze ATP through a complete E2-E1 cycle.

(1) McKenna et al. Science. 2020

The Interrelationships of the Structure and Dynamics of Ion Transport in Disease-Causing Mutations of the Brain-Specific Na/K-ATPase.

Miguel Holmgren

National Institutes of Health, Bethesda, MD USA

Na^+/K^+-ATPase, which creates transmembrane electrochemical gradients by exchanging 3 Na^+ for 2 K^+, is central to the pathogenesis of neurological diseases such as alternating hemiplegia of childhood. Here we will discuss that binding of Na^+ ions in the human α3 Na^+/K^+-ATPase can be resolved using extracellular Na^+-mediated transient currents. When Na^+/K^+-ATPase is constrained to bind and release only Na^+, three kinetic components: fast, medium, and slow, can be isolated, presumably corresponding to the protein dynamics associated with the binding (or release depending on the voltage step direction) and the occlusion (or deocclusion) of each of the 3 Na^+. Patient-derived mutations of residues which coordinate Na^+ at site III exclusively impact the slow component, demonstrating that site III is crucial for deocclusion and release of the first Na^+ into the extracellular milieu. These results advance understanding of Na^+/K^+-ATPase mutation pathogenesis and provide a foundation for study of individual ions' binding kinetics.

Dimerization of the Sodium Pump in Health and Disease.

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The sodium-potassium ATPase (NKA) is the ion motive ATP-dependent transporter that establishes Na^+ and K^+ gradients across the cell membrane, facilitating many physiological processes. In the heart, NKA activity is regulated by phospholemman (PLM). PLM inhibits NKA by reducing the pump's apparent affinity of the pump for Na^+. In this study, we used time-correlated single-photon counting, FRET-microscopy, molecular dynamics simulations, and electrophysiology to investigate the structure/function mechanisms that govern the NKA-PLM regulatory complex. The data suggest a regulatory complex composed of two α subunits associated with two β subunits and decorated with two PLM regulatory subunits. Protein-protein docking and molecular dynamics simulations generated a structural model of the regulatory complex. This model was characterized by contacts between cytoplasmic domains of α subunits and β subunit extracellular domains. We also observed contacts between the highly-conserved α subunits' M3 helix and the N-termini of the opposing β subunit. We noted that mutations in this region of the M3 helix are associated with alternating hemiplegia of childhood, renal hypomagnesemia, and familial hemiplegic migraine type 2. We observed that a hypomagnesemia-causing variant, G301R, was unable to correctly localize in the membrane, but less severe mutation G301A can correctly localize. Molecular dynamics simulations of the homo- (G301A-G301A, G301R-G301R) and hetero- (WT-G301A, WT-G301R) dimeric complexes revealed changes in the Na^+ binding at position II. Two electrode voltage clamp revealed affected affinity to the transported ions and decrease the turn-over rate of the G301A mutant. We hypothesize that the observed trafficking and functional defects of G301 mutants contribute to the disease phenotype.

Contribution of Intracellular Ca^{2+}-ATPases to the Functional Modulation of Microglia: Implication in Neuropathologies.

A.M. Bhojwani-Cabrera, J.M. Morales-Ropero, V.E. Neubrand, D. Martín-Oliva, M.A. Cuadros, J.L. Marín-Teva, **Maria Rosario Sepúlveda**

University of Granada, Granada, Spain

Microglial cells constitute the first and main line of defense against injury in the central nervous system. They are continuously surveilling nervous parenchyma and react in response to many triggers adopting diverse activated phenotypes that are characterized by morphological transformation, increased migration, proliferation, release of different factors, and phagocytic activity. Although many activators of microglia are known, the underlying subcellular mechanisms

involved are not clear yet. We have studied how Ca^{2+} stores and intracellular Ca^{2+} transporters can play essential roles in modulating microglial functions. We found an upregulation of the sarco(endo)plasmic reticulum Ca^{2+}-ATPase SERCA2b in activated microglia in Alzheimer´s disease-affected brains, and in vitro in microglia cultures after activation with lipopolysaccharide (LPS), which is known to induce an increase of cytosolic Ca^{2+}. Under these conditions, enhanced SERCA2b expression might restore the maintained high cytosolic Ca^{2+} caused by cell activation. We used the specific SERCA inhibitor thapsigargin, which affected microglial migration and phagocytosis in an opposite manner, pointing towards a role of SERCA2b as major player to orchestrate microglial functions. On the other hand, we have also focused on the secretory pathway Ca^{2+}/Mn^{2+}-ATPase SPCA1, which was also upregulated after LPS-induced microglial activation. Considering one of the SPCA function as Mn^{2+} transporter and that Mn^{2+} overexposure produces manganism pathology, sharing symptoms with Parkinson's disease, we have studied Mn^{2+} toxicity in microglia. Results showed a clear effect of Mn^{2+} excess on the viability and morphology of microglial cells. Subcellular analysis showed Golgi fragmentation with alteration of SPCA1 distribution at early stages of toxicity. Mn^{2+} removal did not effectively reverse the Golgi fragmentation, but curcumin pre-treatment maintains cell viability and prevented Mn^{2+}-induced Golgi fragmentation, suggesting curcumin as a potential protective agent against Mn^{2+} toxicity in microglia.

This work was supported by A1-CTS-324-UGR18 from FEDER-Junta de Andalucía, Spain.

Post-Translational Regulation of the K^+ Pump KdpFABC.

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The high-affinity K^+ pump KdpFABC from *Escherichia coli* is a K^+ uptake system essential for survival at K^+ limitation. The phosphorylation of $KdpB_{S162}$ has been shown to prevent the transition from the high-energy E1-P state to the E2-P conformation. How the complex can be stalled in the E1-P intermediate remained unclear. We generated a series of cryo-EM structures of KdpFABC in the catalytic and inhibited state, under turnover conditions and stabilized by nucleotide and phosphate analogs, to identify the structural basis for KdpFABC inhibition in the E1-P state. We find that phosphorylated KdpFABC adopts a conformation not observed for other P-type ATPases. It appears that this conformation represents an off-cycle state that is adopted when phosphorylated KdpFABC tries to relax from the high-energy E1-P state, but cannot fully transition to the E2-P conformation. This represents the first structural characterization of a biologically relevant P-type ATPase off-cycle state, and supports the emerging discussion of P-type ATPase regulation by off-cycle states.

The expression of *kdpFABC* is controlled by the two-component system KdpDE. At low K^+ conditions, the histidine kinase KdpD phosphorylates KdpE, which initiates the transcription of *kdpFABC*. At viable K^+ conditions, KdpD dephosphorylates KdpE, ending further protein production. However, only regulating gene expression is not sufficient. To prevent excessive K^+ accumulation, KdpFABC already present in the membrane is inactivated by phosphorylation of KdpB. Here, we show that this inhibition is also facilitated by KdpD. Phospho-transfer to KdpB is mediated directly by a Walker A motif in the N-terminal KdpD domain. Thus, KdpD fully controls the activity of the Kdp system on a transcriptional and a post-translational level. The inhibition of KdpFABC by KdpD for the first time shows a post-translational regulation of a transporter by its sensor kinase, adding another layer to the growing functional diversity of two-component systems.

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Poster Abstracts

Poster #1 - Sustainability from a Cell Perspective

Do Trang Le[1], Charles Cranfield[1], Evelyne Deplazes[2], **Ronald J. Clarke**[3]

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Sustainability is a worldwide problem, for cells as it is for human beings and our whole planet. Key to solving the sustainability problem is recycling. In cells, this is accomplished within the lysosome. All biological macromolecules, such as proteins and nucleic acids, which are past their use-by date, are transported into the lysosome, where enzymes degrade them into their monomeric building blocks. These enzymes are only active at the low pH of ~4.7 of the lysosome lumen. After degradation, these building blocks are transported out of the lysosome into the cytoplasm, where they are used for the biosynthesis of new proteins and nucleic acids. Because of the need to encapsulate the degrading enzymes within the lysosome to avoid damage to functionally active proteins and nucleic acids, the lysosome membrane is a powerhouse of solute transport within the cell.

This recycling process is crucial for the survival of cells, particularly neurons, which are no longer capable of cell division. Defects in this process cause many hereditary neurodegenerative diseases. But because of their rarity, pharmaceutical companies are not particularly interested in finding cures. However, the diagnosis of neurodegenerative disease is devastating for the family of any afflicted child. One group of neurodegenerative diseases is termed Batten's disease. While symptoms vary, all are caused by lysosomal dysfunction, resulting in the steady build-up of waste material in the cell. Disease progression is typically characterised by the development of blindness, followed by the onset of seizures and early death, typically in the 20s.

Understanding the disease would not only help its sufferers but also provide valuable information on a crucial cell function, i.e., how cells recycle their waste. We will describe how biophysical measurements are helping to provide some insights into the molecular physiology of lysosome function and the pathology of Batten's disease.

Poster #2 - Unravelling Regulatory Mechanisms of the Human Lipid Flippase ATP8B1

Michelle Juknaviciute Laursen, Thibaud Dieudonné, Poul Nissen

Aarhus University, Department of Molecular Biology and Genetics

The human flippase ATP8B1 is a member of the P4 ATPase family of phospholipid flippases. ATP8B1 with its chaperone CDC50 protein is localized at the plasma membrane and is involved in the regulation of the asymmetric lipid distribution of the plasma membrane. ATP8B1 is directly involved in bile homeostasis, and mutations in ATP8B1 are linked to several bile disorders, such as Progressive Familial Intrahepatic Cholestasis.

In vitro assays revealed that full-length ATP8B1 is inactive, but truncation of the C-terminal region results in activation of the ATPase activity of the complex. This activity is further increased

when the N-terminal region of ATP8B1 is also truncated, indicating a cooperative autoinhibition mechanism.[1] The activation mechanism *in vivo*, however, remains poorly understood.

To explore C-terminal autoinhibition, a C-terminally truncated ATP8B1 was treated with a peptide equivalent to the missing C-terminal segment showing clear inhibition with an IC50 of 59 nM. When repeated with a peptide phosphorylated at the residue corresponding to Ser-1223 of ATP8B1, the inhibition was decreased to an IC50 of 2.9 µM, indicating that phosphorylation of Ser-1223 may affect the autoinhibition of ATP8B1.

To identify kinases capable of phosphorylating the C-terminal region, a peptide construct of the C-terminal region linked to GFP was expressed and purified for a kinase screen provided by Reaction Biology. From the results of the kinase screen, Protein Kinase C (PKC), among other kinases, was found to phosphorylate the C-terminal peptide. Due to PKC's known involvement in bile homeostasis and that kinase target site prediction proposes PKC to phosphorylate ATP8B1 at Ser-1223, we find PKC to be a possible regulator of ATP8B1. Further studies remain to establish the effect of PKC on ATP8B1 *in vivo.* Identifying the activation mechanism of ATP8B1 may reveal new strategies for drug development against bile disorders.

[1]Dieudonné et al. (2022) DOI: 10.7554/eLife.75272

Poster #3 - A Fever-Induced Neurological Syndrome is Caused by the Temperature-Sensitivity of an ATP1A3 Mutation, p.Arg756His.

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ATP1A3 is the gene encoding the α3 isoform of Na,K-ATPase. In the CNS it is expressed only in neurons. Human mutations produce a wide spectrum of phenotypes, but particular syndromes are associated with unique substitutions. For arginine 756, three different substitutions produce encephalopathies that manifest during febrile infections. Here we tested the pathogenicity of p.Arg756His (R756H) expressed in mammalian cells. R756H had sufficient transport activity to support cells when endogenous ATP1A1 was inhibited. It had approximately half of the turnover rate of WT with reduced affinity for Na^+ and increased affinity for K^+. There was evidence for moderate ER retention during biosynthesis at 37ºC, indicating misfolding, and only modest benefit from the folding drug phenylbutyrate (4-PBA). When cells were incubated at 39ºC, however, in the febrile range, α3 protein level dropped significantly without loss of the beta subunit, paralleled by an increase of endogenous α1. Elevated temperature resulted in internalization of α3 from the surface with some β subunit, accompanied by cytoplasmic redistribution of LAMP1, a marker of lysosomes and endosomes. After a return to 37ºC, there was recovery of α3 protein levels, sensitive to cycloheximide. Heating in vitro showed mutation-specific activity loss at a rate 20- to 30-fold faster than the wild type, indicating a temperature-dependent destabilization of the protein structure. The observations are consistent with fever-induced variable symptoms in patients followed by recovery, with some accumulation of persistent symptoms. Arg756 appears to confer thermal resistance by forming hydrogen bonds among four separate parts of the complex Na,K-ATPase structure.

Poster #4 - Heterozygous ATP1A1 Knockout Mice Lack a Neuropathic Phenotype.

Kerri Spontarelli, Ryan Sweazey, Alexandria Padro, Jeannie Lee, Tulio Bueso, Roberto Hernandez, Jongyeol Kim, Jeremy Bailoo, Pablo Artigas

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Charcot-Marie-Tooth disease (CMT), the most common heritable peripheral neuropathy, presents with distal muscle atrophy and weakness, loss of sensation, absent reflexes, and *pes cavus*. The two major types of CMT are due to demyelination (CMT1) and axonal degeneration (CMT2). CMT2 has been linked to heterozygous germline mutations in *ATP1A1* (the gene encoding the α1 subunit of the Na^+/K^+-ATPase (NKA)) which are reported to cause loss of function. NKA actively transports Na^+ and K^+ to establish electrochemical gradients essential for proper action potential propagation and is an αβ heterodimer. The α1 isotype of the catalytic α subunit is expressed in all tissues and is the predominant ortholog in peripheral axons. To further investigate the role of *ATP1A1* haploinsufficiency in CMT2 pathophysiology, heterozygous *ATP1A1* knockout mice (*ATP1A1*$^{+/-}$) were studied by comparing performance of *ATP1A1*$^{+/-}$ and WT littermates in a series of behavioral tests evaluating strength, coordination, dexterity, and balance, performed regularly up to 18 months old to determine if a neuropathic phenotype was present. No significant differences between WT and *ATP1A1*$^{+/-}$ mice were observed throughout their lifespans. We also evaluated if increasing the demand on peripheral neurons via exercise would induce a CMT2-like phenotype in *ATP1A1*$^{+/-}$ mice. *ATP1A1*$^{+/-}$ and WT littermates were exercised on a treadmill at 14m/min, 30 min/day, 5 days/week, for 5 months. Behavioral tests performed up to 9 months old revealed no significant differences between *ATP1A1*$^{+/-}$ or WT mice. Likewise, no significant differences in behavioral performance were observed at any time point between exercised and non-exercised mice. Electromyography was used to determine if subclinical disease was present in *ATP1A1*$^{+/-}$ mice which detected no difference in the compound neuromuscular action potentials of WT and *ATP1A1*$^{+/-}$ mice. The absence of a neuropathic phenotype in *ATP1A1*$^{+/-}$ mice suggests a complex pathophysiology of *ATP1A1*-driven CMT2. 1R03-NS116433-01

Poster #5 - Mechanism of K^+ Transport Along the Intersubunit Tunnel of kdpFABC.

Hridya Valia Madapally[1], **Himanshu Khandelia**[1], David Stokes[2]

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Bacteria such as *E. Coli* employ a unique channel-pump protein complex called kdpFABC to import K^+ into the cell in low external K^+ environments. The exact mechanism by which the channel subunit, kdpA, and the pump subunit, kdpB, participate together to transport the ion remains contentious. Current research indicates that K^+ ions diffuse from the kdpA ion-binding site to the kdpB ion-binding site through a ~40 Å long tunnel which passes through the channel-pump interface. However, there is no clear consensus on whether K^+ ion or water populate the tunnel in the cryo-EM studies. Though molecular dynamics simulations, we find that not more than two K^+ ions can coexist in the tunnel between S4 site of the selectivity filter and the channel-pump interface. We also employ well-tempered metadynamics to calculate the energetics of ion

transport along the intramembrane tunnel. The high energy barrier required for the K^+ ion to cross the channel-pump interface suggests that the K^+ transport along the tunnel may not occur in the E1×ATP state of the pump. Further investigations are ongoing to understand the mechanism of transport of K^+ ions across the tunnel.

Poster #6 - Novel Mechanisms for Voltage Sensing

Konark Bisht, **Himanshu Khandelia**

University of Southern Denmark

Ion channels and pumps regulate electrical signaling in cells, essential for many biological processes. Regulation is imparted by the ability of channels and pumps to sense transmembrane voltage differences (dVTM). Current theories postulate that charged voltage-sensing molecular motifs move in direct response to changes in dVTM to control ion conduction. We propose that channels can also sense dVTM by utilizing the reorientation of electrical dipoles in their transmembrane helices due to an external electric field. The idea draws inspiration from the concept of "flexoelectricity" (FE) in liquid crystal physics, which describes electromechanical coupling in systems with orientational order. This concept allows an ion channel to harness the electric-field induced dipole reorientations of its transmembrane helices to generate torque and sense dVTM.

We consider model peptides having electrical dipole moments in a lipid bilayer and perform molecular dynamics simulations to study the effect of variation of TM voltage on their tilt angles and ascertain the optimal parameters for designing a sensitive membrane voltage sensor.

Poster #7 - Reconstitution of the Na,K-ATPase for Structural Analysis.

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The Na,K-ATPase is a membrane embedded primary active transporter, responsible for maintaining the electrochemical gradient of sodium and potassium across the plasma membrane. Progressing through its catalytic cycle the pump alternates between inward (E1, high sodium affinity) and outward (E2, high potassium affinity) open conformations, exchanging three intracellular Na^+ with two extracellular K^+. The pump is an obligate oligomer composed of 3 subunits denoted α (catalytic), β (accessory) and FXYD (regulatory). Each subunit exists as multiple isoforms of which all combinations are active and characterized by different kinetic properties, allowing regulation by tissue specific expression. $\alpha_1\beta_1$ is ubiquitously expressed and can be seen as the housekeeping complex. α_2 is primarily found in muscle and glia cells while α_3 is expressed in neurons.

Here we present recently obtained structural data of the different isoforms of the catalytic α subunit. Recombinant human Na,K-ATPase was expressed in *Pichia pastoris*, purified and reconstituted into Saposin-lipoprotein nanoparticles for structural characterization by Cryo-EM.

Utilizing ADP-AlF_4^- to trap the protein in an E1P-ADP•$3Na^+$ state, structures of $\alpha_1\beta_1$FXYD1, $\alpha_2\beta_1$FXYD1 and $\alpha_3\beta_1$FXYD1 was solved to resolutions of 2.8, 2.8 and 2.6 Å, respectively. Throughout the catalytic cycle of the Na,K-ATPase, several conformational states remain elusive in regards to structural characterization. Utilizing the salipro reconstituted protein in the presence of Na^+ and ATP, we took advantage of the ability of cryo-EM to handle somewhat heterogeneous samples. From the dataset of ATP activated protein, we obtained structural data at approx. 3.3 Å resolution of three distinct conformational states, representing E1-ATP•$3Na^+$, E2P and an intermediate state containing characteristics of both E1 and E2.

Poster #8 - Na/K-ATPase Signaling Tonically Inhibits Sodium Reabsorption in the Renal Proximal Tubule.

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Two-thirds of the filtered sodium, potassium, chloride, bicarbonate, phosphate, and water, as well as virtually all the filtered glucose and amino acids, are reabsorbed in the renal proximal tubule (RPT). Basolateral Na/K-ATPase (NKA) in the RPT is classically known for driving sodium reabsorption through its enzymatic ion-pumping function, and its stimulation is associated with anti-natriuresis. In contrast, physiological concentrations of cardiotonic steroids (CTS), the specific ligands of NKA, activate non-enzymatic signaling function of NKA that stimulates cellular redistribution of basolateral NKA and apical Na/H-Exchanger (NHE3), resulting in inhibition of transepithelial sodium flux in RPT cells.

We tested the physiological relevance of the enzymatic and non-enzymatic functions of NKA in RPT Na^+ reabsorption using genetic approaches. Knockdown of 90% of NKA α1 in RPT LLC-PK1 cells activated NHE3 (decreased phospho/total ratio) and increased Na/HCO_3 cotransporter (NBCe1A) expression. In hypomorphic RPT NKA α1 -/- mice obtained by SGLT2-Cre/LoxP targeting, a 70% decrease in RPT NKA α1 decreased the inhibitory phosphorylation of NHE3 and increased membrane abundance of NHE3 and NBCe1A. Strikingly, a 65% decrease in urine output and absolute Na^+ excretion was observed, without evidence of renal injury, driven by increased RPT Na^+ reabsorption as indicated by a 65% decrease in lithium clearance and unchanged GFR. The hyper-reabsorptive and anti-natriuretic phenotype of these mice was rescued upon crossing with RPT NHE3 -/- mice, consistent with a role of NKA/NHE3 coupling.

Hence, NKA signaling exerts a tonic inhibitory action on Na^+ reabsorption by regulation of key apical and basolateral Na^+ transporters, which is lifted upon NKA genetic suppression in cells and in vivo. The proposed NKA signaling on RPT Na+ transporters and Na^+ handling is therefore

physiologically relevant and functionally dominant. NKA signaling therefore provides a long sought-after mechanism for the natriuretic action of endogenous NKA ligands such as CTS.

Poster #9 - Substrate Specificity of Drs2p-Cdc50p from Saccharomyces cerevisiae.

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P4-ATPases define a eukaryotic subfamily of P-type ATPases, and function as lipid flippases driving the transverse flip of lipids from the extracellular or luminal leaflet to the cytosolic leaflet of cell membranes. Structural studies have revealed insights into substrate lipid binding and translocation[1,2], however structural insights pertaining to substrate selectivity has been hampered by the narrow lipid specificity of studied lipid flippases.

Drs2p-Cdc50p from *Saccharomyces cerevisiae* has been reported as being a phosphatidylserine- and phosphatidylethanolamine-specific lipid flippase. To validate the substrate specificity of Drs2p-Cdc50p, we have screened its glycerophospholipid stimulated ATPase activity, identifying a broader range of anionic lipid substrate candidates than initially reported. Structural studies by cryo-EM of detergent solubilized Drs2p-Cdc50p in complex with these lipids have revealed new insights into lipid binding and selectivity.

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Poster #10 - Structural and Functional Studies of ATP13A2 in Nanodiscs.

Ronja Driller[1], Joseph Lyons[1], Peter Vangheluwe[2], Poul Nissen[1]

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ATP13A2 is a human lysosomal P5B ATPase transporting polyamines. Malfunctions cause severe neurodegenerative diseases, such as familial early-onset parkinsonism. Recently, several structures of ATP13A2 in different states have been solved, all of them in detergent. Interestingly, none of the published structures could identify a clear polyamine exit site. With now several hypothetical exit sites and modes, it remains to be proven, how exactly ATP13A2 transports polyamines. Furthermore, ATP13A2 possesses a unique membrane-anchoring domain at its N-terminus and it is not yet clear, what its precise function is and how it interacts with lipids.

To answer these open questions, we aim to characterize ATP13A2 in a more native-like lipid environment. Therefore, we reconstituted ATP13A2 in various nanodiscs and different lipid compositions to obtain pure protein sample for functional studies and cryo-EM driven structural studies.

Poster #11 - Towards Production & Purification of Human Copper(I)-ATPase ATP7B.

Tomas Heger, Charlott Stock, Christine Juul Fælled Nielsen, Thibaud Dieudonné, Poul Nissen

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Human copper(I)-transporting ATPase *ATP7B* is expressed mainly in the liver and brain. It localizes to the trans-Golgi network and intracellular vesicles. ATP7B is involved in copper homeostasis; therefore, its genetic defects result in copper accumulation leading to a hepato-neurological disorder called Wilson's disease (WD). Current treatments of WD are based on copper-chelating agents or zinc salts. However, other medications that might be better tolerated by the patients or more effective are needed.

Our strategy in WD drug discovery aims to find new small-molecule ligands of the WD mutant of ATP7B (H1069Q). As the first step towards a protein-compound interaction screening, we introduce the protocol for ATP7B expression and purification. We demonstrate the versatility of the eGFP fusion partner in a series of methods enabling optimization of expression and detergent-mediated extraction as well as affinity purification of ATP7B.

Based on fluorescence microscopy and SDS-PAGE in-gel fluorescence, we selected a suitable yeast (*S. cerevisiae*) strain as an expression host. We assessed and optimized the conditions of yeast membrane solubilization by detergents using fluorescence-detection size-exclusion chromatography (F-SEC). We prepared resins with non-covalently and covalently attached anti-GFP nanobodies for affinity purification and compared the performance of both resin types. After a detergent exchange step, ATP7B was proteolytically eluted from the resin, and finally, we obtained a highly pure sample of ATP7B from SEC.

Poster #12 - Structural and Function Clues on a P4B-ATPases Suggest Autoinhibition.

Line Marie Christiansen[1, 2], Thibaud Dieudonne[1], Joseph Lyons[1, 3], Poul Nissen[1, 2]

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The lipid composition of eukaryotic cellular membranes is tightly controlled by three groups of membrane proteins: flippases, floppases and scramblases. These ensure a correct lipid distribution for cell-to-cell signaling, vesicle formation and apoptosis throughout different cellular membranes. The majority of lipids flippases are P4-ATPases and are further divided into two main groups: those who function in a complex with a CDC50-family protein and those that do not. An overall understanding of the first group has emerged within recent years, whereas our understanding of the latter group (P4B-ATPases) is still in the initial stages. The group includes *Saccharomyces*

cerevisiae Neo1p and mammalian ATP9A and ATP9B, whereof ATP9A dysfunction has recently been linked to neurodevelopmental phenotypes.

By a combined biochemical and structural approach, Neo1p has been utilized as a model for P4B-ATPases. Biochemical studies reveal a stabile E2P phosphoenzyme in absence of substrate, insensitive to addition of suggested phospholipid substrates. Single particle Cryo-EM lead us to determine two states: an E2P and an E1P state. The aluminium flouride inhibited 2.7 Å E1P-like state reveals a new orientation of the A-domain and supports the existing evidence of P4-ATPases adopting an E1P-like state in absence of substrate. This regardless of addition of inhibitors at the point of solubilization.

Recently, a 2.2Å structure in presence of beryllium flouride adopted an E2P state, with an only partially open substrate binding pocket. The position of transmembrane helix two and conserved P4B-ATPase residues are seen to block the proposed substrate binding site, suggesting an autoinhibited E2P state. Unlike other autoinhibited P4-ATPases (Timcenko 2018, Dieudonne 2022) no structural clues suggesting inhibition by termini were evident in the data.

Together the data suggests that the P4B-ATPase Neo1p accumulates in an autoinhibited E2P form with a yet unknown activation mechanism.

Poster #13 - Phospholamban Inhibits the Cardiac Calcium Pump by Reversing the Allosteric Effect of ATP on Calcium Affinity.

Sean Cleary, Jaroslava Seflova, Ellen Cho, Seth Robia

Loyola University Chicago

Phospholamban (PLB) is a transmembrane micropeptide that regulates the sarcoplasmic reticulum Ca^{2+} ATPase (SERCA) in cardiac muscle. PLB reduces the apparent Ca^{2+} sensitivity of SERCA, increasing the Ca^{2+} concentration required for pump cycling. However, PLB does not decrease Ca^{2+} binding to SERCA when ATP is absent and the pump is not cycling. One possible explanation for these seemingly conflicting results is PLB may slow the Ca^{2+} binding step in the SERCA enzymatic cycle, altering the Ca^{2+} dependence of cycling without affecting true Ca^{2+} affinity. Alternatively, we speculated that equilibrium measurements of Ca^{2+} binding to non-cycling SERCA may overlook important allosteric effects of bound nucleotide. Specifically, ATP is known to have an allosteric effect on SERCA that is distinct from its role as a catalytic substrate and source of energy. When ATP binds, it *increases* the affinity of SERCA for Ca^{2+} - exactly the opposite of the PLB effect. We speculated that PLB may inhibit SERCA by reversing pre-activation of SERCA Ca^{2+} affinity by ATP. To test this hypothesis, we used a 2-color SERCA biosensor expressed in microsomal membranes to measure Ca^{2+} binding to the pump reported by changes in intramolecular FRET. We quantified the Ca^{2+} affinity of non-cycling SERCA in the absence of ATP, then mimicked ATP binding while preventing enzymatic cycling using the non-hydrolyzable ATP-analog AMPPCP. Interestingly, nucleotide activation by AMPPCP increased the Ca^{2+} affinity of 2-color SERCA, but this effect was reversed by co-expression of PLB. PLB had no effect on Ca^{2+} affinity in the absence of nucleotide. These results may reconcile the seemingly paradoxical effects of PLB. We propose that PLB reduces the true Ca^{2+} affinity of SERCA by reversing allosteric activation of the pump by ATP.

Poster #14 - Characterization of a Conserved Motif Within the C-Terminal Auto-Regulatory Domain of the P4-ATPase, ATP8A2.

Eli Matsell[1], Jens Peter Anderson[2], Robert Molday[1]

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ATP8A2 is a P4-Type ATPase that, in association with its accessory subunit CDC50A, flips phosphatidylserine (PS) and to a lesser extent phosphatidylethanolamine (PE) from the exoplasmic to the cytoplasmic leaflet of cellular membranes. This phospholipid transport is responsible for the generation and maintenance of transmembrane lipid asymmetry, a property that plays a crucial role in a wide variety of cellular processes including protein trafficking and cell signaling. The mechanism of phospholipid transport for ATP8A2 and other P4-ATPases follows the Post-Albers cycle, however their regulatory properties remain elusive. Previous studies using deletion mutagenesis have shown that the C-terminal segment plays an important role in the expression and functional activity of ATP8A2. More recently, structural studies have identified a conserved motif, 'GYAFS', within the C-terminal segment of P4-ATPases that interacts directly with residues within the ATP-binding pocket. The focus of this study is to further define the role of the 'GYAFS' motif in modulating the function of ATP8A2 as a PS-dependent phospholipid flippase. Each residue within the 'GYAFS' motif was replaced with either an alanine residue or a related amino acid using site-directed mutagenesis, and the PS-dependent ATPase activity of the immunoaffinity purified ATP8A2-CDC50A complex was measured using established assays. The mutants were found to have variable impact on protein expression and activity. Numerous ATP8A2 variants expressed at exceeding low levels and as a result the effect of the mutation on protein folding and PS-activated ATPase activity was difficult to distinguish. To resolve this issue, constructs are being developed in which protease cleavage sites are introduced at various locations within the C-terminal tail. This should allow WT-like expression of ATP8A2 in its uncleaved state and evaluation of the functional activity of ATP8A2 after cleavage and removal of C-terminal segments including the conserved 'GYAFS' motif followed by the reintroduction of selected C-terminal peptides.

Poster #15 - The Unique Functional Effects of "Monomeric" Forms of Phospholamban on SERCA Activity.

Vinh Nguyen and Howard S. Young

University of Alberta

Phospholamban (PLN) is the canonical peptide inhibitor that binds to the sarco(endo)plasmic reticulum calcium ATPase (SERCA) at the inhibitory groove composed of transmembrane helices M2, M6, and M9. PLN can primarily adopt two different oligomeric states, a monomer and pentamer, where the former is considered the active state of PLN that binds to SERCA's inhibitory groove. At high calcium concentrations, PLN increases SERCA's activity despite being an inhibitor, which is a phenomenon that has yet to be explained. Recent studies have found evidence that pentameric PLN can also interact with SERCA at an accessory site separate from the inhibitory groove, transmembrane helix M3. The goal of this study is to further elucidate the influence of

PLN's oligomeric state on SERCA's activity. So-called monomeric variants of PLN were generated to determine if the reduction in the PLN pentamer would affect SERCA's activity. Two mutants, dubbed AFA-PLN and SSS-PLN, were generated through mutations of three cysteines in PLN that were previously shown to influence oligomerization. Activity measurements of SERCA in the presence of AFA-PLN or SSS-PLN showed decreased maximal activity at high calcium concentrations, instead of increasing SERCA's activity as seen with wild-type PLN. The result of this study shows that monomeric versus pentameric forms of PLN have different effects on SERCA's activity, challenging the paradigm that pentameric PLN is simply an inactive storage unit.

Poster #16 - Micropeptide Hetero-Oligomerization Adds Complexity to the Calcium Pump Regulatory Network.

Taylor Phillips, Garrett Hauck, Marsha Pribadi, Ellen Cho, Sean Cleary, Seth Robia

Loyola University Chicago

The sarco(endo)plasmic reticulum calcium ATPase (SERCA) is an ion transporter that creates and maintains intracellular calcium stores. SERCA is inhibited or stimulated by several membrane micropeptides including another-regulin (ALN), dwarf open reading frame (DWORF), endoregulin (ELN), phospholamban (PLB), and sarcolipin (SLN). We previously showed these micropeptides assemble into homo-oligomeric complexes with varying affinity. Here we tested whether different micropeptides can interact with each other, hypothesizing that co-assembly into hetero-oligomers may affect micropeptide bioavailability to regulate SERCA. We quantified the relative binding affinity of each combination of candidates using automated fluorescence resonance energy transfer (FRET) microscopy. All combinations were capable of interacting with good affinity, similar to that of to self-binding (homo-oligomerization). Testing each pair at a 1:5 ratio and a reciprocal 5:1 ratio, we noted that the affinity of hetero-oligomerization of some micropeptides depended on whether they were the minority or majority species. In particular, SLN was able to join oligomers when it was the minority species, but did not readily accommodate other micropeptides in the reciprocal experiment when it was expressed in 5-fold excess. The opposite was observed for ELN. PLB was a universal partner for all other micropeptides tested, forming avid hetero-oligomers whether it was the minority or majority species. Increasing expression of SERCA decreased PLB-DWORF hetero-oligomerization, suggesting that SERCA-micropeptide interactions compete with micropeptide-micropeptide interactions. Thus, micropeptides populate a regulatory network of diverse protein assemblies. The data suggest that the complexity of this interactome increases exponentially with the number of micropeptides that are coexpressed in a particular tissue.

Poster #17 - Dwarf Open Reading Frame (DWORF) is a Direct Activator of the Sarcoplasmic Reticulum Calcium Pump SERCA.

M'Lynn Fisher and Howard S. Young

University of Alberta

The sarco/endoplasmic reticulum calcium pump (SERCA) plays a critical role in the contraction/relaxation cycle of muscle. In cardiac muscle, SERCA is regulated by the inhibitor phospholamban (PLN). A new regulator, dwarf open reading frame (DWORF), has been reported to displace PLN from SERCA. Here, we show that DWORF is a direct activator of SERCA[1], increasing its turnover rate in the absence of PLN. Measurement of in-cell calcium dynamics supports this observation and demonstrates that DWORF increases SERCA-dependent calcium reuptake. These functional observations reveal opposing effects of DWORF activation and PLN inhibition of SERCA. To gain mechanistic insight into SERCA activation, fluorescence resonance energy transfer experiments revealed that DWORF has a higher affinity for SERCA in the presence of calcium. Molecular modeling and molecular dynamics simulations provide a model for DWORF activation of SERCA, where DWORF modulates the membrane bilayer and stabilizes the conformations of SERCA that predominate during elevated cytosolic calcium.

Poster #18 - Structural & Functional Characterization of Glucosylceramide Transporting ATP10A-D.

Filip Pamula, **Klara Scholtissek**, Joseph Lyons

Aarhus University

Lipid flippases (P4-ATPases) transport lipids from the outer leaflet to the inner leaflet of the membrane, therefore playing a vital role in lipid homeostasis. Since lipid asymmetry is crucial to the function of cells and their organelles, the role of flippases in disease pathways and therapy possibilities are of great interest. Recent structural and biochemical studies have shed light on phospholipid recognition and transport by lipid flippases.

Human ATP10A-D have been shown to transport glucosylceramide and phosphatidylcholine *in vivo*. ATP10A and ATP10D are localized in the plasma membrane, whereas ATP10B is present in the lysosomes. Mutations or deletions of ATP10A and ATP10D are associated with diabetes and atherosclerosis, respectively while mutations in the gene encoding ATP10B are shown to increase the risk of developing Parkinson's disease (PD). In contrast to phosphatidylserine (PS) selective flippases, the regulation and molecular details of substrate specificity of our targets are not fully understood. We focus on elucidating the structural and molecular basis of ATP10A-D function and regulation as well as the substrate specificity of P4-ATPases by using cryo-electron microscopy supported by biochemical activity assays.

We are able to co-express human ATP10B, ATP10D and ATP10A with CDC50A subunit in mammalian HEK-FS cells. Complex formation with CDC50A has been confirmed and an optimized purification protocol yields pure heterodimer in modest amounts. Assessment of the protein quality was performed by means of FSEC, SEC, negative stain EM and initial single-particle cryo-EM analysis. The next steps will include acquiring high-resolution cryo-EM data of proteins at different stages of catalytic cycle and with different substrates. We will further characterize protein activity in the presence of the structural insights using available activity assays.

Poster #19 - Neuroprotective Effect of Gamma-Benzylidene Digoxin Derivative Against Brain Ischemia.

Bruno S Gonçalves, Carla PS Santos, Matheus V Machado, Marina M Toledo, Cristiane Q Tilelli, Isabella F Silva, Helio B Santos, Ralph G Thome, Luciana ED Carvalho, Vanessa F Cortes, Jose AFP Villar, Herica L Santos, Maira C Lima, **Leandro A Barbosa**

Universidade Federal de Sao Joao del-Rei

BD-15 is a new a non-toxic benzylidine digoxin derivative that was demonstrated to direct increase of the alpha3-Na,K-ATPase activity, and to prevent chemical ischemia in N2a cells model, but it was never tested in a ischemia model in animal. Wistar rats were submitted to transient brain ischemia through bilateral occlusion of both common carotid arteries for 30 minutes, and after that the animal were followed by three days of reperfusion, with or without BD-15 treatment (one dose per day IP, 100 µg/kg). Hippocampus and cortex were collected to perform biochemical analysis such as: membrane lipid profile and peroxidation and membrane enzyme activities (PMCA, SERCA, and acetylcholinesterase); histological analysis and behavioral parameters assessed by the open field behavior test were also evaluated. In histological analysis, the ischemia provoked a brain tissue damage after 3 days reperfusion, and BD-15 treatment was able to impair this damage. Ischemia provoked an decrease in the total Na,K-ATPase activity and an increase of the expression levels of the pump and BD-15 prevent that changes caused by ischemia. The same profile was found for total PMCA activity and PMCA4 expression levels. BD-15 was also able to prevent the decrease of activity of SERCA, Mg-ATPase and acetylcholinesterase caused by ischemia. The membrane lipid peroxidation was increased in the ischemic group, and again BD-15 was able to prevent this effect. Regarding the lipid profile, we observed an increase in the content of phospholipids and cholesterol in the ischemic group, and a decrease in the groups treated with BD-15. The behavioral parameters demonstrated that BD-15 prevents the motor damage caused by ischemia. In conclusion, BD-15 is a non-toxic CTS that demonstrate the prevention of brain damages provoked by ischemia, and appear to promote neuroprotection in animals exposed to global ischemia.

Poster #20 - ATP10A has a Protective and Sex-Specific Role in Lipid Metabolism.

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Genetic predisposition and environment play substantial roles in obesity, type 2 diabetes and cardiovascular disease (CVD). Genetic association studies have linked *ATP10A*, encoding a type IV P-type ATPase (P4-ATPase), to human metabolic disease. ATP10A is a lipid flippase that catalyzes the membrane translocation of phosphatidylcholine and glucosylceramide. These lipids and their respective metabolites have been independently implicated in metabolic dysfunction. To explore the role of this flippase in metabolism, we created a novel *Atp10a* knockout (KO) mouse model. *Atp10a* KO mice display a female-specific weight gain during high-fat diet feeding and this is attributable to increased adiposity. Female *Atp10a* KO mice also exhibit elevated plasma

free fatty acids, cholesterol, and triglycerides, as well as a depletion in eicosanoid species compared to the wild type (WT) littermates. Additionally, female *Atp10a* KO mice exhibit elevated fasting blood glucose levels without compensatory elevation of insulin. We also found that the liver of female *Atp10a* KO mice displays larger lipid droplets, which was associated with increased diacylglycerol acyltransferase-2 (DGAT2) expression and an attenuation of the insulin signaling pathway compared to the WT littermates. Thus far, our studies have shown that knocking out *Atp10a* in mice on a high fat diet results in sex-specific perturbations to body composition, plasma lipid levels, glucose homeostasis, and liver metabolism. We have recently found that ATP10A is specifically expressed in the endothelial cells of multiple tissues. We are now exploring these metabolic phenotypes through the lens of endothelial cell dysfunction. These studies suggest mechanisms by which this flippase contributes to the development of CVD with obesity.

Poster #21 - A Switch in FXYD Proteins Dysregulates NKA in Dilated Cardiomyopathy.

John Yap, Taylor Phillips, Jaroslava Seflova, Jacob Cunningham, Carson Miller, Seth Robia

Loyola University Chicago

In the heart, Na^+ homeostasis is established by the Na^+/K^+-ATPase (NKA). Because NKA is functionally linked to the Na^+/Ca^{2+} exchanger, changes in NKA activity influence Ca^{2+} levels and cardiac contractility. It is well known that NKA in the heart is inhibited by FXYD1, a member of the FXYD protein family. However, the cardiac expression and function of other FXYDs have not been determined. In this study, western blot analysis revealed that FXYDs 5, 6 and 7 are also expressed in human myocardium. Furthermore, in patients with dilated cardiomyopathy (DCM), FXYD1 expression was decreased and FXYD6 expression was increased. Like FXYD1, FXYD6 inhibits NKA. Thus, the switch from FXYD1 to FXYD6 in DCM may be compensatory. We also observed that FXYD6 was expressed at a molecular weight consistent with a FXYD6-FXYD6 dimer. This is an important phenomenon since FXYD6 binds to NKA as monomers. Because FXYD6 contains three cysteine residues (C9, C60 and C62), we hypothesized that FXYD6 dimerization may occur due to oxidative crosslinking of cysteine residues. To test our hypothesis, we exposed cells expressing FXYD6 to the oxidizing agents diamide or H_2O_2. Oxidation of FXYD6 increased dimerization, as measured by FRET and western blotting, and this was reversed by subsequent addition of the reducing agent dithiothreitol (DTT). Additionally, FXYD1 oligomerization did not change with the addition of diamide nor DTT. Together, these results suggest that in DCM, NKA is dysregulated by a switch from FXYD1 to FXYD6, which can become oxidized to form dimers, sequestering monomeric FXYD6 away from NKA. Decreased inhibition of NKA would lead to decreased Ca^{2+} levels and cardiac contractility, both of which are features of DCM.

Poster #22 - Comparative Study of Transmembrane Peptides in the Regulin Family that Modulate Calcium Transporting Activity of SERCA.

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The Regulin family of micropeptides consists of several small transmembrane peptides that modulate the calcium transporting activity of sarco-endoplasmic reticulum calcium ATPase (SERCA). SERCA is ubiquitously expressed in all living cells and plays a vital role in calcium homeostasis and cellular function. Here, we characterize all of the different micropeptides in the Regulin family and their differences due to their distinct tissue expression profiles, their specialized functional regulation of SERCA, and their unique structural interactions with SERCA. Phospholamban (PLN) and sarcolipin (SLN) are the earliest identified micropeptides in the Regulin family, expressed in cardiac and skeletal muscles, respectively. PLN and SLN are also more extensively studied than the other micropeptides in the Regulin family. Both PLN and SLN are inhibitors of SERCA, i.e., they both decrease the apparent calcium affinity of SERCA, however, PLN is found to increase maximal activity while SLN decreases maximal activity at higher physiological calcium concentrations. Myoregulin (MLN), dwarf open reading frame (DWORF), endoregulin (ELN), and another-regulin (ALN) are relatively new members of the Regulin family. MLN is expressed in skeletal muscles, DWORF in cardiac muscle, ELN in endothelial cells, and another-regulin is ubiquitously expressed in all cells. MLN, ELN and DWORF do not affect calcium affinity but affect the maximal activity of SERCA, i.e., MLN and ELN decrease maximal activity while DWORF increases maximal activity at higher physiological calcium concentrations. Similar to SLN, ALN also decreases calcium affinity and maximal activity of SERCA. Previously, structural studies have identified PLN and SLN to bind to the inhibitory groove of SERCA formed by transmembrane helices M2, M6 and M9. Here, we also demonstrate from molecular dynamics simulations that distinctive functional regulation of SERCA by these micropeptides can be attributed to unique structural interactions with the helices in the inhibitory groove of SERCA.

Poster #23 - A Novel Super-Efficient Rescue Mutation Restoring the Functional Capability of Na,K-ATPase α3 in Neurological Disease.

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Beginning with the discovery of Na,K-ATPase α3-mutations as the cause of Rapid-onset Dystonia Parkinsonism (RDP), several neurological movement disorders are now known to be caused by mutation of the Na,K-ATPase α3-isoform including the more frequent Alternating Hemiplegia of Childhood (AHC). These disorders are closely related, and some patients display symptoms from more than one disorder or an intermediate phenotype (1). Presently, no effective therapeutic strategies for AHC or other α3-related encephalopathies exist. In 2015 we described a unique second-site mutation (E314D) that resulted in gain-of-function in an α1-mutant D928N equivalent to the RDP/AHC-mutant D923N in α3 (2), where it improved (rescued) the reduced Na affinity and activity (2). In further experiments along this line, we have found that the >100-fold reduction of Na affinity in D923N was rescued to 43-fold reduction by the second-site mutation E309D in α3, equivalent to E314D in α1. This intriguing finding raises the prospect that an efficient drug development strategy to treat α3-related encephalopathy, could emerge from using insight from

the structural basis for the rescue effect. Here we report on a new rescue mutation, which is a much more efficient rescuer than E309D. Excitingly, our study shows that the >100-fold reduction of Na affinity in the disease mutant D923N was rescued to only 2-fold reduction of Na affinity, when D923N was combined with the new rescue mutation. Furthermore, the two rescue mutations together led to a complete rescue of the reduced Na affinity of D923N to wild type-like Na affinity.

1. Holm R., Toustrup-Jensen M., Einholm A.P., Schack V.R., Andersen, J.P., Vilsen, B. (2016) Biochim. Biophys. Acta 1857, 1807-1828 (Review).
2. Holm R., Einholm A.P., Andersen J.P., Vilsen B. (2015) J. Biol. Chem. 290, 9801-9811.

Poster #24 - Exploring the Association Energy Landscape of Presenilin 1 with the Calcium Pump SERCA

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Presenilin 1 (PS1) is a nine-transmembrane protein that acts as the catalytic component of the γ-secretase, a membrane-embedded aspartyl protease involved in the development and progression of Alzheimer's disease. The regulation of calcium transport by PS1 in the endoplasmic reticulum of different organs recently led to study its role in cardiac cells. Specifically, a recent study showed that PS1 binds to the calcium pump (sarcoplasmic reticulum Ca^{2+}-ATPase, SERCA) in a 1:1 stoichiometry complex and exerts a complex stimulatory/inhibitory profile on this pump. Here, we employed the protein association energy landscape (PANEL) protocol to determine the possible conformations of SERCA-PS1 complex by combining stochastic samplings and coarse-grained (CG) simulations. The exhaustive sampling of this CG model approach allowed us to identify three energetically favorable SERCA-PS1 complexes. These SERCA-PS1 complexes were subsequently subjected to atomistic molecular dynamics simulations to study intermolecular interactions in greater detail. Interestingly, we found that the interaction of these proteins involves the regulatory and accessory protein recognition sites of SERCA and PS1, respectively. This represents the first study to understand the interactions of SERCA with PS1 at the molecular level, and will be key to understand the effect of PS1 mutations in the development of Alzheimer's disease, cardiomyopathies and other degenerative diseases.

Poster #25 - The Monomeric P4-ATPase Neo1 Controls Phosphoinositide Membrane Asymmetry and Neomycin Resistance in Budding Yeast.

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The plasma membrane of cells is characterized by an asymmetric distribution of lipid species across the exofacial and cytofacial aspects of bilayer and is established by P4-ATPases.

Phosphoinositides are assumed to be restricted to the cytosolic leaflet by virtue of their synthesis on this side of the membrane. After synthesis, however, phosphatidylinositol-4-phosphate (PI4P) is delivered by oxysterol binding proteins to ER where lipids can flip-flop spontaneously between the luminal and cytosolic leaflet. PI4P in the cytosolic leaflet of the ER can be degraded by the Sac1 phosphatase but any PI4P in the luminal leaflet would be resistant to degradation and available for transport through the Golgi complex and potential exposure on the outer leaflet of the plasma membrane (PM). *NEO1* was first identified in a screen for overexpressed genes that confer resistance to the aminoglycoside neomycin, an antibiotic that specifically binds to PI4P. Here we test hypothesis that Neo1 confers resistance to neomycin by flipping PI4P from the Golgi luminal leaflet to the cytosolic leaflet, thereby preventing PI4P exposure on the extracellular leaflet of the PM. The GFP- SidC biosensor for PI4P was purified from *E. coli* and used to probe the outer leaflet of WT and *neo1-ts* cells. Strikingly, temperature inactivation of *neo1-ts* caused exposure of PI4P on the PM outer leaflet of intact cells. Structure-guided mutagenesis of residues in substrate translocation path of Neo1 identified both entry and exit gate mutations that cause exposure of PI4P. These mutants were also extremely sensitive to neomycin while other *neo1* mutants that expose PS and/or PE, but not PI4P, were resistant to neomycin. In addition, the ATPase activity of purified Neo1 is stimulated by PI4P in the absence of other substrates. These observations suggest that Neo1 is a novel PI4P flippase and that neomycin enters eukaryotic cells through surface exposed PI4P.

Poster #26 - Activation and Substrate Recognition Mechanism of ATP8B1/CDC50A Revealed by Cryo-EM.

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Lipid flippases (P4-ATPases) mediate active transport of phospholipids from the exoplasmic leaflet to the cytoplasmic one, in order to establish and maintain phospholipid asymmetry in eukaryotic membranes. This asymmetry is tightly regulated, and important for numerous cellular processes such as membrane trafficking, signaling, apoptosis, cytokinesis and cell fusion. The human genome encodes 14 different P4-ATPases, a subset of which are linked to severe diseases including cognitive disorders e.g. Alzheimer's and Parkinson's disease, diabetes, cancer or liver disorders. For example, mutations of the human lipid flippase ATP8B1, are responsible for inherited intrahepatic cholestasis, a rare liver disease and KO of *Atp8b1* in beta cells inhibits glucose-stimulated insulin release. Little is known about the exact involvement of ATP8B1 in these diseases, mainly because of the lack of consensus regarding its transported substrate and on how its activity is regulated. Previously, we have demonstrated that ATP8B1 is autoinhibited by an evolutionary conserved mechanism involving its N- and C-terminal tails and that the activity of ATP8B1 is tightly regulated by phosphoinositide [1].

Here, we present our most recent results on the structural and functional characterization of the ATP8B1/CDC50A transport cycle. Our single-particle cryo-EM analysis reveals the structural changes happening upon ATP binding, autophosphorylation, relief of the autoinhibition, substrate binding and substrate occlusion. Interestingly, our functional data suggest that ATP8B1 has a much

broader substrate specificity than initially anticipated and that surprisingly the substrate specificity is not driven by the lipid head group.

1. Dieudonné et al., 2020, eLife

Poster #27 - Ouabain Promotes Cystogenesis in a Slowly Progressive ADPKD Mouse Model.

Jordan Trant, Gladis Sanchez, Jeffrey McDermott, Gustavo Blanco

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Ouabain induced Na,K-ATPase (NKA) signaling influences many different functions in the cell. We have previously shown that ouabain enhances the proliferation and fluid secretion of autosomal dominant polycystic kidney disease (ADPKD) renal tubular epithelial cells and embryonic kidneys. These effects, which are essential to ADPKD cystogenesis, were observed *in vitro*. Here, we determined the effects of ouabain *in vivo* using a slowly progressive ADPKD mouse model, the RC/RC mouse. Ouabain at doses of 0.3 mg/g or saline (used as a control) was injected intraperitoneally to wildtype (WT) and RC/RC mice. This followed a protocol that started at postnatal day 9 and was repeated daily for 1-5 months. As readout parameters for ADPKD, kidney weight to body weight ratio (KW/BW), percent of cystic area relative to total kidney area (% cyst area), blood urea nitrogen (BUN), and kidney fibrosis were measured. Ouabain treatment significantly increased the percent cyst area in RC/RC mice compared to saline-injected controls at all time points tested. The KW/BW was also augmented in RC/RC mice at 3, 4, and 5 months of treatment. These effects were not seen in WT mice. BUN values were not affected by ouabain in either of the groups studied. Between 1 and 5 months, ouabain significantly increased fibrosis in the RC/RC ouabain-injected mice compared with the saline-injected controls. These findings demonstrate that ouabain stimulates kidney cyst progression and fibrosis in ADPKD not only *in vitro*, but also *in vivo*. These results support the idea that physiologic circulating levels of ouabain constitute an epigenetic factor contributing to worsening the kidney damage caused by ADPKD.

Supported by NIH R01 HD080423 award.

Poster #28 - Na,K-ATPase α4 is Involved in Sperm Energetics.

September Numata, Jeffrey McDermott, Gustavo hBlanco

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The Na,K-ATPase alpha 4 isoform (NKAα4) is expressed specifically in the male germ cells of the testes and is particularly abundant in mature spermatozoa. Genetic ablation of NKAα4 in mice (NKAα4 KO mice) results in complete infertility of male mice, due to severe impairments in sperm motility and capacitation. Here, we show that deletion of NKAα4 also leads to major defects in sperm metabolism and energetics. Sperm from NKAα4 KO mice displayed a significant reduction in the extracellular acidification rate (ECAR) compared to wild-type sperm, indicative of impaired glycolytic flux. Accordingly, glucose uptake was reduced in sperm lacking NKAα4. In addition, mitochondrial function was disrupted in NKAα4 KO sperm, as demonstrated by a decrease in the

mitochondrial membrane potential and lower oxygen consumption rate (OCR). Moreover, the ratio of NAD/NADH was increased in NKAα4 KO sperm, indicating a shift in the cellular redox state of the cells. Concomitant with these metabolic changes, we found a decrease in ATP production, an increase in the production of reactive oxygen species, and higher lipid peroxidation in NKAα4 KO sperm. Altogether, these findings reveal a novel link between NKAα4 activity and sperm metabolism and establish a new mechanism for the control of sperm function and male fertility by NKAα4.

Poster #29 - Essential Roles of Aromatic Residues at the Extracellular Entry of the Ion Binding Sites in Gating Function of the Na,K-pump.

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The Na,K-ATPase pumps $3Na^+$ from the cytoplasm toward the extracellular side of the plasma membrane in exchange for $2K^+$ for each ATP hydrolyzed, in a mechanism involving the binding, occlusion and release of the ions in consecutive steps. This process can be described in terms of two occlusion gates that alternately open and close, toward the cytoplasmic and extracellular sides. At the extracellular entry of the path between transmembrane helices M3, M4, M5, and M6 leading to the ion binding sites I and II, several aromatic residues candidate to form stacking interactions that might contribute to close the extracellular gate in the occluded state. Here we have examined the roles in Na^+ and K^+ interaction of the aromatic residues Phe296, Phe301, Phe302, Tyr310, Phe318, and putative interaction partners Arg882 and Asn792, which seem to hydrogen bond to Tyr310 (rat α1 numbering of residues). Mutants were generated and expressed in COS-1 cells, from which the plasma membrane fraction was harvested and examined in biochemical experiments. Mutants Phe318Ala and Phe318Leu were unable to support cell growth, whereas Phe318Trp and Phe318Tyr supported growth, indicating that the aromatic function at this position is essential for pump function. Partial ATPase activity was retained following replacement of Tyr310 with Ala, Leu, Phe, Arg, or Glu, but the mutants showed anomalous high apparent affinity for K^+, which inhibited enzyme turnover. This behavior reflects a high stability of the E2 form with bound K^+, which could be the result of K^+ having better access to the sites due to incomplete closure of the external gate. Mutants Arg882Leu and Phe318Trp also exhibited the peculiar K^+ interaction characteristic of the Tyr310 mutants, suggesting that Tyr310, Phe318, and Arg882 all contribute to the closing of the external gate leading to K^+ occlusion in the E2 form during the pump cycle.

Poster #30 - Deep Learning Identification of Cardiac Transporters as Targets for Approved Drugs.

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We re-tasked a novel ligand-centered deep learning drug discovery method developed by us to identify molecular targets for approved drugs in the heart. This approach uses information about small-molecule effectors (rather than target structures) to map and probe the pharmacological space of functionally relevant targets in the heart. Here we studied the calcium pump SERCA as a proof-of-principle target for our method. We chose SERCA because it plays a central role in the excitation-contraction-relaxation cycle in normal and pathological cardiac muscle and because it represents a primary pharmacological target in the heart. We applied this method to demonstrate that SERCA is a pharmacological target for statins, a group of FDA-approved HMG-CoA inhibitors used as lipid-lowering medications. Complementary experiments and atomistic simulations showed that these approved drugs are micromolar inhibitors of SERCA by interacting with two different effector sites located in the transmembrane domain of the pump. These proof-of-concept studies support the applicability of our approach for off-target identification and drug repurposing, ultimately minimizing the translational gap in drug development targeting the heart.

Poster #31 - Roles of Intra-Protein and Protein-Lipids Interactions Including Arg324 of Sarcoplasmic Reticulum Ca^{2+}-ATPase.

Kazuo Yamasaki, Takashi Daiho, Satoshi Yasuda, Stefania Danko, Jun-ichi Kawabe, Hiroshi Suzuki

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Sarcoplasmic reticulum Ca^{2+}-ATPase is one of the most investigated pump proteins. As this pump is a membrane protein, the lipid environment surrounding the pump is critical to the pump's properties. However, the effects of lipid are investigated not so extensively. Especially, studies on the effects of direct interactions between protein residues and lipid molecule are still few. In 2017, Toyoshima's group clearly showed the locations of lipid headgroups surrounding Ca^{2+}-ATP in the structures of four major reaction intermediates. In those structures, several direct interactions between amino acid residues and headgroups of phosphatidylcholine are observed. Arg324 of the Ca^{2+}-ATPase forms electrostatic interaction with phosphate moiety of phospholipids in 3 of 4 structures except in $E1Ca_2$ state. In $E1Ca_2$ state, this arginine potentially forms hydrogen bound with OH-moiety of Tyr122. To explore the roles of these interactions, we conducted experiments using wild-type and mutant Ca^{2+}-ATPase expressed in COS-1 cells. The results using microsomes from COS-1 show that the hydrogen bond between Arg324 and Tyr122 forms transiently during the step forming activated $E1Ca_2$ state and facilitates this step. And also, it was revealed that the electrostatic interaction between Arg324 and lipid headgroup is necessary to reduce affinity for luminal Ca^{2+}.

For further exploration, expressed Ca^{2+}-ATPase were reconstituted in nanodiscs harboring various types of phospholipids. A plot of the logarithm of EP transition rate versus square of mean activity coefficient quantifies the contribution of electrostatic interactions between Arg324 and lipid headgroups in the EP transition step. The properties observed in microsomes was not reproduced with any single-kind phospholipid, but was with a mixture of phospholipids that mimics the native membrane. This result suggests that Arg324 plays the role by changing its lipid partner among different kinds of lipid (e.g. swinging motion between PE and PC) during EP transition step.

Poster #32 - Approach to a Mechanistic Understanding of the Na,K-ATPase.

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For the F-type ATPase the molecular mechanism of active ion transport is well understood and it can be reconstructed how the chemical energy provided by ATP hydrolysis is transduced into ion movement across a membrane against the electrochemical potential gradient of the translocated ions. In contrast, this mechanism is still unknown in the case of P-type ATPases. For the Na,K-ATPase, on the one hand, most reaction steps executed during ion transport were identified kinetically and compiled in the so-called Post-Albers pump cycle that summarizes the transport process. On the other hand, a series of protein structures became available which reveal the spatial arrangement of the amino acids of a number of P-type ATPases in various states of the pump cycle at an Ångström resolution. Both data bases are, however, at the present state not sufficient to explain how energy is converted. An approach using thermodynamic principles and experimental data on the reaction kinetics of the Na,K-ATPase rendered it possible to achieve at least mechanistic constraints that have to be satisfied by any mechanistic model: (1) Terrell L. Hill derived from thermodynamical principles that energy transduction in molecular machines does not occur in a single step of the reaction cycle, a so-called power stroke, but is distributed over the whole reaction cycle. (2) The empirical quantity 'activation energy' indicates the contribution of entropic effects in various partial reactions and allows the discrimination between different kinds of processes performed such as major conformational rearrangements, ligand coordination and diffusion-controlled processes. (3) So-called 'basic free energies' of the states in the pump cycle, calculated from experimental rate constants, allow the determination of energy consumption or release of single reaction steps. Based on the restrictions derived from these findings, a possible mechanistic model is proposed of how energy transduction may be explained.

Poster #33 - Elucidating the Mechanism of ATP13A2 Regulation and Transport Activation.

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ATP13A2 is a P-type ATPase that transports polyamines from lysosomal lumen to the cytosol maintaining lysosomal health and integrity. It is genetically implicated in PD causing a rare early-onset parkinsonism associated with dementia called Kufor-Rakeb syndrome (KRS), thus making it a potential therapeutic target for the treatment of PD. Cryo-EM structures of the transport cycle of ATP13A2 have been recently published by several groups, however, not much is known about how it is regulated.

In this project, we aim to elucidate the mechanisms of regulation and transport activation of ATP13A2. We hypothesize that N-/C-terminus of ATP13A2 are involved in the regulation by interacting with interactors such as lipids, kinases and other domains of the protein. To this end,

we affinity purified recombinant protein fragments and full length ATP13A2 from bacteria and yeast respectively. We also purchased synthetic peptides corresponding to the N-/C- termini and candidate regions for N-/C-terminal interaction. These were subjected to a variety of biochemical experiments such as protein-lipid overlays, ATPase and auto-phosphorylation assays to study the polyamine dependent activity, lipid interaction and activation. We will present the findings from the above mentioned biochemical experiments. This will also provide insights into the activation/regulation of other members of the family.

Poster #34 - Role of M2 and M6 Helices of Sarcoplasmic Reticulum Ca Pump in Ca Transport.

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Ca pump transport Ca coupled with ATP-hydrolysis. The catalytic site consists of the three cytoplasmic domains N, P, and A and the motion of A-domain functions in regulating Ca gating at transmembrane region (TM). The long M2 helix of TM linked with A-domain changes its structure during transport cycle. We have previously shown that Ala substitution of Gly105 at the border of transmembrane part (M2m) and cytoplasmic part (M2c) results in slow EP isomerization and uncoupling. Gly105 functions as a flexible hinge of M2m/M2c, which is critical for Ca transport. We have found that the defects of G105A could be restored by an additional mutation at M2m. In this study, we will report functional consequences of mutations on M2m and M6 residues.

Poster #35 - Crystal and Cryo-EM Structures of a Plasma Membrane Flippase ATP11C.

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ATP11C, a plasma membrane phospholipid flippase, maintains the asymmetric distribution of phosphatidylserine accumulated in the inner leaflet. Caspase-dependent inactivation of ATP11C is essential for an apoptotic “eat me” signal, phosphatidylserine exposure, which prompts phagocytes to engulf cells. We show one crystal structure (3.9 Å) and six cryo-EM structures (3.0-4.0 Å) of ATP11C in five different states of the transport cycle. Three structures of phospholipid-bound states visualize phospholipid translocation accompanied by the rearrangement of transmembrane helices and an unwound portion at the occlusion site, and thus they detail the basis for head group recognition and the locality of the protein-bound acyl chains in transmembrane grooves. Invariant Lys880 and the surrounding hydrogen-bond network serve as a pivot point for helix bending and precise P domain inclination, which is crucial for dephosphorylation. The structures detail key features of phospholipid translocation by ATP11C, and a common basic mechanism for flippases is emerging (Nakanishi et al., 2020, *JBC*, *Cell Rep*).

We also determined cryo-EM structure of ATP11C embedded in DOPS nanodisc (3.4 and 3.9 Å), in which the surface of inner leaflet is distended around transmembrane helix 2, possibly

reflecting the perturbation needed for phospholipid release to the lipid bilayer (Nakanishi et al., 2022, *JBC*).

Poster #36 - Spherical Oligo-Silicic Acid (SOSA) as an Endogenous Regulator of P-type ATPases.

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Despite intensive research work the regulation of the protein phosphorylation and ion transport steps in P-type ATPases remained unresolved. We identified[1] the ATPase inhibitory Spherical Oligo-Silicic Acid (SOSA) as ancient regulators of these key pump steps. SOSA is endogenous because it may be generated by controlled spherical growth of mono-silicic acid (mSA) present in almost all cells. In the protein phosphorylation step the active SOSA with φ~1.6 nm is stabilizing the phosphorylated state by e.g.: interaction with free and ADP complexed Mg^{2+} ions. Similar stabilization of phosphorylated substrates by SOSA have been identified in several ATP-driven phosphorylation reactions such as in glycolysis. The analogy with Vanadates indicates an evolutionary race won by SOSA. For improved understanding of the ion translation, we assume the presence of at least one ion-channel segment filled with gel-like structured oligo-silica. This nano-silicagel medium can explain the gating of the Na ions or the ping-pong like translation of the K^+ and Na^+ ions. The silica filling is stabilized by basic amino acids from the protein but, not covalently bond to it. The space between M4/M5 helices with five Lys and three Arg rests could be optimal for the filling with SOSA and assuring the selective ion translation from the cytosolic space into the transmembrane channel. The Na^+ ion concentration in cytosol controls the ratio of SiOH group salified by Na^+ in the nano-silica filling and thus the ionic conductance of silica gel. This can explain why the pump is switched on at 5.0 mM concentration of cytosolic Na^+. In conclusion: the SOSA concept explains the regulation mechanisms of the phosphorylation and ion translation in ATPases and could open the road for therapy applications.

1. F Kerek, V. Voicu, Spherical oligo-silicic-acid SOSA, Front.Endo. 2015.00233

Poster #37 - Quantification of Na,K-ATPase Oligomers in The Plasma Membrane of Living Cells by FRET-FCS.

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We have used a combination of genetic code expansion, Fluorescence Correlation Spectroscopy (FCS), and FRET to quantify Na,K-ATPase in the plasma membrane of living cells and to analyze oligomerization of the alpha/beta heterodimer.

Genetic code expansion and click chemistry was used to introduce Alexa488 and Alexa647 as a FRET pair in alpha and beta subunits of Na,K-ATPase. HEK 293T cells were transiently transfected, and care was taken to keep the expression at a low level.

FCS measurements in cells where both alpha and beta subunits were labeled was used to calculate the absolute density of endogenous and exogenous Na,K-ATPase in the plasma membrane. To detect oligomerization, we used cells where the beta subunit was labeled with a 50/50 mix of Alexa488 and Alexa647. We successfully detected FRET signals and an analysis of the FRET-FCS data revealed a high density of oligomers. FRET-FCS is, compared to conventional cross-correlation FCCS, one to two orders of magnitude more sensitive for detection of oligomers. FRET-FCS is also inherently insensitive to unbalanced labeling, which is a great advantage during live cell measurements

In conclusion we demonstrate a high density of Na,K-ATPase in oligomer conformation in the plasma membrane, and we propose that oligomerization can have a regulatory function for the net efficiency of Na,K-ATPase in the cell.

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Poster #38 - An Electrophysiological Approach for Low Turnover and Intracellular Transport Proteins: Improving Amplification and Accessibility.

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Functional characterization of transport proteins using conventional electrophysiology can be challenging, especially for low turnover transporters or transporters from bacteria, or proteins expressed in intracellular compartments. Solid-supported membrane (SSM)-based electrophysiology is an emerging technique which allows for sensitive and cell-free assays for the characterization of electrogenic membrane proteins.

The method allows the adsorption of any membranes to a lipid coated sensor (solid supported membrane, SSM) for subsequent electrical measurements. Samples can be in the form of purified proteins reconstituted into proteoliposome or membrane vesicles from cell culture or native tissues whether cell culture-derived or organellar. The large (3 mm diameter) sensor generates a 1000-fold or higher amplification compared to conventional patch clamp. These two features render the method ideal for electrogenic transporters and channels which are difficult to access by other electrophysiological methods due to their low current amplitude or because of their expression in intracellular membranes.

Here we present case studies from different protein target classes demonstrating the properties of the method: Transport and binding signals of electrogenic sugar transporters (SGLT, XylE) and kinetic parameters like rate constants, KD, KM were resolved, pharmacological properties of inhibitors were tested in PepT1 and EAAT2, and the suitability for intracellular membranes was demonstrated by activity measurements from TMEM175 in lysosomes. Measurements of membrane proteins in the ATPase class was also performed using this technology.

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