Biochemistry of Membrane Traffic: Secretory and Endocytic Pathways
Granlibakken Resort, Tahoe City, CA

Program and Abstract Book

Meeting Organizers:

Suzanne Pfeffer, Stanford University School Of Medicine
Vivek Malhotra, Center for Genomic Regulation, Barcelona, Spain

www.asbmb.org/MembraneTraffic
# Schedule At-A-Glance

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Symposium Agenda

Thursday, October 28, 2010

6:00 p.m. – 7:00 p.m.  Dinner

7:00 p.m. – 7:15 p.m.  Opening Remarks and Announcements

7:15 p.m. – 8:00 p.m.  Keynote: An ESCRTs View of Receptor Endocytosis and Down-Regulation: A Journey from Genetics to Biochemical Mechanism

Scott Emr, Cornell University

8:10 p.m. – 8:30 p.m.  ER structure regulates the organization of COPII assembly sites and thus the biogenesis of the Golgi apparatus

Akihiko Nakano, RIKEN ASI

8:30 p.m. – 8:40 p.m.  Discussion

8:40 p.m. – 8:55 p.m.  A structural analysis of the ER in budding yeast by high-resolution EM reveals how multiple ER domains are organized by membrane shaping proteins

Gia Voeltz, University of Colorado, Boulder

8:55 p.m. – 9:00 p.m.  Discussion

9:00 p.m. – 9:15 p.m.  Molecular analysis of homotypic membrane fusion by the GTPase atlastin

James McNew, Rice University

9:15 p.m. – 9:20 p.m.  Discussion

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Nature Cell Biology  •  Wiley-Blackwell
Friday, October 29, 2010

7:30 a.m. – 9:00 a.m.  Breakfast

9:00 a.m. – 9:20 a.m.  A vesicle carrier that mediates peroxisomal membrane protein traffic from the endoplasmic reticulum

**Randy Schekman**, University of California at Berkeley

9:20 a.m. – 9:30 a.m.  Discussion

9:30 a.m. – 9:45 a.m.  Sec16 defines a new layer of regulation in the secretory pathway

**Benjamin Glick**, University of Chicago

9:45 a.m. – 9:50 a.m.  Discussion

9:50 a.m. – 10:05 a.m.  Sec31p as a sole component of the COPII cage: understanding the bst phenotype

**Elizabeth Miller**, Columbia University

10:05 a.m. – 10:10 a.m.  Discussion

10:10 a.m. – 10:25 a.m.  Cstage5 is involved in the endoplasmic reticulum to Golgi trafficking in mammalian cells.

**Kota Saito**, University of Tokyo, Graduate School of Pharmaceut

10:25 a.m. – 10:30 a.m.  Discussion

10:30 a.m. – 11:00 a.m.  Coffee Break

11:00 a.m. – 11:15 a.m.  TRAPP is required for ER exit of procollagen by controlling the Sar1 cycle

**Rossella Venditti**, TIGEM

11:15 a.m. – 11:20 a.m.  Discussion

11:20 a.m. – 11:35 a.m.  125A as part of mammalian COPII machinery in ER export

**Wanjin Hong**, Institute of Molecular and Cell Biology

11:35 a.m. – 11:40 a.m.  Discussion

11:40 a.m. – 12:00 p.m.  Mechanism of membrane fusion and the release of hormones and transmitters

**James Rothman**, Yale University

12:00 p.m. – 12:10 p.m.  Discussion
12:10 p.m. – 4:00 p.m. Lunch and Free Time

4:00 p.m. – 4:20 p.m. Sorting in the Golgi Apparatus
Christopher Burd, University of Pennsylvania School of Medicine

4:20 p.m. – 4:30 p.m. Discussion

4:30 p.m. – 4:50 p.m. Mechanisms for selective activation of Arfs within the Golgi complex
Paul Melancon, University of Alberta

4:50 p.m. – 5:00 p.m. Discussion

5:00 p.m. – 5:20 p.m. Mechanism of secretory cargo sorting at the TGN
Vivek Malhotra, CRG: Center for Genetic Regulation

5:20 p.m. – 5:30 p.m. Discussion

5:30 p.m. – 5:45 p.m. GOLPH3 Bridges PtdIns(4)P and Myosin18A to Stretch and Shape the Golgi to Promote Vesicle Budding
Seth Field, University of California, San Diego

5:45 p.m. – 5:50 p.m. Discussion

6:00 p.m. – 7:00 p.m. Dinner

7:00 p.m. – 8:00 p.m. Poster Presentations (Last Names A-L)

8:00 p.m. – 8:20 p.m. Phosphatidylinositol 4-phosphate controls both membrane recruitment and a regulatory switch of the Rab GEF, Sec2p
Peter Novick, UCSD

8:20 p.m. – 8:30 p.m. Discussion

8:30 p.m. – 8:45 p.m. Ypt/Rab GTPases and Traffic Coordination
Nava Segev, University of Illinois at Chicago

8:45 p.m. – 8:50 p.m. Discussion

8:50 p.m. – 9:05 p.m. PI(4)P Controls the Sequential Assembly of Clathrin Adaptors at the TGN
Greg Payne, UCLA School of Medicine

9:05 p.m. – 9:10 p.m. Discussion
Saturday, October 30, 2010

7:30 a.m. – 9:00 a.m.  Breakfast

9:00 a.m. – 9:20 a.m.  Regulation of Rab GTPases by exchange factors
                       **Francis Barr**, University of Liverpool

9:20 a.m. – 9:30 a.m.  Discussion

9:30 a.m. – 9:50 a.m.  Control of Golgi function by Rab GTPases and Golgin proteins
                       **Suzanne Pfeffer**, Stanford University School of Medicine

9:50 a.m. – 10:00 a.m. Discussion

10:00 a.m. – 10:20 a.m. CATCHR-Family Tethering Complexes: Structure and Mechanism
                         **Frederick Hughson**, Princeton University

10:20 a.m. – 10:30 a.m. Discussion

10:30 a.m. – 11:00 a.m. Coffee Break

11:00 a.m. – 11:15 a.m. Regulation of SNARE complex assembly by the yeast exocyst complex and Sec1p
                         **Mary Munson**, UMass Medical School

11:15 a.m. – 11:20 a.m. Discussion

11:20 a.m. – 11:35 a.m. The role of COG subcomplexes in the intra-Golgi vesicular trafficking
                         **Vladimir Lupashin**, University of Arkansas for Medical Sciences

11:35 a.m. – 11:40 a.m. Discussion

11:40 a.m. – 12:00 p.m. Exocytosis of large cargo: a lesson from coronaviruses
                         **Carolyn Machamer**, Johns Hopkins Univ School of Medicine

12:00 p.m. – 12:10 p.m. Discussion

12:10 p.m. – 4:30 p.m. Lunch and Free Time

4:30 p.m. – 4:50 p.m. A novel coat complex traffics membrane proteins to the primary cilium
                       **Maxence Nachury**, Stanford University School of Medicine
4:50 p.m. – 5:00 p.m.  Discussion

5:00 p.m. – 5:15 p.m.  Cell cycle-regulated Golgi stack assembly and function  
**Yanzhuang Wang**, University of Michigan

5:15 p.m. – 5:20 p.m.  Discussion

5:20 p.m. – 5:35 p.m.  Significant divergence in mammalian host genes requirements for  
A-B toxins traffic revealed by genome-wide RNAi screens  
**Frederic Bard**, IMCB

5:35 p.m. – 5:40 p.m.  Discussion

6:00 p.m. – 7:00 p.m.  Dinner

7:00 p.m. – 8:00 p.m.  Poster Presentations (Last Names M-Z)

8:00 p.m. – 8:20 p.m.  From synthetic endosomes to a systems analysis of endocytosis  
**Marino Zerial**, MPI-CBG

8:20 p.m. – 8:30 p.m.  Discussion

8:30 p.m. – 8:50 p.m.  Understanding the mechanism of GEEC (GPI-AP Enriched Endosomal Compartment) formation using single molecule visualization techniques  
**Satyajit Mayor**, National Centre for Biological Sciences-Bangalore

8:50 p.m. – 9:00 p.m.  Discussion and More Posters

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**Sunday, October 31, 2010**

7:30 a.m. – 9:00 a.m.  Breakfast

9:00 a.m. – 9:20 a.m.  Structural Insights into Dynamin-Catalyzed Membrane Fission  
**Sandra Schmid**, Scripps Research Institute

9:20 a.m. – 9:25 a.m.  Discussion

9:25 a.m. – 9:40 a.m.  Regulation of Lland-Independent EGFR Endocytosis by a Phosphatidic Acid-Pka Pathway  
**Alfonso Gonzalez**, Pontificia Universidad Catolica De Chile
9:40 a.m. – 9:50 a.m.  Discussion
9:50 a.m. – 10:10 a.m. Membrane Fusion: 5 Lipids, 4 SNAREs, 3 Chaperones, 2 Nucleotides, and a Rab, All Dancing in a Ring
William Wickner, Dartmouth Medical School
10:10 a.m. – 10:20 a.m. Discussion
10:20 a.m. – 10:50 a.m. Coffee Break
10:50 a.m. – 11:05 a.m. hRME-6, a rab5GEF that integrates endocytosis and signalling
Liz Smythe, University of Sheffield
11:05 a.m. – 11:10 a.m. Discussion
11:10 a.m. – 11:25 a.m. The Drosophila peripheral Golgi transmembrane protein Ema promotes autophagosomal growth
Sungsu Kim, Washington University in St. Louis School of Medicine
11:25 a.m. – 11:30 a.m. Discussion
11:30 a.m. – 11:50 a.m. Regulation and physiological functions of clathrin-mediated membrane traffic
Frances Brodsky, University of California, San Francisco
11:50 a.m. – 12:00 p.m. Discussion and Departure

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Poster Presentations
Last Names A – L Manned on Friday and Last Names M – Z Manned Saturday

Conserved function of higher eukaryote specific HOPS homologues -vps33b and vps16b in endosome phagosome fusion: Insight into ARC syndrome pathology and immune defense
Mohammed Akbar, UT Southwestern Medical Center at Dallas

The dynamics of SNARE complexes in intracellular membrane fusion
Kannan Alpadi, Baylor College of Medicine

Phosphorylation of a Golgi Epsin by Protein Kinase A Regulates TGN-endosome trafficking
Quyen Aoh, University of North Carolina- Chapel Hill

The Tumoricidal Action of the Adenoviral E4ORF4 Protein Involves SRC-Family Kinases-Dependent Changes in Endocytic Traffic
Marie-Chloe Boulanger, Centre de recherche en cancérologie de l'Universit

Role of actin in clathrin-coated pit formation: additional force to counteract membrane tension
Steeve Boulant, Harvard Medical School

LMTK2 Regulates CFTR Recycling in Polarized Epithelial Cells
Neil Bradbury, Chicago Medical School

The clathrin adaptors AP1A and AP1B directly interact with the tight junction component CAR dictating its surface localization
Jose Carvajal-Gonzalez, Weill Cornell Medical College

Negative regulation of the endocytic adaptor Dab2 in mitosis
David Chetrit, Tel-Aviv University

Dysferlin and the ferlin family; an ancient family of calcium-sensitive vesicle fusion proteins
Sandra Cooper, The Children's Hospital at Westmead

Mechanistic details of sorting nexin dependent retrograde endosome-to-Golgi transport
Chris Danson, Bristol University
An evolutionarily conserved protocoatomer-related complex containing Sec13 and Seh1 dynamically associates with the vacuole in *Saccharomyces cerevisiae*

**Svetlana Dokudovskaya**, Institut Gustave Roussy

A potential role for Rab10 in ER network assembly

**Amber English**, University of Colorado at Boulder

Regulation of O-glycosylation through Golgi to ER relocation of initiation enzymes

**David Gill**, Institute of Molecular and Cell Biology

Novel fluorescent probes to study sphingomyelin dynamics in living cells

**Claudio Giraudo**, Yale University

Caveolin-1 is ubiquitinated and targeted to intralumenal vesicles in endolysosomes for degradation

**Arnold Hayer**, Stanford University

A Fifth Adaptor Protein Complex

**Jennifer Hirst**, University of Cambridge

The conserved Bardet-Biedl Syndrome proteins assemble a coat that traffics membrane proteins to cilia

**Hua Jin**, Stanford University

Rab7 localization and activity are regulated by palmitoylation

**Felix Jules**, Université de Montréal-Hôpital Maisonneuve-Rosemon

Inactivation of clathrin heavy chain inhibits synaptic recycling but allows bulk membrane uptake

**Jaroslaw Kasprowicz**, VIB/KULeuven

The Drosophila Golgi transmembrane protein Ema promotes autophagosomal growth

**Sungsu Kim**, Washington University in St. Louis School of Medicine

Sec31p as a sole component of the COPII cage: understanding the bst phenotype

**Catherine Latham**, Columbia University

Cbl Amino Acids at a Putative Dimer Interface Regulate EGF Receptor Trafficking

**Nancy Lill**, The Ohio State University

Cytoskeletal “Wheels” for the Rapid Recycling Pathway

**Elizabeth Luna**, Univ of Mass Medical School
The Role of Ceroid Lipofuscinosis Neuronal Protein-5 (CLN5) in lysosomal Sorting and Trafficking
Aline Mamo, Hopital Maisonneuve-Rosemont

Membrane trafficking in cytokinesis: a role for the Exocyst
Helia Neto, University of Glasgow

Isoform specific properties of Dynamin 2
Sylvia Neumann, The Scripps Research Institute

SNARE motif: a common motif used by pathogens to manipulate membrane fusion
Fabienne Paumet, Thomas Jefferson University

Protein targeting to the silica deposition vesicle in diatoms
Nicole Poulsen, Georgia Institute of Technology

Adenosine mediated modulation of membrane trafficking is through transactivation of EGF receptors
Herman Prakasam, University of Pittsburgh

Retromer-dependent STxB and CIM6PR Trafficking Passes Through the Recycling Endosome
Brent Raisley, University of Iowa

Syp1 regulation of actin polymerization and cargo recognition in Clathrin Mediated Endocytosis
Amanda Reider Apel, Johns Hopkins University

AnnexinA6-mediated disintegration of SNAP23 and syntaxin-4 in CHO cells
Meritxell Reverter-Martín, Universidad de Barcelona-Facultad Medicina

Rap1A: A Novel Interactor Involved in CFTR Traffic (and in Chloride Secretion in wt-CFTR CFBE Monolayers)
Francisco Romeiras, Faculty of Sciences, University of Lisboa, Portuga

Glycolipid flip-flop across the ER during protein N-glycosylation
Sumana Sanyal, Whitehead Institute/MIT

Ang2/Fat-free is a Conserved Subunit of the Golgi-associated Retrograde Protein (GARP) Complex
Christina Schindler, National Institutes of Health
Characterization of the endocytic pathway of the cytokine MIF and its receptors
Verena Schwartz, RWTH Aachen University

Identification and interaction mapping of three novel TRAPP components suggests a distinct organization of the mammalian TRAPP complex
James Scrivens, Concordia University

Deregulation of ceramide chain-length composition affects trafficking and Golgi morphology
Stefka Spassieva, Medical University of South Carolina

Assembly of caveolin-1 and cavins during the caveolar life-cycle
Miriam Stoeber, Swiss Federal Institute of Technology Zurich

Rab6 Regulates Golgi Vesicle Release and Cisternal Homeostasis
Brian Storrie, University of Arkansas for Medical Sciences

Opposing roles of Annexin A1 and A2 in trafficking of Shiga toxin
Lionel Tcatchoff, Center for Cancer Biomedicine

Skywalker is a novel GTPase activating protein that restricts synaptic endosomal traffic and neurotransmitter release
Valerie Uytterhoeven, KU Leuven

The role of SNX-BAR proteins in tubular/vesicular endosomal sorting
Jan van Weering, University of Bristol

Structure of a C-terminal fragment of its Vps53 subunit suggests similarity of GARP to a family of tethering complexes
Neil Vasan, Yale University School of Medicine

Rac1 interaction with CaM. Importance of the activation state, the plasma membrane localization and the c-terminal polybasic domain
Maite Vidal-Quadras, Universidad de Barcelona- Facultad de medicina

Ligand induced internalization and recycling of the human Y2 receptor is regulated by its C-terminal tail
Cornelia Walther, Leipzig University

Rab 14 regulates tight junction integrity and epithelial polarity
Jean Wilson, University of Arizona
A novel Golgi-localized protein involved in the trafficking of dense core vesicle cargos
Tao Xu, Institute of Biophysics

Dissecting the roles of O-glycosylation and dimerization in the apical sorting of a model
raft-independent protein
Robert Youker, University of Pittsburgh

As of 9.28.10

Notes
Conserved function of higher eukaryote specific HOPS homologues -vps33b and vps16b in endosome phagosome fusion: Insight into ARC syndrome pathology and immune defense

Mohammed Akbar¹, Charles Tracy¹, Walter Kahr³, Helmut Kramer¹, ².

Department of Neuroscience, UT Southwestern Medical Center At Dallas¹, Department of Cell Biology, UT Southwestern Medical center at Dallas², Department of Paediatrics, The Hospital for Sick Children, University of Toronto³

Abstract:

Removal of dead cells and foreign particles (microbes) is an interminable process for survival of multi cellular organisms. Defective clearance (due to impaired phagocytosis or phagosome maturation) causes severe diseases due to over or under active immune response. Several conserved proteins have been described for their role in clearance, particularly in phagosome maturation but ambiguity remained because other vital cellular processes such as autophagy, endocytic trafficking and lysosomal related organelles biogenesis also rely on them. Here we describe functions of only higher eukaryotic specific conserved proteins, vps16b and its binding partner sec1/munc18 homologue vps33b, specifically in phagosome-late endosome/lysosome fusion. vps16b null mutant and vps33b knock down flies are hypersensitive to infections with normal nonpathogenic bacteria. At the cellular level mutant phagosome undergo early steps of maturation, i.e., PI3P generation, rab5 effector recruitment and SNARE (avl) present on phagosome membrane. Additionally, acquisition of rab7 is normal but the MVB/late endosome protein hook is not present on phagosome membrane. Biochemical and EM studies suggest that mutant phagosome inadequately acidify and do not mature to phagolysosome through fusion with late endosome / lysosome. This defect is specific to fusion of phagosome with late endosome/lysosome as the cargo of autophagosomes and endosomes exhibit normal lysosomal delivery. Importantly, loss of function of either vps16B or vps33b gene causes a fatal autosomal disorder known as Arthrogryposis, renal dysfunction and cholestasis syndrome (ARC). Experiments with patients’ (mutation in vps33b gene) cell showed identical defects in phagosome maturation. Our finding suggests that defective phagosome maturation may be an underlying mechanism of pathogenesis of ARC syndrome and could explain the recurring infections observed in ARC patients.
The dynamics of SNARE complexes in intracellular membrane fusion

Kannan Alpadi¹, Andreas Mayer¹ ², Christopher Peters¹.

Department of Biochemistry & Molecular biology, Baylor College of Medicine, Houston, Texas-77030, USA¹, Department of Biochemistry, University of Lausanne, Epalinges, CH-1066, Switzerland²

Abstract:

Intracellular membrane fusion is central to all secretory and endocytic vesicular traffic. Membrane fusion events proceed via distinct stages of priming, docking, hemifusion and fusion pore opening. The factors needed for membrane fusion involve N-ethylmaleimide sensitive factor (NSF)/α-Soluble N-ethylmaleimide sensitive factor attachment protein (α-SNAP), Soluble N-ethylmaleimide sensitive factor attachment protein receptors (SNAREs), Sec1-Munc18 protein (SM) and Rab GTPases and their effectors. SNARE complexes are essential for membrane fusion. They exist in two different forms depending on the stage of fusion; SNARE complexes residing on the same membrane by certain topology called as cis-SNARE complexes and SNAREs from two opposing membrane form complexes prior to fusion by specific topology called as trans-SNARE complexes. The dynamics of assembly, disassembly, reassembly and stability of SNARE complexes are tightly regulated by known and unknown factors. This process is likely to be critical and evolutionary conserved. We addressed the dynamics of SNARE complexes formation by using vacuoles from S. cerevisiae.
Phosphorylation of a Golgi Epsin by Protein Kinase A Regulates TGN-endosome trafficking

Quyen Aoh¹, Christine Phu¹, Mara Duncan¹.

University of North Carolina- Chapel Hill¹

Abstract:

The efficient sorting and packaging of many cargoes into clathrin-coated vesicles (CCVs) at the trans-Golgi Network (TGN) and endosomes is vital to cellular homeostasis and to how a cell responds to environmental cues. The clathrin adaptors, adaptor complex-1 (AP-1), GGA, and Ent/epsin proteins, play key roles in coat formation and cargo recognition however little is known about how they are regulated to prevent aberrant assembly. In a biochemical screen to identify candidate regulatory kinases of TGN-endosome trafficking, we found that overexpression of individual Protein Kinase A (PKA) catalytic subunits (Tpk1p, Tpk2p, Tpk3p) induces hyperphosphorylation of the clathrin adaptor Ent5. Importantly, we found that phosphorylation of Ent5 by PKA occurs following the diauxic shift strongly suggesting a novel link by which PKA signaling can remodel TGN-endosome trafficking in response to global changes in cell metabolism. We demonstrate that PKA phosphorylates Ent5 in vitro and in vivo and that overexpression of PKA catalytic subunits causes a synthetic defect in carboxypeptidase processing in ent3Δ cells. Similarly, phosphomimetic mutation of Ent5 PKA consensus sites negatively regulates Ent5 function ent3Δ cells. In cells overexpression PKA catalytic subunits or phosphomimetic mutant Ent5, Ent5 is mislocalized. Based on these results we propose a model in which PKA regulates clathrin coat assembly through phosphorylation of Ent5.
Significant divergence in mammalian host genes requirements for A-B toxins traffic revealed by genome-wide RNAi screens

Frederic Bard

Institute of Molecular and Cell Biology

Abstract:

Retrograde traffic flows from the plasma membrane to the ER via the endosomes and the Golgi apparatus. It is essential to maintain cellular homeostasis and is also subverted by various pathogens and toxins, such as Ricin and Pseudomonas Exotoxin (PE). To molecularly characterize the retrograde traffic, we screened human cells by RNAi at the genome-wide level for factors required for Ricin and PE intoxication. The host genetic requirements appear strikingly different between both toxins with only about 13% common factors out of the 2038 genes identified. Among known regulators of membrane traffic, the VPS35-Retromer complex is specifically required for PE and the TRAPP complex for Ricin. Similarly, at the ER level, Ricin and PE interact with largely different subsets of potential ERAD factors, including the Derlins for Ricin and the Sec61 translocon for PE. Off-target effect was excluded for 178 of the most potent regulators, which have for the most part no or little prior link with retrograde traffic, such as several factors of the Cullin-Ring Ligases family. ERGIC2 is among the small set of factors common for both toxins and is essential for toxin and general Golgi to ER traffic. In addition to an increased understanding of the retrograde traffic pathways, our study provides a unique resource for the future development of toxins antidotes.
Regulation of Rab GTPases by exchange factors

Francis Barr1, Shin-ichiro Yoshimura1.

University of Liverpool, Liverpool, L3 9TA, UK.1

Abstract:

Rab GTPases are used to encode information about the state of a membrane or membrane domain, in order to control specific membrane trafficking events. Rabs are activated by specific guanine nucleotide exchange factors (GEFs) promoting the release of GDP and binding of GTP. According to the prevailing model GEFs together with other regulatory factors localize to and act at specific membrane surfaces, and thus provides a mean to locally activate their target Rabs. This system allows vesicles derived from a particular organelle to be tagged with a specific Rab GTPase, and their movement along the cytoskeleton and tethering to a specified domain on a target membrane to be controlled. A key requirement for Rab function in membrane trafficking is site-specific activation by GDP-GTP exchange factors (GEFs), but the majority of the 63 human Rabs have no known GEF. In this presentation I will discuss our recent data on the identification of novel Rab GEFs and their cellular functions.
The Tumoricidal Action of the Adenoviral E4ORF4 Protein Involves SRC-Family Kinases-Dependent Changes in Endocytic Traffic

Marie-Chloé Boulanger¹, Claudia Champagne¹, Marie-Claude Landry¹, Andréane Sicotte¹, Josée N. Lavoie¹.

Centre de recherche en cancérologie de l’Université Laval. Hôtel-Dieu de Québec, CRCHUQ¹

Abstract:

Multiple cell death mechanisms operate in response to stress, particularly in cancer cells bearing dysfunctions in apoptotic pathways. They seem to share overlapping signaling pathways directing inter-organellar communication and remodeling of cellular organelles through the trafficking of signaling proteins and lipids. Yet the mechanisms underlying such changes in intracellular membrane flow are largely undefined. The adenovirus E4orf4 protein was proposed to behave as a tumoricidal factor that would target oncogenic pathways upon which cancer cells have become addicted. We have shown that E4orf4 provides a key, probing tool to tackle stress-responsive trafficking pathways in cancer cells. It acts by modifying the activity of Src-family kinases (SFKs) to promote cell polarization and organelle-based actin remodeling leading to cell death. Such process was found to depend on increased retrograde transport between recycling endosomes and the Golgi complex through a pathway involving Src, Yes, Cdc42, actin, Rab11a and syntaxin-6. We further found that the tumoricidal action of E4orf4 was manifested in human breast cancer cell lines showing qualitative changes in SFK-dependent signaling, but not in normal breast epithelial cells. E4orf4 killing was associated with mixed cell death phenotypes including caspase-independent apoptosis and mitotic catastrophe upon defective cytokinesis, which depended on Cdc42 and Rab11a. Furthermore, v-Src-induced morphological transformation selectively sensitized epithelial cells to E4orf4 tumoricidal activity that inhibited assembly of actin-rich invasive structures through its binding to SFKs. We propose that a SFK-endosomal pathway at the recycling endosome-Golgi interface would contribute to polarized endocytic transport during cell activation processes including cell death mechanisms and could play a role in cell transformation. Supported by the Canadian Institutes of Health Research.
Role of actin in clathrin-coated pit formation: additional force to counteract membrane tension

Steeve Boulant¹, Comert Kural¹, Jean-Christophe Zeeh¹, Tom Kirchhausen¹.

Harvard Medical School, Boston MA 02115¹

Abstract:

The role of actin during clathrin-coated pit (CCP) formation in mammalian cells differs with cellular context. In non-polarized cells, the importance of actin for CCP formation remains controversial. However, clear evidence indicates actin is necessary for clathrin-mediated endocytosis in polarized cells. To clarify the role of actin during clathrin-mediated endocytosis in polarized cells we asked whether: 1) the mechanism by which CCPs form in the apical and basolateral membranes of polarized cells are the same or different and 2) actin involvement in CCP formation is a response to a cellular property of polarized cells. To address these questions, we used ultra-fast fluorescent 3D imaging of CCP formation in live-cells. We found that formation of CCPs from the basolateral membrane of polarized cells does not require actin dynamics. In contrast, apical endocytic clathrin coats absolutely require actin dynamics for budding and scission from the cell surface. Actin recruitment is ARF6-, Rac1- dependent and promotes constriction of the neck of the pits and allows recruitment of dynamin-2, which further drives membrane scission. Using knock-out and constitutive expression experiments, we have demonstrated that the “border-brush” at the apical side of polarized cells is responsible for the actin dependency of CCPs. Additionally, direct physical stretching of cells and increase of turgor pressure induce a switch from an actin-independent to an actin-dependent mechanism for CCP formation in both the basolateral membrane of polarized cells and in non-polarized cells. This study provides, for the first time, an explanation for the selective actin dependency during clathrin-mediated endocytosis in mammalian cells. We demonstrate that the requirement for actin is a direct response from the cell to counteract local membrane tension. Actin provides a strong scaffold supporting the generation of forces needed to direct the inward invagination of the plasma membrane.
LMTK2 Regulates CFTR Recycling in Polarized Epithelial Cells

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Abstract:

Cystic Fibrosis (CF) is a lethal genetic disease that affects some 30,000 individuals in the US alone. CF is caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene that encodes a chloride ion channel expressed in the apical membranes of polarized epithelial cells lining the airways and gastrointestinal tract where it is responsible for regulating salt and water transport. CFTR is rapidly endocytosed from the apical cell membrane. The majority of internalized CFTR is then recycled efficiently back to the cell surface through the endosomal recycling compartment (ERC). The aim of the present study was to investigate the role of lemur tyrosine kinase (LMTK2) in regulating the recycling of CFTR in polarized human epithelia. RT-PCR analysis of human lung, intestine and pancreatic tissues revealed the presence of LMTK2 transcript in all tested tissues. siRNA mediated knockdown of LMTK2 in T84 human colonic epithelia caused CFTR to be trapped in endosomes, reducing apical membrane CFTR levels and dramatically reducing forskolin-stimulated CFTR activity. In contrast, overexpression of LMTK2 led to enhanced CFTR recycling and an increased in apical CFTR activity. Since LMTK2 is a kinase, we generated a point-mutation in the active site, rendering the kinase catalytically inactive. Overexpression of the "dead kinase" resulted in loss of CFTR activity similar to that seen with knockdown of LMTK2 suggesting that the dead kinase acts as a dominant negative construct. CFTR and LMTK2 are colocalized and interact within the same endosomal compartment as shown by co-immunoprecipitation. Furthermore, CFTR is a target substrate for LMTK2 kinase activity. Our data provide support for a novel mechanism for the regulation of ion channel recycling through trafficking-dependent phosphorylation events.
Regulation and physiological functions of clathrin-mediated membrane traffic

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Abstract:

The assembly of clathrin into a protein lattice that coats membrane vesicles represents a paradigm for how coat proteins can mediate sorting. Understanding how this assembly is regulated at the molecular level has been the long-term goal of structural and biochemical studies of clathrin. Recent studies demonstrating how clathrin light chain subunits influence this assembly pathway will be presented. Cellular experiments to manipulate clathrin light chain and heavy chain expression have revealed novel diverse functions for each of the subunits and for the two isoforms of clathrin heavy chain. Experiments defining these unique functions in glucose transporter membrane traffic, cytokinesis and cell migration will be described.
Sorting in the Golgi Apparatus

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Abstract:

The addition of carbohydrate moieties to biosynthetic cargo in the Golgi apparatus is carried out by glycosyltransferases that function sequentially, such that the products of early acting enzymes are substrates for later-acting enzymes. The location of enzymes within the Golgi stack parallels the glycosylation reactions; early-acting enzymes are enriched in cis/medial Golgi compartments while later-acting enzymes are enriched in medial/trans compartments. Phosphatidylinositol 4-kinases (PI4Ks) are key regulators of the sorting reactions that are required to establish and maintain the structure and function of the Golgi, though the downstream effectors of PI4Ks and mechanisms of their actions are poorly characterized. We have identified factors and pathways in yeast that are required to retain a subset of medial Golgi residents. One factor is Vps74, a peripheral Golgi membrane protein that is the ortholog of human GOLPH3, whose over-expression has been correlated with activation of mTOR and oncogenic transformation. In vitro binding studies show that Vps74 recognizes the cytosolic portions of medial Golgi enzymes and that in vps74 mutant cells, these Golgi residents fail to be retained in the Golgi and are delivered via the anterograde pathway to the vacuole. Conversely, over-expression of Vps74 results in sorting of these enzymes into the retrograde trafficking pathway and their accumulation in the endoplasmic reticulum. Synthesis of PtdIns4P by Golgi PI4K is required for the recruitment of Vps74 and GOLPH3 to the Golgi and candidate PtdIns4P binding sites on each protein were identified using lipid binding assays, x-ray crystallography, and cell-based assays. The results suggest that Vps74 is a PI4K effector that is required for sorting of Golgi residents into the retrograde pathway. Genetic interaction studies have led to the identification of additional factors that function with PI4K and Vps74 to maintain the proper identity of Golgi compartments.
The clathrin adaptors AP1A and AP1B directly interact with the tight junction component CAR dictating its surface localization

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Abstract:

Polarized epithelial cells form permeability barriers that separate the organism from the outside medium. The barrier function of epithelial cells depends on their ability to generate and maintain apical and basolateral plasma membrane domains, separated by tight junctions. Recently, we reported that several basolateral proteins, with a broad range of basolateral sorting signals, require clathrin for basolateral transport in the biosynthetic and recycling routes. Some of these basolateral proteins, e.g. VSVG protein, utilize the clathrin adaptor AP1B for biosynthetic transport to the basolateral membrane, whereas other, e.g. the coxsackie adenovirus receptor (CAR) utilizes AP1B in the recycling but not in the biosynthetic route. Here, we demonstrate that the clathrin adaptor AP1A mediates biosynthetic transport of CAR to the basolateral membrane. Yeast two hybrid assays in combination with deletions and directed point mutations showed that CAR interacts directly with the medium subunits of both AP1A (m1A) and AP1B (m1B) via its known basolateral signal, YXXf (Y318-V321). Mutagenesis of m1A and m1B identified the interaction site as the highly conserved pocket also present in the medium subunit m2 of the clathrin adaptor AP2. Knock-down of m1A by siRNA and shRNA resulted in missorting of newly synthesized CAR in its biosynthetic route. In conclusion, we have shown for the first time that the direct interaction of a tyrosine basolateral sorting motif within the context of the whole cytoplasmic tail of a basolateral PM protein with AP1A and AP1B drives its polarized localization in epithelial cells.
Negative regulation of the endocytic adaptor Dab2 in mitosis

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Abstract:

Mitotic cells undergo extensive changes in shape and size through the altered regulation and function of their membrane trafficking machinery. Disabled 2 (Dab2), a multi-domain cargo-specific endocytic adaptor and a mediator of signal-transduction, is a potential integrator of trafficking and signaling. Dab2 binds effectors of signaling and trafficking which localize to different intracellular compartments. Thus, differential localization is a putative regulatory mechanism of Dab2 function. Moreover, Dab2 is phosphorylated in mitosis, and thus regulated in the cell-cycle. However, a detailed description of the intracellular localization of Dab2 in the different phases of mitosis and an understanding of the functional consequences of its phosphorylation are lacking. Here we show that Dab2 is progressively displaced from the membrane in mitosis. This phenomenon is paralleled by a loss of co-localization with clathrin. Both phenomena culminate in metaphase/anaphase and undergo partial recovery in cytokinesis. In accord with an arrest at the spindle assembly checkpoint, treatment with 2-Methoxyestradiol (2ME2) induces the same effects observed in metaphase cells. Furthermore, 2ME2 induced the phosphorylation of Dab2, which was partially reduced upon the mutation of Serines 393, 394 and 401 to Alanines. The lesser phosphorylation of Dab2 correlated with reduced membrane displacement. 2ME2 also reduced Dab2/clathrin interactions, endocytic vesicle motility, clathrin exchange dynamics and the internalization of a receptor endowed with an NPXY endocytic signal. We propose that the negative regulation of Dab2 is part of an accommodation of the cell to the altered physico-chemical conditions prevalent in mitosis, aimed at allowing endocytic activity throughout the cell cycle.
Dysferlin and the ferlin family; an ancient family of calcium-sensitive vesicle fusion proteins

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Abstract:

The ferlin gene family are tail-anchored proteins possessing a rare and identifying feature of multiple tandem C2 domains and a C-terminal transmembrane domain. Ferlins bear structural homology to the synaptotagmin family of vesicle fusion proteins, and mutations in two mammalian ferlins are linked to human diseases characterised by abnormal vesicle fusion. Mutations in dysferlin underlie an autosomal recessive form of muscular dystrophy due to defective vesicle-mediated membrane resealing, and, mutations in otoferlin cause an autosomal recessive form of non-syndromic deafness caused by defective synaptic vesicle fusion within the cochlear. We show that ferlin proteins have ancient origins in eukaryotic biology, with ancestral ferlins identified in simple unicellular eukaryotes that lack synaptotagmin-like homologues of plants and higher order eukaryotes. We are trying to understand the cellular role of dysferlin, and how it regulates calcium-activated vesicle-mediated membrane resealing. Our studies of dysferlin cell biology reveal a short-lived and transitory transmembrane protein, with a propensity for rapid endocytosis when mutated. Accelerated dysferlin endocytosis, and subsequent endosomal-driven degradation, provides a new explanation as to why some patients with single amino-acid mutations, show dysferlin deficiency. Our recent studies have shown that dysferlin regulates the expression of syntaxin-4, and, that dysferlin and syntaxin-4 similarly transit a common endosomal pathway in skeletal muscle cells. Despite its well characterised role in regulated exocytosis, there are significant gaps in current understanding of endosomal trafficking of syntaxin-4. Dysferlin provides a novel marker of syntaxin-4 endosomal transit in muscle cells, perhaps also relevant to dysferlin’s role in the calcium-activated exocytosis of membrane resealing.
Mechanistic details of sorting nexin dependent retrograde endosome-to-Golgi transport

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Abstract:

Compared with our understanding of the molecular details that underlie the processes of endocytosis, how cells regulate retrograde protein and lipid movement between endosomes and the Golgi apparatus is poorly described. One evolutionarily conserved complexes involved in such retrograde transport is the retromer. Mammalian retromer is composed of two sub-complexes: a cargo-selective VPS26-VPS29-VPS35 trimer, this binds to cargo including the CI-MPR, and an endosomal bound coat comprising specific heterodimeric combinations of the sorting nexins (SNXs) SNX1/SNX2 and SNX5/SNX6. Through the presence of a BAR (Bin/amphiphysin/Rvs) domain, these SNX-BAR proteins drive and stabilize the formation of membrane tubules thereby coupling cargo sorting to the process of donor membrane deformation. In recently published work, we have described how this canonical retromer coat associates with the minus-end direct microtubule motor dynein in order to aid the efficiency of carrier scission and allow for long-range carrier movement. In addition, we have established a role for the retromer coat in the recognition of incoming carriers at the Golgi complex. Here we describe the molecular details of a SNX8-dependent tubular-based endosome-to-Golgi retrograde transport pathway that regulates sorting of a specific set of SNARE proteins. Using a combination of in vitro liposome assays, yeast two-hybrid analysis, proteomics and biochemistry, advanced light and electron microscopy coupled with RNAi-studies in mammalian cells, we establish how this pathway functions independently of the retromer, define the mechanistic details through which the BAR domain-containing SNX8 and the canonical retromer SNX-BARs reside on, and drive the formation of distinct endosomal tubules, and elucidate the molecular basis through which recognition of the SNAREs are achieved.
An evolutionarily conserved protocoatomer-related complex containing Sec13 and Seh1 dynamically associates with the vacuole in Saccharomyces cerevisiae

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Abstract:

The presence of multiple membranous intracellular compartments is a major feature of eukaryotic cells. Many of the proteins required for formation and maintenance of these endomembrane systems share an evolutionary history. Retention of identical components, common secondary and quaternary structure, architecture and function in membrane curvature support the hypothesis that some membrane coats evolved from a common ancestral protocoatomer. Here, we identify the SEA (Seh1-associated) protein complex in Saccharomyces cerevisiae that contains the nucleoporin Seh1 as well as Sec13, a subunit of both the nuclear pore complex and COPII coated vesicles. In addition to Npr2 and Npr3 (upstream regulators of TORC1 kinase), the SEA complex also contains four uncharacterized proteins (Sea1-Sea4). Combined computational and biochemical approaches indicate that the SEA complex proteins possess structural characteristics similar to components of other membrane coating complexes, including COPI, COPII, the nuclear pore complex, and the Vps class C complex HOPS/CORVET. Homologues of SEA complex proteins are well conserved within Opistokhonts, undetectable in Planta, and only partially retained in other supergroups. The SEA complex dynamically associates with the vacuole membrane in vivo. Synthetic genetic interaction data and functional assays suggest a role for the SEA complex in intracellular trafficking, amino acid biogenesis and response to nitrogen starvation. These data demonstrate that the SEA complex is a new member of a family of coating assemblies, thus providing additional support for the protocoatomer model of the endomembrane system evolution.
An ESCRTs View of Receptor Endocytosis and Down-Regulation: A Journey from Genetics to Biochemical Mechanism

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Abstract:

The Endosomal Sorting Complex Required for Transport (ESCRT) complexes are essential for the sorting of receptors into Multi-Vesicular Bodies (MVBs). ESCRTs play a critical role in receptor down-regulation, HIV budding and cytokinesis. Consequently, ESCRT machinery dysfunction contributes to many diseases ranging from cancer to neuro-degeneration. Monoubiquitination (Ub) of plasma membrane proteins by the Rsp5 (Nedd4 in humans) Ub ligase in yeast serves as a critical signal for sorting into MVBs. We have identified five distinct protein complexes (ESCRT-0, -I, -II, -III and Vps4) that function in the recognition and sorting of ubiquitinated transmembrane cargoes. ESCRT-II functions as an activation scaffold during ESCRT-III assembly on endosomes. The monomeric ESCRT-III subunits Vps20, Snf7, Vps24 and Vps2 assemble into an oligomeric complex. Despite their biochemical and structural similarity, each subunit contributes a specific function. Vps20 nucleates oligomerization of Snf7, which appears to sequester MVB cargo. Vps24 terminates Snf7 oligomerization by recruiting Vps2, which subsequently engages the AAA-ATPase Vps4 that disassembles ESCRT-III back into its individual monomers. We propose that the ordered assembly and disassembly of ESCRT-III delineates an MVB sorting domain to sequester cargo, deform the membrane and complete the last steps of MVB vesicle formation and fission. A diversity of plasma membrane proteins are subject to ESCRT-mediated down-regulation. We recently identified a new family of proteins, the ARTs for Arrestin-Related Trafficking adaptors, that function upstream of the ESCRTs. We propose that ARTs provide specificity in the selection of PM proteins for modification with Ub which then directs them into the general ESCRT-mediated MVB sorting system.
A potential role for Rab10 in ER network assembly

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Abstract:

The Endoplasmic Reticulum (ER) is a membrane bound organelle that includes the nuclear envelope and the peripheral ER. The structure of the ER is quite dynamic; throughout the cell cycle the ER membrane network is constantly rearranging in the cytoplasm using a fusion mechanism that has not been characterized. We are interested in identifying the fusion machinery required for ER network formation and maintenance. To identify the fusion machinery, we are using an ER formation assay derived by fractionation of Xenopus laevis eggs. In this system, ER vesicles fuse to form tubular networks in a GTP-dependent manner. To isolate the GTP-dependent fusion machinery, we purified GTP binding proteins from the ER extract on a GTP-agarose column and then identified the bound proteins by Mass Spectrometry. Several Rab proteins were identified; Rab proteins are known GTPases that are involved in membrane fusion of vesicles. Previous studies have shown that in vitro ER formation is disrupted by the addition of a Rab GDP-dissociation inhibitor (GDI); indicating a Rab could be involved in ER fusion. Of the several Rab GTPases identified only Rab8/10 has no previously characterized function. We demonstrate that Rab 8/10 is tightly associated with the Xenopus ER membrane vesicles but can be dissociated by the addition of recombinant RabGDI. Current in vitro studies are focused on determining if Rab8/10 depletion from ER extracts affects ER network formation. We find that a transient transfection of mCherry-Rab10 (human Rab8/10 homolog) into COS-7 cells co-localizes with an ER marker throughout the ER, consistent with a potential role in ER fusion. Furthermore, transfection of a dominant negative form of Rab10, mCherry-Rab10 (T23N), into COS-7 cells disrupts the ER in a manner consistent with a fusion defect. Current studies are aimed at measuring the effects of Rab10 depletion and overexpression on ER morphology to confirm a role for Rab10 in ER fusion.
GOLPH3 Bridges PtdIns(4)P and Myosin18A to Stretch and Shape the Golgi to Promote Vesicle Budding

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Abstract:

Golgi membranes, from yeast to humans, are uniquely enriched in PtdIns(4)P, although the role of this lipid has been poorly understood. Using a proteomic lipid binding screen we discovered that GOLPH3 (yeast VPS74p) is a major direct effector of PtdIns(4)P at the Golgi. Furthermore, we provide overwhelming evidence that GOLPH3 also interacts with MYO18A, providing a link from the Golgi to F-actin. We demonstrate that this linkage is required for normal Golgi morphology, ultrastructure, and vesicle budding for anterograde trafficking. These results can be explained simply by a role for this motor-containing, membrane-binding complex to deliver a tensile force to the Golgi membrane to assist in vesicle abstraction, and that this force consequently helps to flatten the stacks and to stretch the ribbon around the nucleus. The model makes testable predictions that have been experimentally confirmed. GOLPH3 is also the first example of a Golgi protein that is an oncogene, frequently overexpressed in human solid tumors. Our new data addresses the regulation of GOLPH3 function and its role in promoting oncogenic transformation.
Regulation of O-glycosylation through Golgi to ER relocation of initiation enzymes

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Abstract:

Following growth factor stimulation, kinases are activated to regulate multiple aspects of cell physiology. Activated Src is present on Golgi membranes but its function here remains unclear. We find that Src regulates mucin-type protein O-glycosylation through redistribution of the initiating enzymes, polypeptide N-acetylgalactosaminyl transferases (GalNac-Ts), from the Golgi to the ER. Redistribution occurs after stimulation with EGF or PDGF in a Src dependent manner. Similarly, constitutive relocation of GalNac-Ts to the ER is present in cancerous cells with perpetually elevated Src activity. All GalNac-T family enzymes tested are redistributed to the ER upon transient Src activation whereas multiple other glycosylation enzymes are not displaced from the Golgi. Furthermore, upon Src activation, the COP-I coat is also redistributed in punctate structures that co-localize with GalNac-Ts. Expression of a dominant negative Arf1 isoform, Arf1(Q71L), efficiently blocks GalNac-Ts redistribution under Src activation, indicating that Src regulates a COP-I dependent trafficking event. Transient Src activation under EGF stimulation and constitutive Src activation in cancerous cells both increase the efficiency of cellular O-glycosylation initiation by GalNac-Ts. Targeting GalNac-Ts to the ER independently of Src activation recapitulates Src effect on increasing O-glycosylation initiation efficiency, indicating that GalNac-Ts redistribution to the ER is both necessary and sufficient for regulating O-glycosylation upon Src activation. Finally, exogenous expression of GalNac-Ts in the ER but not the Golgi promotes increased cellular adhesion and invasion. We propose that growth factor stimulation regulates O-glycosylation initiation in a Src dependent fashion by GalNac-Ts redistribution to the ER. In addition, our results suggest that the subcellular distribution of GalNac-Ts as well as their expression levels plays a critical role in driving development of cancer.
Novel fluorescent probes to study sphingomyelin dynamics in living cells

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Abstract:

Sphingomyelin is a highly abundant lipid in eukaryotic membranes and has been implicated in modulating signal transduction, enzymatic reactions and viral budding from infected cells. Sphingomyelin, cholesterol and glycosphingolipids share the ability to decrease membrane fluidity by segregating laterally in the plane of the membrane and give rise to domains ranging from 20-100nm that are enriched in these lipids and specific proteins. The synthesis of most of these lipids is carried out in the Golgi Complex and from there, they are transported and concentrated at the plasma membrane. To investigate the dynamics of sphingomyelin in living cells and its ability to form nanodomains within the Golgi membranes we generated a fusion construct coding for a signal sequence, a fluorescent protein and the non-toxic region of lysenin toxin, which specifically binds to sphingomyelin. Expression of this construct in cultured cells showed a spotted distribution mainly concentrated in the perinuclear area with a scarce co-localization with Golgi markers. Temperature blockage at 20°C for 3 h considerably increased the level of co-localization with Golgi markers. Preliminary results showed that knocking down the expression of the sphingomyelin synthase-1 and -2 genes by using siRNA, and thus inhibiting sphingomyelin synthesis, resulted in a dispersal of the fluorescent marker throughout the cells. Further studies are required to determine whether Golgi components and cargo molecules are differentially included/excluded from sphingomyelin rich domains within the secretory pathway. Altogether, these results set the stage for future experiments to study the dynamics of sphingomyelin in living cells and can be used as springboard to study the dynamics of other lipids by using appropriate toxins that bind to them.
Sec16 defines a new layer of regulation in the secretory pathway

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Abstract:

COP II vesicles bud from specialized ER domains termed transitional ER (tER) sites, but the mechanism that creates tER sites is unknown. Prior studies of the yeast Pichia pastoris revealed that a point mutation in the central conserved domain (CCD) of Sec16 causes thermosensitive growth and dispersed tER sites. Surprisingly, we show here that the CCD is not essential for normal growth or tER organization. This apparent paradox reflects a dual role for the CCD. First, the CCD enhances membrane association of Sec16. The membrane-bound form of Sec16 restraints COP II dynamics, thereby stabilizing tER sites. These functions are disrupted by the point mutation. Second, a beta-propeller blade from the CCD was predicted to bind the COP II protein Sec13, and this interaction was confirmed by crystallography. Binding to Sec13 drives release of Sec16 into the cytosol, thereby relaxing the restraint on COP II dynamics. The combined data imply that the CCD helps Sec16 to regulate the COP II system.
Regulation of Ligand-Independent EGFR Endocytosis by a Phosphatidic Acid-PKA Pathway

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Abstract:

Phosphatidic acid (PA) has been involved in ligand-induced endocytosis of a variety of receptors, including the EGFR. We recently showed that PA generated by phospholipase D increases endocytosis and decreases recycling of empty/inactive EGFR, leading to receptor accumulation in juxtanuclear recycling endosomes (Norambuena et. al Mol Biol Cell-2010). The pathway involves PA-mediated activation of type 4 phosphodiesterases (PDE4) with subsequent down regulation of cAMP/PKA. Here, using siRNA, FRET and a PDE4’s PA binding site we identified PDE4D as a PDE4 isoform controlling this ligand-independent EGFR internalization. We also identified MAPK p38 as a downstream element of the PKA pathway. When PKA activity is down regulated, p38 becomes activated and promotes selective internalization of EGFR without affecting endocytosis or recycling of LDLR. Propranolol used as an inhibitor of PA phosphohydrolase to increase the PA levels also enhances endocytosis of empty/inactive EGFR but not of TfR and LDLR. However, propranolol inhibits recycling of empty/inactive EGFR as well as of TfR and LDLR. The PA/PKA pathway provides new possibilities for trans-modulating the subcellular distribution of EGFR by heterologous stimuli (Financed by Basal Project PFB 12/2007 from CONICYT and FONDECYT grant #1100747).
Caveoli are stable, long-lived plasma-membrane microdomains composed of caveolins, cavins, and a cholesterol-rich lipid membrane. Little is known about how they disassemble, and how the coat components are degraded. We studied the degradation of caveolin-1 (CAV1), a major caveolar coat protein, in CV1 cells. CAV1 turned over very slowly, unless caveolae assembly was compromised, which increased turn-over and changed the cellular distribution of CAV1. Now CAV1 reached the plasma membrane in unassembled form, and remained mobile and diffusely distributed. It also accumulated in late endosomes and lysosomes where it was eventually degraded. Targeting to the degradative pathway involved ubiquitination and inclusion into intra-lumenal vesicles in endosomes. A dual-tag strategy allowed us to monitor exposure of CAV1 to the acidic lumen of individual, maturing late endosomes in living cells. Our findings also led to a revised model of caveolar trafficking because we could show that ‘caveosomes’ most likely correspond to late endosomes and lysosomes in which over-expressed CAV1 accumulated for degradation.
A Fifth Adaptor Protein Complex

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Abstract:

In addition to adaptor protein (AP) complexes there are several other families of proteins that contain µ homology domains (MHDs), including the monomeric stonins, and a family that includes the mammalian proteins FCHO1, FCHO2, and SGIP1. The least characterised of the MHD proteins is encoded in humans by a gene on chromosome 14 (C14orf108). Because so far all MHD proteins have been shown to be involved in membrane traffic, it seemed likely that the same would be true for C14orf108. We have been able to show that that C14orf108 has more in common with the µ-adaptins than with other MHD-containing proteins. C14orf108’s homology to the µ-adaptins extends upstream beyond the MHD, and secondary structure predictions indicate that the µ-adaptins and C14orf108 adopt very similar folds. In addition, C14orf108 binds to a previously uncharacterised protein which is homologous to the β-adaptins and has an almost identical predicted secondary structure. Further evidence for the interaction between C14orf108 and the β-like adaptin comes from the similar distribution of the two genes in eukaryotes from five different supergroups, and from their similar knockdown phenotypes. Our results suggest the possibility of a fifth adaptor complex, and we are trying to determine if ‘AP-5’ like the other AP complexes exists as a heterotetramer of 4 subunits, and whether it associates with clathrin.
125A as part of mammalian COPII machinery in ER export

Wanjin Hong

Institute of Molecular and Cell Biology

Abstract:

Not Provided
CATCHR-Family Tethering Complexes: Structure and Mechanism

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Abstract:

We seek to understand the protein machinery that guides the movement and fusion of intracellular transport vesicles. Several of our current projects entail structural and mechanistic studies of large multi-subunit tethering complexes of the CATCHR (Complex Associated with Tethering Containing Helical Rods) family. CATCHR-family tethering factors (COG, Dsl1, exocyst, and GARP) function in the capture of cargo-laden vesicles and may orchestrate the activities of other components of the trafficking machinery, including SNAREs and coat proteins. We are investigating the structure and function of these complexes using x-ray crystallography, electron microscopy, site-directed mutagenesis, in vitro reconstitution, and a suite of spectroscopic techniques. Our results for the COG and Dsl1 complexes help elucidate their architecture and flexibility, their interaction with SNARE and coat proteins, and the mechanisms through which they regulate SNARE complex assembly and membrane fusion.
The conserved Bardet-Biedl Syndrome proteins assemble a coat that traffics membrane proteins to cilia

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Abstract:

The molecular etiology of Bardet-Biedl Syndrome (BBS), a retinopathy-obesity-nephropathy disorder, has so far remained elusive. Previously, we have identified a stable complex of seven highly conserved BBS proteins, the BBSome, that implicated in vesicular trafficking to the cilium. To further our understanding of the molecular pathway that underlies BBS, we characterized the Arf-like GTPase Arl6/BBS3, a highly conserved BBS protein that is not part of the BBSome. The best characterized Arf-like GTPases, Arf1-GTP and Sar1-GTP, initiate COPI and COPII coat assembly, respectively. Interestingly, our structural analyses predict that the BBSome consists of β-propeller and α-solenoid domains that form the core geometrical elements of COPI, COPII and clathrin coats. Affinity chromatography of retinal extract over Arl6 columns revealed that BBSome is the major effector of Arl6-GTP. In addition, Arl6, like the BBSome, localizes to the primary cilium in mammalian cells, and Arl6-GTP is required to target the BBSome to the ciliary membrane. Moreover, Arl6-GTP is necessary and sufficient to efficiently recruit highly purified BBSome to chemically defined liposomes. Strikingly, recruitment of BBSome to membrane resulted in electron dense coat on the surface of liposome. Finally, the ciliary membrane protein SSTR3 is mislocalized in mouse hippocampal neurons depleted of Arl6. Furthermore, ciliary targeting signal of SSTR3, intracellular loop 3 (i3) domain targeted a non-ciliary membrane protein to cilia in a BBSome dependent manner. Together, our data suggests that the BBSome represents a novel coat complex that mediates vesicular trafficking of ciliary proteins to the primary cilium.
Rab7 localization and activity are regulated by palmitoylation

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Abstract:

Ras-like protein in brain (Rab) GTPases are involved in key events controlling the formation and fusion of transport vesicles. 60 Rab proteins have been identified in the human genome and few have been intensely studied. Mutations in the Rab7 gene have been implicated in Marie-Charcot Tooth syndrome, a neuropathy characterized by a loss of muscle tissue and touch sensation. Rab7 is localized to late endosomes and was recently implicated in the recruitment of retromer, a protein complex that mediates the endosome-to-Golgi trafficking of cationic independent mannose 6-phosphate receptor (CI-MPR). In this work we demonstrate that Rab7 is palmitoylated on cysteines 257 and 259. Immunofluorescence experiments using an RFP tagged Rab7 protein (RFP-Rab7) and palmitoylation deficient mutants (RFP-Rab7C257S, RFP-Rab7C259S and RFP-Rab7C257, 259S) revealed that the palmitoylation of Rab7 is necessary for its correct cellular localization. Membrane isolation assays demonstrate that the palmitoylated c-terminal cysteines are necessary for Rab7 interactions with membranes. Furthermore we identified DHHC-1 and DHHC-8 as palmitoyltransferases that interact with Rab7 and can affect its level of palmitoylation. We show more specifically, using membrane separation, that Rab7 is not recruited to membranes in cells depleted of DHHC-1 or DHHC-8. We also found that retromer was not recruited to membranes in cells depleted of DHHC-1 or DHHC-8, and that CI-MPR was degraded, suggesting that it was retained in endosomes and subsequently degraded rather then recycling back to the Golgi. Our studies suggest a novel role of palmitoylation for the proper localization and function of Rab7, a post-translational modification that may play a role in the localization and function of other Rab proteins.
Inactivation of clathrin heavy chain inhibits synaptic recycling but allows bulk membrane uptake

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Abstract:

Synaptic vesicle cycling depends on clathrin, an abundant protein that polymerizes around newly forming vesicles and dynamin a protein that mediates fission of newly formed vesicles. However, how clathrin is involved in synaptic recycling in vivo remains unresolved. We test clathrin function during synaptic endocytosis using clathrin heavy chain (chc) mutants combined with chc photoinactivation to circumvent early embryonic lethality associated with chc mutations in multicellular organisms. Acute inactivation of chc at stimulated synapses leads to substantial membrane internalization visualized by live dye uptake and electron microscopy. Our data not only indicate that chc is critical for synaptic vesicle recycling but also show that in the absence of the protein, bulk retrieval mediates massive synaptic membrane internalization. Furthermore, inactivation of chc in the context of other endocytic mutations like dap 160, synj as well as shibire ts1 (dynamin mutant) results in membrane uptake. Qualitative similarities between phenotypes of inactivated chc and recently published data on Dynamin 1 KO mice stimulated neurons (Hayashi et al. 2009) may suggest that dynamin and clathrin directly cooperate in vivo. To further scrutinize these interactions in vivo we will employ the FALI technique to inactivate dynamin in shi null mutant flies. Our work will shed light onto the regulatory mechanisms of SV formation and the role of clathrin and dynamin in this process.
The Drosophila Golgi transmembrane protein Ema promotes autophagosomal growth

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Abstract:

Autophagy is a self-degradative process in which cytosolic components are engulfed in double membrane autophagosomes and transported to lysosomes for degradation. Autophagy allows the cell to respond to a wide spectrum of stressful conditions such as starvation, growth factor deprivation, protein aggregation and pathogen invasion. Identification of the highly conserved Atg genes has revealed many of the molecular details of autophagosome formation, however the source of the membrane that is added to autophagosomes as they grow is unclear. Here we demonstrate the Drosophila membrane protein Ema is required for the growth of autophagosomes in the Drosophila fat body. In an ema mutant, autophagosomes still form in response to starvation and developmental cues, and these autophagosomes can still mature into autolysosomes, however these autophagosomes are very small. In fat cells, Ema localizes to peripheral Golgi elements and is recruited to the periphery of autophagosomes in response to starvation. The peripheral golgi protein Lava Lamp also is recruited to the periphery of autophagosomes. The recruitment of these Golgi-derived vesicular elements to the membranes of autophagosomes in response to starvation does not occur in the ema mutant. Therefore, we propose that Golgi membranes contribute to autophagosomal growth and that this process requires the transmembrane protein Ema.
Sec31p as a sole component of the COPII cage: understanding the bst phenotype

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Abstract:

Nascent proteins that enter the secretory pathway via the endoplasmic reticulum (ER) are packaged into vesicles for forward transport. Vesicles are sculpted from the ER membrane by the cytosolic COPII protein coat - the G-protein, Sar1p, Sec23/Sec24p and Sec13/Sec31p - in a process that is highly conserved among eukaryotes. In budding yeast, these COPII proteins are essential for viability. However, in certain mutants, called bypass of Sec thirteen (bst), cells can survive without SEC13 (¹). All known bst mutations affect biogenesis or trafficking of GPI-anchored proteins. We are currently exploring the mechanistic basis of the bst phenotype. One hypothesis is that Sec31p can form the outer coat without Sec13p. In support of this model, structural studies suggest that Sec31p forms many of the critical contacts of the outer coat, including the intermolecular contacts of the Sec13/31p cage and interactions with the inner coat proteins (²,³). We have purified monomeric Sec31p from an insect cell expression system and demonstrate that Sec31p is specifically recruited by Sar1p and Sec23/24p to synthetic liposomes in the absence of Sec13p. Monomeric Sec13p is not recruited to liposomes under similar experimental conditions, whereas a Sec31p mutant that does not bind to Sec13p retains the ability to form a coat complex with Sar1/Sec23/Sec24p. We are currently investigating the structural morphology of vesicles produced by this Sec13p-deficient COPII coat, as well as the functionality of Sec31p in an in vitro budding assay without Sec13p. Defining the mechanistic basis of the bst phenotype contributes to our understanding of the link between the membrane environment and COPII coat function. 1. Elrod-Erickson et al, Mol Biol Cell(1996)7:1043-58. 2. Bi et al. Dev Cell(2007)13:635. 3. Stagg et al. Cell(2008)134:474.
Cbl Amino Acids at a Putative Dimer Interface Regulate EGF Receptor Trafficking

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Abstract:

The ubiquitin ligase c-Cbl controls epidermal growth factor receptor (EGF-R) signaling by enhancing receptor ubiquitination, downregulation, and degradation. Cbl residues 1-434 are the minimal evolutionarily conserved sequences sufficient to regulate EGF-R. They contain a variant SH2 domain, linker region, RING finger (RF), and a subset of the RF tail amino acids 420-436. Data from a solved Cbl/UbcH7 crystal structure suggest that Cbl dimerizes through its RF tail. Our objective was to perform structure/function studies to identify amino acids at the putative Cbl dimer interface that regulate EGF-R fate. Constructs encoding the Cbl RF tail and opposing interface alanine substitution mutants were developed by oligonucleotide-directed mutagenesis. Wild type and mutant proteins were expressed in HEK 293 and COS-7 cells, which were then assayed for EGF-R downregulation, trafficking, and degradation. Trafficking was followed by fluorescence localization of GFP-tagged Cbl proteins, EGF and markers of endocytic compartments in fixed and live cell imaging studies. The integrity of RF tail residues V431 and F434 is essential for Cbl-dependent EGF-R degradation in lysosomes. Full-length Cbl V431A failed to effect the ubiquitin-dependent degradation of hSprouty2 and blocked EGF-R internalization. Full-length Cbl F434A compromised EGF-R degradation by stabilizing phosphorylated Hrs and retarding sorting endosome fusion. Using these mutants with different epitope tags, we determined that Cbl forms dimers and that dimer levels are altered by RF tail amino acid substitutions. The mutation of residues on the opposite side of the putative Cbl dimer interface revealed that only some interface residues are critical for EGF-R regulation. Our results suggest that Cbl dimers can regulate EGFR fate at multiple trafficking checkpoints.
Cytoskeletal “Wheels” for the Rapid Recycling Pathway

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Abstract:

Cell migration and signaling require coordination between the cytoskeleton and membranes. Supervillin is a tightly bound lipid raft-associated membrane protein that regulates cell migration, focal adhesion dynamics, matrix invasion, and cytokinesis. We show here that supervillin also prolongs phosphorylation of extracellular signal-regulated kinases in response to epidermal growth factor activation and associates with markers for early and sorting endosomes and with overexpressed components of the Arf6 recycling pathway in the cell periphery. Supervillin tagged with the photoswitchable fluorescent protein, tdEos, moves both into and away from dynamic structures resembling pre-invadopodial complexes at the basal cell surface. Rapid recycling of beta1- and beta-3 integrins is inhibited in supervillin-knockdown cells, but the rates of integrin endocytosis and recycling from perinuclear recycling endosomes are unchanged. A lack of synergy between supervillin knockdown and the actin filament barbed-end inhibitor, cytochalasin D, suggests that both treatments affect actin-dependent rapid recycling. Supervillin interaction partners include several kinesins, the myosin II heavy chain, and the contractility regulator, HAX1. The working model is that supervillin-associated scaffolds regulate signaling at, and the dynamic reorganization of, cholesterol-rich membranes engaged in rapid recycling. Supported by NIH GM033048 (EJL).
The role of COG subcomplexes in the intra-Golgi vesicular trafficking

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Abstract:

The Conserved Oligomeric Golgi (COG) complex is a ubiquitously expressed membrane-associated protein complex that consists of eight different subunits (Cog1-8). COG functions in the retrograde intra-Golgi trafficking through association with coiled-coil tethers, SNAREs, Rabs and COPI proteins. Yeast genetic studies indicated that the COG complex consists of two functional subcomplexes: LobeA (Cog1-4) and LobeB (Cog5-8), but the exact relationship between these subcomplexes was not known. Quantitative IP experiments and gel-filtration analysis demonstrated that the cytoplasmic COG complex is a stable octamer, while half of the membrane-bound COG complex partitions into at least two sub-complexes. Depletion of the Golgi SNARE Syntaxin5 augments partitioning of the COG complex into the sub-complexes, indicating that Syntaxin5 positively regulates intra-COG complex assembly. Live cell microscopy of HeLa cells stably expressing CFP-Cog6/YFP-Cog3 reveals that Cog6 (Lobe B) is associated with both the Golgi membrane, and small vesicles carrying Golgi enzyme GlcNAcT1. GlcNAcT1 vesicles were also positive for Cog8, and likely correspond to the intra-Golgi trafficking intermediates. In contrast, Cog3(LobeA) was not found on vesicles and was exclusively associated with Golgi membranes. This hints that the transient assembly of COG sub-complexes is the initial step in intra-Golgi vesicle tethering. Relocalization of the siRNA resistant COG complex subunits to the mitochondria, using either mitochondria-specific membrane anchor or a “knocksideways” strategy, was employed to divert COG-specific vesicular trafficking from the secretory pathway and elucidate additional specific features of the COG subcomplexes.

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Exocytosis of large cargo: a lesson from coronaviruses

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Abstract:

Coronaviruses are an interesting model for studying exocytosis of large cargo. These enveloped viruses assemble by budding into the lumen of the ER-Golgi intermediate compartment, and are released by exocytosis. However, the virions (~100 nm) are larger than the typical secretory vesicle. How do cells handle this large cargo? We have evidence that one of the viral envelope proteins, E, modifies the microenvironment of the Golgi complex to enhance release of infectious virus. The E protein helps orchestrate budding and is incorporated into the virus envelope at low levels, but is made in excess infected cells and is thought to have additional functions. E is a small protein with a single hydrophobic domain, and has ion channel activity in vitro when inserted into planar bilayers. To test the role of the potential ion channel function, we constructed a recombinant coronavirus containing E protein with a heterologous hydrophobic domain. The mutant virus assembles normally, but release of infectious virus is severely compromised. Virions accumulate intracellularly in vacuolar structures where partial proteolysis leads to release of non-infectious particles. When overexpressed from cDNA, the coronavirus E protein induces morphological changes in the Golgi complex and alters cargo traffic. There are two critical residues in the hydrophobic domain required for these effects, suggesting that ion channel function may be critical for productive exocytosis of infectious virions. Elucidation of the mechanism by which the E protein promotes the release of infectious virions should help dissect the process by which cells handle other large cargo.
Mechanism of secretory cargo sorting at the TGN

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Abstract:

Knockdown of actin severing protein Cofilin by siRNA and, over expression of inactive Cofilin or Cofilin-inactivating kinase (LIMK), arrested secretion of an exogenously expressed soluble secretory protein in the Trans Golgi Network (TGN) of mammalian cells. A SILAC-mass spectrometry based protein profiling revealed that a large number of endogenous secretory proteins were not secreted under these conditions. Surprisingly, a population of proteins normally retained in the Golgi was secreted. There was a similar defect in the delivery of integral membrane proteins to the cell surface. Overall, these findings indicate defective cargo sorting at the TGN. We suggest that Cofilin dependent actin trimming generates a sorting domain at the TGN, which filters secretory cargo for export; uncontrolled growth of this domain traps proteins not destined for secretion and excludes secretory proteins. I will present new data on the downstream effectors of Cofilin that are required for secretory cargo sorting at the TGN.
The Role of Ceroid Lipofuscinosis Neuronal Protein-5 (CLN5) in Lysosomal Sorting and Trafficking

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Abstract:

The trafficking of proteins to the lysosomes is an important cellular function with significant implications in human health. Three trans-membrane receptors function in the sorting and trafficking of cargo from the Golgi apparatus to lysosomes: the cationic-dependent mannose 6-phosphate receptor (CD-MPR), the cationic-independent mannose 6-phosphate receptor (CI-MPR) and sortilin. For anterograde traffic (Golgi-to-endosome trafficking), cargo binds to the receptors in the Golgi and is packaged into clathrin coated vesicles. When the receptor/cargo complex reaches the more acidic environment of the endosomes, the cargo dissociates from the receptor and the majority of the receptor is recycled back to the Golgi for another round of sorting while a percentage is degraded in lysosomes. Retromer, PACS-1 and TIP47 have been implicated in the efficient retrograde traffic (endosome-to-Golgi) of CI-MPR and sortilin.

Neuronal Ceroid Lipofuscinosis (NCL) constitutes a group of common recessive disorders of childhood. The precise function of most of these proteins is still unknown however most encode for either soluble or transmembrane proteins located in either endosomes/lysosomes or the ER. Mutations in the gene encoding CLN5 are the cause of Finnish variant late infantile NCL. Although CLN5 localizes to the lysosomal compartment, its function is unknown. Our results show that CLN5 interacts with, but is not a cargo of sortilin. We demonstrate that CLN5-depleted cells and cells expressing CLN5Y392* (a common mutation in this disease) have a defect in the recruitment of retromer which results in the degradation of sortilin and CI-MPR. Taken together, our results support a role for CLN5 in controlling the itinerary of the lysosomal sorting receptors by regulating retromer recruitment. Most importantly, we propose a possible molecular mechanism explaining the pathological dysfunction leading to Finnish variant late infantile NCL.
Understanding the mechanism of GEEC (GPI-AP Enriched Endosomal Compartment) formation using single molecule visualization techniques

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Abstract:

The CLIC/GEEC endocytic pathway which is responsible for the internalization of GPI-APs (glycosyl-phosphatidylinositol-anchored protein) and the bulk fluid phase into most cells, is a CDC42 regulated, clathrin and dynamin independent endocytic pathway. A few molecular players involved in the functioning of this pathway have been identified, however, the precise roles of these players in the generation of CLICs and their consumption into GEECs have not been clearly identified. The activity of CDC42 at the membrane is indirectly regulated by activated ARF1 through ARHGAP10. It has been speculated that the recruitment of actin polymerization machinery, which is CDC42 dependent, takes over from the CDC42 action in the development of endocytic vesicles. At the same time at the membrane surface, GPI-APs are complexed in form of dynamic nanoclusters, non-randomly distributed spatially. These nanoclusters are dependent on the underlying actin architecture. But, how these nanoclusters are related to the formation of CLICs/GEECs has not been established. These questions can be addressed if the live formation of GEECs can be spatially and temporally mapped with high resolution using single molecule visualization techniques and studying association of molecular players in this process. We have employed a single molecule visualization strategy coupled with the visualization of the state of GPI-AP nanoclustering and endocytic vesicle formation, for understanding this system. Preliminary results indicate that endosome formation with occurs to higher extent in A7 melanoma cells which are capable of undergoing migration compared to CHO cells. These initial results implicate that membrane dynamics and endocytosis are closely related but the exact relation will require further experiments and study of other molecular players involved in CLIC/GEEC formation.
Molecular analysis of homotypic membrane fusion by the GTPase atlastin

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Abstract:

Homotypic membrane fusion is essential for the formation and maintenance of the endoplasmic reticulum (ER). We have shown that the large GTPase atlastin is important for regular ER formation and maintenance in flies and is able to promote homotypic fusion of liposomes in vitro. Traditional membrane fusion proteins such as viral fusion proteins and SNAREs use energy derived from metastable protein folding intermediates to drive fusion. The use of chemical energy in the form of nucleotide hydrolysis at the point of membrane fusion is unique to atlastin, and perhaps mitofusin, defining a new class of membrane fusion proteins. Detailed analysis of this type of mechanism has yet to be explored. Our current studies aim to determine the mechanism by which Drosophila atlastin drives the merging of the lipid bilayers. To that end, we are determining the enzymatic parameters of atlastin and analyzing how the GTPase reaction mechanism is coupled to protein state changes and membrane fusion. Additionally, we are conducting a structure function analysis of atlastin to determine domains required for membrane fusion. Carboxy-terminal truncations result in an impaired ability to fuse membrane. Mutations designed to disrupt a predicted juxtamembrane coiled-coil domain reduce GTPase activity and prevent the ability of atlastin to fuse liposomes. Furthermore, acute addition of a cytoplasmic domain of atlastin to fusion assays inhibits the fusion of atlastin proteoliposomes while the cytoplasmic domain lacking the predicted coiled-coil domain has no effect. These results indicate that atlastin forms homo-oligomers through a juxtamembrane coiled-coil to promote homotypic membrane fusion.
Mechanisms for selective activation of Arfs within the Golgi complex

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Abstract:

Two classes of guanine nucleotide exchange factors (GEFs) for Arfs regulate recruitment of coat proteins on the Golgi and both are targets of BFA. GBF1 colocalizes with COP1 at the cis-Golgi, while BIGs localize at the trans side of the Golgi and overlap with clathrin and its adaptors. In contrast to this clear specialization of the GEFs, it is widely assumed that Class I and II Arfs function interchangeably throughout the Golgi. I will present recent observations demonstrating that Arf4 and Arf3 display several unexpected properties. For example, in contrast to other Arfs, Arf4 associates in the GDP-bound state selectively with the ER-Golgi Intermediate Compartment. Furthermore, unlike other Golgi-localized Arfs, Arf3 associates selectively with membranes of the trans-Golgi network (TGN) in a manner that is both temperature-sensitive and uniquely dependent on guanine nucleotide exchange factors of the BIGs family. BIGs knockdown redistributed Arf3 but not Arf1 from Golgi membranes. Furthermore, shifting temperature to 20°C, a temperature known to block cargo in the TGN, selectively redistributed Arf3 from Golgi membranes. Arf3 redistribution occurred slowly, suggesting it resulted from a change in membrane composition. Arf3 knockdown and overexpression experiments suggest that redistribution is not responsible for the 20°C block. To investigate in more detail the mechanism for Arf3 recruitment and temperature-dependent release, we characterized several mutant forms of Arf3. This analysis demonstrated that those properties are readily separated and depend on pairs of residues present at opposite ends of the protein. Furthermore, phylogenetic analysis established that all 4 critical residues were absolutely conserved and unique to Arf3. These results suggest that both Arf3 and Arf4 play a unique functions that likely involves recruitment by a specific receptor.
Regulation of SNARE complex assembly by the yeast exocyst complex and Sec1p

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Abstract:

 Trafficking of protein and lipid cargo through the secretory pathway is mediated by membrane-bound vesicles. Secretory and recycling vesicles are targeted to sites of exocytosis on the plasma membrane by a conserved multi-subunit protein complex termed the exocyst, and fuse through the action of SNARE proteins on the vesicle and target membranes. Prior to vesicle fusion, SNAREs are held in uncomplexed, inhibited conformations that must be released at the right time and place. In addition to a putative role in tethering vesicles to the plasma membrane, the exocyst complex may directly regulate assembly of the SNARE complex. Previously, we showed that the yeast exocyst subunit Sec6p directly interacts with the plasma membrane t-SNARE Sec9p, and that residues in the N-terminal domain of Sec6p are critical for this interaction. We also determined the crystal structure of the C-terminal domain of Sec6p, which is comprised of helical bundles that are structurally similar to other tethering complexes such as COG, Dsl1 and GARP. Patches of conserved surface residues on the C-terminal domain mediate exocyst anchoring at sites of secretion. Moreover, we recently discovered that Sec6p binds to the SNARE regulatory protein Sec1p, indicating functional interplay between Sec6p and Sec1p in the regulation of SNARE complex assembly and membrane fusion.
A novel coat complex traffics membrane proteins to the primary cilium

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Abstract:

The BBSome is a complex of Bardet-Biedl Syndrome (BBS) proteins that shares common structural elements with COPI, COPII, and clathrin coats. Here, we show that the BBSome constitutes a coat complex that sorts membrane proteins to primary cilia. The BBSome is the major effector of the Arf-like GTPase Arl6/BBS3, and the BBSome and GTP-bound Arl6 colocalize at ciliary punctae in an interdependent manner. Strikingly, Arl6•GTP-mediated recruitment of the BBSome to synthetic liposomes produces distinct patches of polymerized coat apposed onto the lipid bilayer. Finally, the ciliary targeting signal of somatostatin receptor 3 needs to be directly recognized by the BBSome in order to mediate targeting of membrane proteins to cilia. Thus, we propose that trafficking of BBSome cargoes to cilia entails the coupling of BBSome coat polymerization to the recognition of sorting signals by the BBSome.
ER structure regulates the organization of COPII assembly sites and thus the biogenesis of the Golgi apparatus

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Abstract:

In yeast, plant and lower animal cells, the entry of cargo into the Golgi apparatus occurs via direct transport from the ER, because they lack the ER-to-Golgi intermediate compartment. The site of cargo delivery, the ER exit site (ERES), has been defined as the special site within the continuity of the ER, where COPII coat (Sec23/24 and Sec13/31) and Sec16 accumulate. We have been investigating the dynamic properties of yeast ERES by high-speed and high-resolution live imaging. We have recently found that some mutations that affect the ER structure cause remarkable aberration of the organization of ERES and accordingly the formation of the Golgi compartments. These findings provide new insights into how the ER-to-Golgi system is organized.
Membrane trafficking in cytokinesis: a role for the Exocyst

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Abstract:

It has been previously recognised that the exocyst complex localises to the midbody and that some of the subunits are required for cytokinesis. However, the role of each individual exocyst subunit during cytokinesis and the mechanisms by which they are delivered to the midbody have not been fully explored. We show that all the eight exocyst subunits localise to the midbody ring at the final stages of cytokinesis, however the different exocyst components reach the abscission site at different stages of the cell cycle possibly via distinct trafficking pathways. The exocyst subunits sec5 and Exo84, but none of the others, accumulate at the midzone from the beginning of furrow formation and co-localise with Rab35 vesicles. On the other hand, sec3 and Exo70 co-localise with Rab11 labelled vesicles and localize to the midbody-ring only in late cytokinesis. Furthermore, looking into the role of SNARE and Sec1/Munc proteins in cytokinesis, we found that Vps45 and its SNARE interactor – syntaxin16 are not only required for cytokinesis but also for the localisation of the exocyst at the midbody-ring. A direct interaction between SNARE proteins and the exocyst has not been reported. Nonetheless, the role of tethers in the recruitment of SNAREs is becoming more a coherent picture. Tethers, such as COG and Dsl1, which share significant structural similarities with the exocyst, recruit SNARES and interact with vesicle coat proteins bringing close membranes together for final fusion. The results reported here represent a valuable contribution to a better understanding of how various protein families such as: Rab-GTPases, tethers and SNAREs coordinate functions during cytokinesis.
Isoform specific properties of Dynamin 2

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Abstract:

Dynamin is best studied for its role in clathrin-mediated endocytosis (CME) and most of the studies have employed the neuronal isoform, dynamin-1 (Dyn1). However, there is a second major, ubiquitously expressed isoform Dyn2 in mammals. Contrary to previous assumptions, findings in knockout mice and cell lines have suggested that Dyn1 and 2 are not functionally redundant for CME either at the synapse (Ferguson et al., 2007) or in non-neuronal cells (Liu et al., 2008). We have performed comparative analyses of dynamin’s in vitro activities to define the biochemical differences that account for these differential in vivo activities. Using an in vitro fission assay (Pucadyil et al., 2008) we found that Dyn1 could efficiently catalyze membrane fission and vesicle formation from planar membranes, while Dyn2 could not. However if applied to membrane tethers, which resemble highly curved membranes formed at the necks of clathrin coated pits, both isoforms were capable of scission. While basal GTPase activities were identical, liposome stimulated GTPase activities were distinct. Dyn2 GTPase activity was only stimulated in the presence of highly curved membranes, whereas the stimulated GTPase activity of Dyn1 was less sensitive to membrane curvature. These findings suggest that Dyn2 is less effective in membrane binding and/or curvature generation than Dyn1, which is a powerful curvature generator (Ramachandran et al., 2009). This was confirmed by direct measurements of these activities. Using Dyn1/Dyn2 chimeras, we identified the PH domain as being responsible for the differential biochemical properties of the two isoforms. The relative abilities of these chimeras to reconstitute CME are currently being tested in non-neuronal cells. We speculate that these biochemical differences reflect the differential requirements for Dyn1 in mediating rapid endocytosis at the synapse vs. the role of Dyn2 in regulating slower, clathrin-mediated endocytic events in non-neuronal cells.
Phosphatidylinositol 4-phosphate controls both membrane recruitment and a regulatory switch of the Rab GEF, Sec2p

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Abstract:

Sec2p is the guanine nucleotide exchange factor (GEF) that activates the Rab GTPase Sec4p on secretory vesicles. Sec2p also binds a Rab acting earlier in the secretory pathway, Ypt32p-GTP, forming a RabGEF cascade. Ypt32p and the Sec4p effector Sec15p (a component of the exocyst complex) compete for binding to Sec2p. Indeed Ypt32p initially recruits Sec2p, but subsequently allows a handoff of active Sec2p/Sec4p to Sec15p. Intriguingly, Golgi-associated phosphatidylinositol 4-phosphate (PI4P) works together with Ypt32-GTP in this context. PI4P inhibits Sec2p-Sec15p interactions, promoting recruitment of Sec2p by Ypt32p as secretory vesicles form. However, PI4P levels appear to decline as vesicles reach secretory sites, allowing Sec15p to replace Ypt32p as vesicles mature. In this way, the regulation of PI4P levels may switch Sec2p/Sec4p function during vesicle maturation, from a RabGEF recruitment cascade involving Ypt32p, to an effector positive feedback loop involving Sec15p.
SNARE motif: a common motif used by pathogens to manipulate membrane fusion

Fabienne Paumet¹, Jordan Wesolowski¹.

Thomas Jefferson University¹

Abstract:

Although pathogens use a variety of molecular components to penetrate host cells through their membranes, the presence of heptad repeat motifs seems to be a common element. Heptad repeats are characterized by a pattern of seven, generally hydrophobic residues. To initiate membrane fusion, viruses for instance use glycoproteins-containing heptad repeats that are structurally and functionally similar to the SNARE proteins known to be involved in eukaryotic membrane fusion and also in possession of a heptad repeat motif. As bacterial genomes are being sequenced, more and more microorganisms appear to be carrying membrane proteins resembling eukaryotic SNAREs. This category of SNARE-like proteins might therefore share similar functions and could be used by microorganisms either to promote or block membrane fusion. Such a recurrence across pathogenic organisms suggests that this architectural motif was evolutionarily selected because it provides the best solution to ensure the survival of pathogens within the particular eukaryotic environment.
Control of Golgi function by Rab GTPases and Golgin proteins

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Abstract:

The ~70 Rabs encoded by the human genome are localized to distinct membrane compartments where they organize function-specifying, membrane microdomains. Rab microdomains organize the SNARE proteins and tethering proteins required for vesicle docking and fusion. The Golgi complex is decorated with long, coiled-coil proteins that all possess multiple Rab binding sites. At the TGN, so-called GRIP domain proteins function in maintaining Golgi structure and in receipt of transport vesicles from the endocytic pathway. The GCC185 GRIP domain Golgin is required for the receipt of mannose 6-phosphate receptor-containing transport vesicles (1). This Golgin has at least five binding sites that can interact with as many as 14 different Rabs across its length (2). Only the C-terminal binding sites for Rab6 and Arl1 GTPases are sufficient to localize GCC185 to the Golgi. We have generated a series of deletions to map the most important interaction domains on GCC185 and have defined two distinct regions that are key for either Golgi structure maintenance or receipt of mannose 6-phosphate receptor-containing transport vesicles. This Golgin appears to interact with another TGN protein needed for Golgi structure and transport vesicle receipt, RhoBTB3 (3). The importance of all of the Golgins for Golgi structure maintenance, and the existence of so-called Rab GTPase cascades (4) has inspired the generation of a new model for how the Golgi is formed and how proteins pass through it. 1. Reddy, J., et al. (2006) Mol. Biol. Cell 17, 4353-63; 2. Hayes, G.L., et al. (2009) Mol. Biol. Cell 20, 209-217; 3. Espinosa, E., Calero, M., Kambhampaty, S. and Pfeffer, S.R. (2009) Cell 137, 938-948; 4. Rivera-Molina, F.E., and Novick, P.J. (2009) Proc. Natl. Acad. Sci. U.S.A. (2009) 106, 14408-13.
Protein targeting to the silica deposition vesicle in diatoms

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Abstract:

Diatoms are unicellular eukaryotic microalgae that possess intricately nanopatterned cell walls made of SiO2 (silica). The structure and patterns of diatom biosilica are under genetic control and thus represent a particularly fascinating example of biological morphogenesis. Diatom silica formation takes place within a specialized intracellular organelle termed the silica deposition vesicle (SDV), whose connection to other intracellular organelles is unclear. Morphogenesis of the species-specific silica nanopatterns is thought to be controlled by an organic matrix in the lumen of SDV, which includes a unique family of proteins, termed silaffins. Silaffins are derived from precursor proteins that contain N-terminal signal peptides for co-translational import into the ER, and become extensively modified (phosphorylation, glycosylation, sulfation, hydroxylation, alkylation) before they reach the SDV and become entrapped inside the silica. To gain insight into the mechanism of intracellular protein transport to the SDV, truncated versions of silaffin tpSil3 were fused to GFP and their ability for SDV targeting in the diatom Thalassiosira pseudonana was analyzed. Surprisingly, several independent sequence regions of tpSil3 were able to target GFP to the SDV. The shortest sequence region with SDV targeting capability was comprised of 19 amino acid residues containing five serine and five lysine residues. Analysis of site-specific mutations to this 19-mer peptide suggests that post-translational modifications of both serine and lysine residues as well as the clustering of lysine residues are required for efficient SDV targeting. Furthermore, the 19-mer peptide contains a characteristic sequence motif that is present in all other silaffins. We speculate that this sequence motif may represent the recognition site for a receptor protein that mediates the intracellular targeting of silaffins to the SDV.
Adenosine mediated modulation of membrane trafficking is through transactivation of EGF receptors

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Abstract:

Adenosine is a naturally occurring nucleoside, which has gained importance in recent times as a signaling molecule, especially in pathologic conditions like ischemia, hypoxia and cardio-renal diseases. Adenosine binds specific G-Protein coupled receptors (GPCRs) A₁, A₂a, A₂b and A₃. But the precise downstream signaling involved in adenosine mediated modulation of membrane trafficking and cellular activity is not well understood. Using the urinary bladder uroepithelium as a model system, we have shown that adenosine causes exocytosis of discoidal/fusiform vesicles on the apical surface of umbrella cells. Furthermore, my studies show that this effect of adenosine on the umbrella cells is due to transactivation of Epidermal Growth Factor receptors (EGFR). By using specific pharmacologic agents and inhibitors I have shown that adenosine binds to A₁ adenosine receptors present on the apical surface of the umbrella cells and triggers Gi proteins. This in-turn activates membrane bound metalloproteases (ADAMs), which cause cleavage of the membrane bound EGFR ligand HB-EGF. The cleaved HB-EGF binds to EGFRs and drive downstream signaling that results in exocytosis of discoidal/fusiform vesicles. Adenosine mediated EGFR-transactivation could be a common pathway through which adenosine mediates its effect in other tissues. Hence, understanding this cellular pathway will be helpful in designing therapeutics to combat cardio-renal disorders and other diseases where adenosine-mediated signaling is involved.
Retromer-dependent STxB and CIM6PR Trafficking Passes Through the Recycling Endosome

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Abstract:

The retrograde pathway delivers membrane traffic from the plasma membrane and endosomes to the Golgi apparatus. Endogenous proteins such as the cation-independent mannose 6-phosphate receptor (CIM6PR) and exogenous cargo such as Shiga toxin B (STxB) use this pathway. Both of these examples are dependent upon the retromer complex, a cytosolic heteropentamer, to navigate the retrograde pathway. It is well established that retromer-dependent retrograde traffic of CIM6PR passes through both early endosomes (EE) and the trans-Golgi network (TGN), but the route between these organelles is far from clear. This may, in part, be due to the use of HeLa cells, where EE are not readily distinguished from recycling endosomes (RE). Here, we examine whether retromer-dependent retrograde traffic passes through the RE or directly from EE to TGN. Pulse chase experiments suggested that STxB traffics from EE to RE to TGN. Selective HRP ablation of the RE suggested that passage through the RE was required to reach the TGN. Further, using a novel drug-induced inactivation of VAMP-3 or a dominant negative EHD-1 (DN EHD-1) mutant to block exit from the RE, we observed STxB in the RE but not reaching the TGN after passage through the EE. We then used a CD8-CIM6PR (tail) chimera to follow CIM6PR trafficking from the plasma membrane to the TGN. As with STxB (but in contrast to furin), this protein also passed through the RE. Both ablation of the RE and a DN EHD-1 prevented the CIM6PR from reaching the TGN. Additionally, CIM6PR and STxB in BSC1 cells with ablated RE co-localized to a nondescript vesicle adjacent to lysosomes and possessing EEA-1, but not Rab11a, suggesting this structure is EE-like. Knockdown of either EHD-1 or the retromer component VPS26 resulted in a delay of trafficking of both STxB and CIM6PR to the TGN, and notably, of both in the RE rather than EE. We conclude that retromer-based retrograde traffic from the plasma membrane follows an EE to RE to TGN pathway.
Syp1 regulation of actin polymerization and cargo recognition in Clathrin Mediated Endocytosis

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Johns Hopkins University\(^1\)

Abstract:

Endocytosis is a dynamic process that is critical for maintaining membrane composition, regulating transmembrane cargo turnover and nutrient uptake, and controlling signaling networks. The muniscin family of adaptors is conserved from yeast to humans, with domains that contribute to cargo selection, membrane tubulation/constriction and actin polymerization; these activities are coordinated to form an endocytic vesicle. We are currently defining the mechanisms by which each domain functions individually, and cooperatively, to mediate endocytosis. Syp1 is one of the earliest proteins recruited to a nascent endocytic site; interestingly, while Syp1 lacks a classical ‘clathrin box’ motif, its localization to the membrane is partially dependent upon clathrin. We have found that the C-terminal \(\mu\)-homology domain that binds cargo and Ede1 also binds clathrin. We are exploring the role for clathrin in recruiting or stabilizing Syp1 at the membrane, and possible cooperativity with cargo-recognition. We identified the transmembrane protein Mid2 as a cargo selected by Syp1, and are now defining endocytic sorting signals in the Mid2 cytoplasmic tail. Using phage display and recombinant protein binding, we are searching for other Syp1 cargos as well as the signal for Syp1 cargo recognition. These approaches will also better define the mode of interaction between Syp1 and its partner Ede1 (yeast Eps15), and reveal if the interactions of Syp1 with cargo and Ede1 are independent or competitive. We are also examining the role of the membrane-binding proline-rich domain of Syp1 in regulating Las17 (yeast WASp) activity and Syp1 membrane tubulation. We have made a proline-rich domain mutant that ameliorates binding to membranes and are testing it for effects on actin polymerization and endocytic internalization. These studies will reveal insights into the temporal coordination of the endocytic process.
AnnexinA6-mediated disintegration of SNAP23 and syntaxin-4 in CHO cells

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Abstract:

We recently demonstrated that high levels of annexin A6 (AnxA6) are coupled with an imbalance of intracellular cholesterol, sequestration of caveolin-1 in Golgi and the inhibition of cytosolic phospholipase A2. Since alterations of arachidonic acid and cholesterol are crucial for SNARE complex formation and functioning, a subset of t-SNAREs associated with cholesterol and caveolin-1 transport were investigated. The comparison of immunofluorescence patterns and the subcellular distribution of syntaxin-4 and SNAP23 in CHOwt and in overexpressing AnxA6 cells (CHOanx6) showed a significant disintegration of these SNAREs at the plasma membrane of CHOanx6 cells. We also examined syntaxin-6 and showed that overexpression of AnxA6 induces a translocation of syntaxin-6, from the TGN into punctate cytosolic structures and recycling endosomes. Finally, in order to find out whether the overexpression of AnxA6 perturbed the capability to form SNARE complexes, we analyzed the SNARE complex formation ability of CHOanx6 cells. Immunoprecipitation of SNAP23 and syntaxin-4 showed significant increased co-immunoprecipitation of VAMP3 in CHOanx6 cells. In conclusion, AnxA6 induces a specific disintegration of t-SNAREs, disturbing the onward vesicular trafficking of caveolin-1.
**Rap1A: A Novel Interactor Involved in CFTR Traffic (and in Chloride Secretion in wt-CFTR CFBE Monolayers)**

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Abstract:

Impairment of the CFTR Cl- channel causes cystic fibrosis, a fatal genetic disease. Regulation of CFTR traffic and activity involves molecular chaperones, Rab GTPases, PDZ-domain-containing proteins and protein kinases/phosphatases [1]. Previously, affinity chromatography with CFTR-NBD1 was done to capture interacting proteins from epithelial respiratory cell extracts [2], being Rap1A, a paralog of the small GTPase Rap1 [3], thus identified. Our aim here was to functionally validate this interaction. Results obtained confirm that CFTR and Rap1A co-immunoprecipitate in wt-CFTR CFBE cells. Also, transient downregulation of Rap1A reduces CFTR maturation (band C) by 33% while transient Rap1A overexpression augments band C by 57%. Using a pull-down assay, we observe that downregulation of Rap1A caused a 67.5% reduction of GTP-bound active Rap1A, suggesting that CFTR maturation depends on active Rap1A. To gain functional insight into this interaction, we assessed CFTR-mediated chloride transport by Ussing chamber in wt-CFTR CFBE monolayers, under overexpression or downregulation of Rap1A. Forskolin alone and adenosine chloride-mediated currents were not affected by Rap1A overexpression or downregulation. However Rap1A silencing was responsible for an 87% reduction of ATP-sensitive ISC in the presence of forskolin. ATP peak currents were completely abolished in the presence of the Ca2+-activated chloride channel (CaCC) specific inhibitor A01 30 μM, suggesting that Rap1A is important for the function of the recently identified CaCC, TMEM16A [4]. Although not completely understood, these data suggest that Rap1A regulates both CFTR traffic and CaCC function (possibly TMEM16A) in airway epithelial cells. Work supported by TargetScreen2-FP6-2005-LH-7-037365 and FCT PhD fellowships (FMR and MS). [1] Amaral MD (2005) Pediatr. Pulmonol., 39:479-491. [2] Faria et al, submitted [3] Kawasaki H et al (1998) Science. 282:2275-9. [4] Caputo A et al (2008) Science 322:590-594
Mechanism of membrane fusion and the release of hormones and transmitters

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Abstract:

Intracellular membrane fusion is catalyzed by the zipper ing of SNARE proteins into helical bundles (termed trans-SNARE complexes, or SNAREpins) between membranes, forcing their bilayers together. In synaptic transmission, fusion does not occur until calcium enters the pre-synaptic cytoplasm. Recent studies shed light both on the biophysics of fusion and on how it is regulated for precise release of neurotransmitters and hormones.
Ctage5 is involved in the endoplasmic reticulum to Golgi trafficking in mammalian cells

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Abstract:

Secretory proteins are synthesized in the endoplasmic reticulum (ER) and transported to the ER-Golgi intermediate compartment (ERGIC) via COPII-coated vesicles. Although the minimal components required for the coated-vesicle formation are relatively well characterized, less is known about how the cargoes are selected into nascent vesicles. Recently we identified TANGO1, an integral membrane protein localized on the ER exit sites. Although TANGO1 is not required for general protein secretion, it is specifically required for collagen exit from the ER. Thus, TANGO1 is a new type of cargo receptor conserved throughout metazoans. cTAGE5, cutaneous T-cell lymphoma-associated antigen, has been shown to be overexpressed in several cancer tissues including T-cell lymphoma, meningioma and melanoma, and regarded as a tumor antigen candidate. However, the physiological characterization of the protein has not been reported so far. Here we show that endogenous cTAGE5 is specifically localized to the ER exit sites. Knockdown of cTAGE5 by RNA interference disrupts ERGIC and Golgi structure, although it does not affect the Vesicular Stomatitis Virus Glycoprotein-ts045 traffic to the plasma membrane. Northern blot analysis revealed that cTAGE5 is ubiquitously expressed in normal human tissues. cTAGE5 contains a signal anchor motif, single trans-membrane domain, two coiled-coil regions and Proline Rich domain from the N-terminus; structurally resemble to the C-terminal half of TANGO1. Interestingly, knockdown of cTAGE5 results in collagen VII accumulation in the ER. Moreover, cTAGE5 can co-immunoprecipitate with TANGO1. Functional relationship between cTAGE5 and TANGO1 will be discussed.
Glycolipid flip-flop across the ER during protein N-glycosylation

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Abstract:

N-glycosylation is an essential modification for most proteins entering the secretory pathway. The topological split in assembly of the core glycolipid donor (dolichol-PP-GlcNAc2Man9Glc3) required for N-glycosylation suggests that a key intermediate is translocated from the cytoplasmic to the lumenal face of the ER during this process. In addition lipid flip-flop is an essential step in transport of mannosyl phosphoryl dolichol (MPD), a glycolipid donor utilized in the ER lumen for a number of biosynthetic pathways. We reconstituted ATP-independent, protein-mediated, rapid transport of both the dolichol-linked oligosaccharide intermediate, dol-PP-GlcNAc2Man5 (M5-DLO) as well as MPD, in proteoliposomes generated from a Triton X-100 extract (TE) of rat-liver microsomes. The lectin Concanavalin A was used to probe the transbilayer distribution of M5-DLO in intact unilamellar vesicles, while MPD flipping was monitored through chemical oxidation of a full-length radiolabeled version of MPD. We resolved the ER resident M5-DLO and MPD flippase activities using traditional chromatographic separations and fractionated each to ~20-fold enrichment. Using an activity enriched fraction we showed that the M5-DLO flippase displays remarkable specificity in translocating intermediates of the N-glycosylation pathway. Specificity was defined by reconstituting lipid intermediates with varying glycan headgroups, including a structural isomer of M5-DLO (iM5-DLO). Kinetic analyses indicated that DLOs larger than M5-DLO are poorly translocated by the flippase; in addition, its structural isomer iM5-DLO was also transported ~100 fold more slowly, suggesting that they are all suboptimal flippase substrates. Similarly, MPD flippase activity displayed a preference for the physiological substrate (β-MPD) over a non-natural substrate (α-MPD). Our results suggest a simple structural correlation for the interaction between ER flippases and their respective substrates.
A vesicle carrier that mediates peroxisomal membrane protein traffic from the endoplasmic reticulum

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Abstract:

The cellular origin of peroxisomal membrane remains a controversial topic. Some have argued that peroxisomes are autonomous and grow and divide without a membrane contribution from other organelles. An alternative view is supported by recent evidence pointing to an ER origin of at least a subset of peroxisomal membrane proteins. The mechanism of protein transport to the peroxisome via the ER has not been investigated. We have explored the role of Pex19p, a soluble cytoplasmic transport protein, which is required for the traffic of Pex3p and Pex15p from the ER to the peroxisome. We examined Pex15p traffic from the ER using a chimeric protein containing a C-terminal glycosylation acceptor peptide. Pex15G expressed in wild-type yeast cells is N-glycosylated and functions properly in the peroxisome. In contrast, pex19 mutant cells accumulate the Pex15G glycoprotein in the ER. We developed a cell-free pre-peroxisomal vesicle budding reaction in which Pex15Gp and Pex3p are packaged into small vesicles in the presence of cytosol, Pex19p and ATP. COPII-vesicle budding detected by the packaging of a secretory SNARE protein occurs in the same incubation but does not depend on Pex19p. Conversely, a dominant mutant Sar1p which inhibits COPII has no effect on Pex3p packaging. Pex15Gp and Pex3p budded vesicles sediment as low buoyant density membranes on a Nycodenz gradient and co-purify by affinity isolation using native but not Triton X-100-treated budded vesicles. ER-peroxisome transport vesicles appear to rely on a novel budding mechanism that requires Pex19p and additional unknown cytosolic factors.
Ang2/Fat-free is a Conserved Subunit of the Golgi-associated Retrograde Protein (GARP) Complex

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Abstract:

The Golgi-associated Retrograde Protein (GARP) complex mediates tethering and fusion of endosome-derived transport carriers to the trans-Golgi network (TGN). In the yeast Saccharomyces cerevisiae, GARP comprises four subunits named Vps51p, Vps52p, Vps53p and Vps54p. Bioinformatics and biochemical analyses have shown that other eukaryotes have orthologs of these subunits, except for Vps51p. A yeast two-hybrid screen of a human cDNA library identified a phylogenetically conserved protein, Ang2/Fat-free, which interacts with Vps52, Vps53 and Vps54. Human Ang2 is larger than yeast Vps51p, but exhibits significant homology in the N-terminal coiled-coil region that mediates assembly with other GARP subunits. Biochemical analyses show that human Ang2, Vps52, Vps53 and Vps54 form an obligatory 1:1:1:1 complex that strongly interacts with the regulatory Habc domain of the TGN SNARE Syntaxin 6. Depletion of Ang2 or the GARP subunits similarly affects protein retrieval to the TGN, lysosomal enzyme sorting and autophagy. These findings indicate that Ang2 is an integral component of the GARP complex in most eukaryotes.
Structural Insights into Dynamin-Catalyzed Membrane Fission

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Abstract:

Dynamin is a large, atypical GTPase that self-assembles to form rings and stacks of rings and catalyzes membrane fission in the late stages of clathrin-mediated endocytosis. We have recently reconstituted dynamin-catalyzed membrane fission from Supported Bilayers with Excess Reservoir, SUPER templates, formed on silica beads and showed that fission occurs after cycles of assembly and disassembly driven by GTP hydrolysis. Only GTPase restricted, self-limited assemblies of dynamin can catalyze fission: long dynamin spirals pre-assembled in the absence of GTP are inactive. Previous genetic and biochemical studies had implicated the C-terminus of the GTPase effector domain (GED) in regulating dynamin’s GTPase activity, thus we engineered a minimal GTPase-GED fusion protein (GG) that reconstitutes a robust GTPase activity comparable to full-length dynamin and used x-ray crystallography to solve the structure of GG in the presence of the transition-state analog GDP.AlF₄⁻ at 2.0Å resolution. This structure reveals the first high-resolution view of dynamin’s GTPase domain in an activated conformation and suggests a novel mechanism for dynamin stimulation that requires the dimerization of this domain. The structure also provides key insights into the coupling of GTP hydrolysis with dynamin disassembly and thus, into the mechanisms governing dynamin-catalyzed membrane fission.
Characterization of the endocytic pathway of the cytokine MIF and its receptors

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Abstract:

Macrophage migration inhibitory factor (MIF) is a pleiotropic cytokine that plays a role in innate and adaptive immunity. Depending on the cellular context and disease state, MIF signaling is mediated by its receptors CXCR2, CXCR4 and/or CD74. Although it is known that MIF is endocytosed, the exact mechanism has remained unknown. In exploring the mechanism of MIF endocytosis with pathway-specific inhibitors and receptor overexpression and blockade approaches, we identified a clathrin endocytosis pathway as the main track for MIF internalization. Monodansylcadaverine (MDC) and chlorpromazine (CPZ) were used to interfere with this pathway. The role of the GTPase dynamin was analyzed in experiments with a cell-permeable inhibitor of dynamin, Dynasore, and with a dominant-negative dynamin construct. In the presence of MDC, fibroblasts and HEK293 cells showed an impaired endocytosis rate of Alexa546-MIF. Dynasore led to the largest margin in reduction. LDL endocytosis (which is clathrin-mediated) served as a control and was inhibited by MDC or Dynasore to a similar extent as MIF. Next, MIF endocytosis was compared to that of transferrin, acetylated LDL, and cholera toxin B (the latter internalized by a clathrin-independent pathway) by colocalization studies. MIF internalization clearly resembled that of LDL. In an attempt to identify the receptors involved in MIF endocytosis, we first focussed on CD74 and CXCR4 which form a heteromeric complex. Uptake of MIF was analyzed in HEK293 and HeLa cells which don’t express CD74. Ectopic overexpression of CD74 led to an acceleration of MIF endocytosis while blockade of CXCR4, which is endogenously expressed on these cells, with the inhibitor AMD3100 led to a 20% reduction of MIF endocytosis in HEK293-CD74 transfectants, whereas in untransfected cells, which only express CXCR4, a blockade of 40% was observed. This indicates that both CD74 and CXCR4 contribute to MIF endocytosis.
Identification and interaction mapping of three novel TRAPP components suggests a distinct organization of the mammalian TRAPP complex

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Abstract:

The multisubunit tethering complex TRAPP is conserved throughout Eukarya. Saccharomyces cerevisiae TRAPP is organized in discrete subassemblies, TRAPPI and –II, implicated in ER-to-Golgi and late Golgi trafficking, respectively. No such subassemblies have yet been reported in mammalian cells, and little is known about the architecture of mammalian TRAPP. We recently reported a novel TRAPPII-associated protein, YEL048c/TCA17, and its mammalian orthologue TrappC2L (C2L). YEL048c-p/Tca17p and C2L are ancestrally related to Trs20p and TrappC2 (C2). Given that Trs20p is found in both TRAPPI and –II, whereas YEL048c-p/Tca17p appears to be a TRAPPII component, we hypothesized that their mammalian orthologues C2 and C2L might precipitate distinct complexes. Contrary to our hypothesis, we found that TAP-C2 and –C2L complexes appeared indistinguishable by SDS-PAGE and mass spectrometry. Further, both TAP-C2 and –C2L precipitated several novel proteins which we here report as TrappC8, TrappC11, and TrappC12. TrappC8 is the mammalian orthologue of the yeast TRAPP protein Trs85p, whereas TrappC11 and –C12 have no orthologues in S. cerevisiae. RNA interference of any of the novel TRAPP proteins results in Golgi fragmentation, indicating that they are indeed involved in membrane trafficking. Depletion of C11 or C12 resulted in accumulation of VSV-G ts045-GFP in punctae colabeling with ERGIC53, but apposed to Sec31a, suggesting that exit from the ER was not impeded. In order to better understand the organization of mammalian TRAPP, we have mapped all binary interactions between the known and novel TRAPP proteins, and have performed single particle electron microscopy on TAP-C11 complexes precipitated from mammalian cells. What emerges is a portrait of mammalian TRAPP where the fundamental unit is larger than S. cerevisiae TRAPPI, and where functional diversification may be achieved by exchange of individual subunits rather than assembly of discrete subcomplexes.
Ypt/Rab GTPases and Traffic Coordination

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Abstract:

Transport of proteins and membranes between cellular compartments is mediated by vesicles that bud from one compartment and fuse with the next. Ypt/Rab GTPases and their effectors mediate all the known aspects of vesicular transport, from vesicle formation and motility, to their targeting and fusion. An attractive idea is that Ypt/Rabs not only regulate individual transport steps, but also integrate them into whole pathways and coordinate them with other cellular processes. In yeast, three Ypts regulate the different steps of the exocytic pathway: Ypt1 is required for entry to the Golgi, the Ypt31/32 functional pair is necessary for exit from the Golgi and Sec4 controls fusion of trans-Golgi vesicles with the plasma membrane. We have uncovered three types of Ypt-mediated coordination mechanisms. First, we found that Ypt31/32 and Sec4 interact directly in a nucleotide-specific manner. This interaction is important for the localization of Sec4 to trans-Golgi vesicles, thereby coupling Ypt31/32-mediated vesicle formation and motility with Sec4-mediated tethering and fusion of these vesicles. Second, we discovered that sequential activation of Ypt1 and Ypt31/32 by the modular complex TRAPP coordinates Golgi entry and exit, thus integrating these two transport steps into a whole pathway. Third, we identified novel effectors of Ypt1 and Ypt31/32 that serve to coordinate the exocytic pathway with other cellular process like autophagy and ubiquitination. In summary, we propose direct GTPase interactions, sequential activation and interaction with novel effectors as mechanisms by which the highly conserved Ypt/Rab GTPases coordinate trafficking inside cells.
hRME-6, a rab5GEF that integrates endocytosis and signalling

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University of Sheffield¹

Abstract:

Rab5 is a major regulator of the early endocytic pathway, modulating transport vesicle formation, endosomal fusion and motility, and signalling. A key question is how rab5 is spatially and temporally regulated on the endocytic pathway. Emerging evidence suggests that rab5 guanine nucleotide exchange factors (GEFs) are key to the establishment of functional pools of rab5 and our lab has demonstrated how the plasma membrane rab5GEF, hRME-6, establishes a functional pool of rab5 that regulates uncoating of AP2 from clathrin-coated vesicles. An emerging paradigm in intracellular signalling is that, contrary to what was previously thought, signalling receptors can signal throughout the endocytic pathway and, importantly, signalling output may be different depending on the localisation of the signalling molecule. This suggests a tight coupling between endocytosis and signalling. In addition to its Vps9 domain that is essential for GEF activity, hRME-6 has a rasGAP domain at its N-terminus suggesting that it is a good candidate to integrate endocytic trafficking with intracellular signalling. We have explored this possibility by analysis of trafficking and signalling of the Tie2 receptor tyrosine kinase in endothelial cells. Our results indicate that hRME-6 can specifically modulate Tie2 signalling and support a model whereby hRME-6 can establish a Tie2 ‘signalosome’.
Deregulation of ceramide chain-length composition affects trafficking and Golgi morphology

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Abstract:

Ceramides are building blocks of the biological membranes and precursors of complex sphingolipids. Ceramides differ in the chain-length of their fatty acid moiety, which influences their biophysical properties but the significance of that for cellular homeostasis is largely unknown. In mammals, CoA dependent ceramide synthesis is catalyzed by a family of six ER localized isoenzymes with different specificity towards the fatty acid CoA substrate. In our recent work we found that down-regulation of CerS2 (very long-chain (VLC) fatty acid (C22, C24) specificity) resulted in shift in the fatty acid chain-length makeup of cellular ceramides. Down-regulation of VLC ceramide synthesis led to over compensatory production of shorter chain-length (C14, C16) ceramides and resulted in pleiotropic cellular effects: ER stress, autophagy and cell cycle arrest. Our current working hypothesis is that the pleiotropic defects in cells with disrupted VLC ceramide synthesis are due to changes in membrane properties, affecting membrane trafficking and Golgi organization. For that purpose we followed the trafficking of the temperature sensitive vesicular stomatitis virus G (VSVG) –GFP fusion protein in cells with down-regulated VLC ceramide synthesis and in controls. The results showed delay in VSVG-GFP trafficking in the cells with disrupted VLC ceramide synthesis. In addition, confocal analysis with ceramide antibody showed abnormal intracellular ceramide distribution in the treated cells compared to control. Moreover, confocal and electron microscopy analyses revealed abnormal Golgi morphology in cells with disrupted VLC ceramide synthesis.
Assembly of caveolin-1 and cavins during the caveolar life-cycle

Miriam Stoeber¹, Arnold Hayer¹, Ari Helenius¹.

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Abstract:

The integral membrane protein caveolin-1 (CAV1) is the major coat protein of caveolae. Recent studies have identified the cavin protein family members cavin-1, cavin-2, and cavin-3 as additional caveolar components. To dissect the assembly of caveolae and the association of cavins, we followed the fate of newly synthesized CAV1 and cavins biochemically and using fluorescent live-cell microscopy in tissue culture cells. We found that CAV1 homo-oligomerized into 8S complexes in the ER. After COPII vesicle mediated transport to the Golgi complex, these basic building blocks of the caveolar coat associated with cholesterol and each other to form homogeneous complexes that sedimented as 70S complexes after delipidation using nonionic detergents. Only after transport of these stable caveolar membrane domains in special Golgi-derived vesicles to the plasma membrane, was cavin-1 recruited from a cytoplasmic pool. The late arrival, the relatively slow kinetics, and stable association argue for involvement of cavin-1 in maintenance, induction of curvature, budding or other late processes in the caveolar life cycle. Both in the presence and absence of CAV1, the majority of cavin-1 in the cell was found in cytosolic complexes sedimenting at 60S. When the other cavin family members cavin-2 and cavin-3 were individually overexpressed or coexpressed, they were present in the same caveolar adaptor complex. To gain more information about differential roles of the cavins, we established cell lines stably expressing CAV1 and one respective cavin family member. This allowed us to study their individual association-/dissociation-dynamics with caveolae by dual color fluorescent microscopy after stimulation of caveolar budding in living cells. Taken together, we identified novel assembly intermediates of CAV1 and cavins and acquired spatio-temporal information on how cavin proteins associate with assembled caveolar domains during the caveolar life-cycle.
Rab6 Regulates Golgi Vesicle Release and Cisternal Homeostasis

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Abstract:

Taking an epistatic approach to characterize critical functions of Golgi associated Rab proteins in mammalian cells, we have shown previously that Rab6 is required for the cytoplasmic accumulation of Golgi-derived vesicles in response to depletion of the putative retrograde tether, COG complex (Sun et al., MBoC, 2007). At the resolution of fluorescence microscopy, siRNA induced depletion of Rab6 has little, if any, effect on the organization of the juxtanuclear Golgi ribbon with the ribbon being, if anything, slightly more continuous. However, by electron microscopy, we now find profound changes in Golgi organization. Pronounced accumulation of arrested, budding structures that remain continuous with Golgi membranes was observed. These structures were coated and found in association with both elements of the trans Golgi network (TGN) and cisternal elements of the Golgi stack. Morphologically, clathrin was identified as the TGN associated coat and COPI as the cisternal coat. In addition, in thin sections, accumulation of “free” Golgi associated coated vesicles were apparent. Tomographic characterization of 300 nm thick sections indicated that some of these are free vesicles devoid of any membrane continuity with the Golgi apparatus. Few, if any, Golgi associated vesicles were observed in control HeLa cells. Furthermore, in survey thin sections, we found a significant increase in the number of cisternae per Golgi stack: 4.2 \( \pm \) 0.32 cisternae per stack in siControl cells versus 6.8 \( \pm \) 0.46 cisternae per stack in siRab6 cells. We suggest that the simplest interpretation of the data is that Rab6 acts at multiple points through multiple effectors to regulate both Golgi vesicle budding and transport. Experiments in progress with single effector knockdowns suggest that the effects on vesicle budding are specific and not a consequence of vesicle accumulation or inhibited factor recycling.
Opposing roles of Annexin A1 and A2 in trafficking of Shiga toxin

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Abstract:

Upon binding to cell surface receptors, the bacterial Shiga toxin (Stx) and the plant toxin ricin are endocytosed and transported retrogradely from endosomal compartment through Golgi apparatus to the endoplasmic reticulum. The enzymatically active part is then translocated to the cytosol where it inactivates protein synthesis. As annexins form a family of calcium and membrane binding proteins with diverse cellular functions, many of them related to membrane trafficking, we initiated this study to investigate the role of annexin A1 and A2 in the uptake and intracellular transport of Stx and ricin. Using several experimental approaches to monitor toxin trafficking in cancerous cells we have shown that annexin A1 and A2 are not involved in toxin internalization. However, they take part in retrograde transport of Stx while ricin transport is not affected by knockdown of annexin A1 or A2. Interestingly, these two members of the annexin family exert an opposite effect on Stx retrograde trafficking. Indeed, knockdown experiments suggest that annexin A1 normally works as a negative regulator of retrograde transport from the endosomes to the Golgi, whereas annexin A2 appears to promote this route of Stx trafficking.
Skywalker is a novel GTPase activating protein that restricts synaptic endosomal traffic and neurotransmitter release

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Abstract:

The exchange of proteins and lipids at sorting endosomes is not only critical to numerous signaling pathways, but also to receptor-mediated signaling and to pathogen entry into cells; however an involvement in the synaptic vesicle cycle remains unexplored. In this work we provide genetic, morphological and biochemical evidence that at Drosophila neuromuscular junction (NMJ) boutons, a single neuronally expressed GAP, Skywalker (Sky), can restrict endosomal trafficking of synaptic vesicles, chiefly by controlling Rab35 GTPase activity. Using acute fluorescein assisted light inactivation (FlAsH-FALI) of a synaptic vesicle protein, our analyses indicate that endosomal trafficking facilitates the replacement of dysfunctional synaptic vesicle components. Consequently, sky mutants harbor a larger readily releasable pool of synaptic vesicles and they show a dramatic increase in basal neurotransmitter release. Thus, the trafficking of vesicles via endosomes controlled by Sky provides an elegant mechanism by which neurons may regulate synaptic vesicle function and neurotransmitter release.
The role of SNX-BAR proteins in tubular/vesicular endosomal sorting

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Abstract:

The endocytic network consists of interconnected tubular-vesicular membranous compartments that together regulate selective sorting of cargo to many different subcellular locations. Although it is clear that defects in endocytic function underlie a variety of human diseases, our understanding of the molecular entities that regulate these sorting events remains limited. Recent research has shown that the protein family of Sorting Nexins containing a BAR domain (SNX-BARs) play a crucial role in the correct sorting of a wide variety of cargo through the tubular/vesicular endosomal network (TEN). Here, we study the molecular mechanism by which SNX-BAR proteins form distinct tubular/vesicular sorting domains in the endosomal system and coordinate cargo sorting with dynamic spatial organization of the endosome using in vitro liposome assays, biochemistry, live cell imaging and immuno-electron microscopy. Our results show that SNX-BARs can reshape membranes into tubular structures depending on a restricted pattern of dimerization within the SNX-BAR protein family, thereby creating spatially and molecularly distinct subdomains for cargo sorting on the TEN. The formation of SNX subdomains is coordinated with the presence of well-established markers of endosomal organization and maturation, the Rab proteins. This type of analysis shows that, for instance, SNX1/Retromer sorting of cargo from endosome to trans-Golgi network predominantly occurs on endosomes that switch from Rab5 to Rab7 positive compartments, indicating that this sorting event coincides with the switch from early to late endosome. Together, our data indicate that SNX-BARs regulate fundamental steps in selective cargo trafficking from the endosomal compartment and that the formation and spatial/temporal organization of specific SNX-BAR subdomains is a critical element required for efficient endosomal sorting.
Structure of a C-terminal fragment of its Vps53 subunit suggests similarity of GARP to a family of tethering complexes

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Abstract:

The Golgi-associated retrograde protein (GARP) complex is a membrane tethering complex that functions in traffic from endosomes to the trans-Golgi network (TGN). Here we present the structure of a C-terminal fragment of the Vps53 subunit, important for binding endosome-derived vesicles, at a resolution of 2.9 Å. We show that the C-terminus consists of two alpha helical bundles arranged in tandem, and we identify a highly conserved surface patch, which may play a role in vesicle recognition. Mutations of the surface result in defects in membrane traffic. The fold of the Vps53 C-terminus is strongly reminiscent of proteins that belong to three other tethering complexes--Dsl1, COG, and the exocyst—thought to share a common evolutionary origin. Thus, the structure of the Vps53 C-terminus suggests that GARP belongs to this family of complexes.
TRAPP is required for ER exit of procollagen by controlling the Sar1 cycle

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Abstract:

The study of inherited human disorders, including those involving membrane trafficking, can provide important hints towards an understanding of the physiological context, molecular pathways, and actual functional role of the gene products affected. On this basis, we have investigated the role of sedlin, a conserved component of TRAPP, a multimolecular complex so far involved in the heterotypic tethering of ER-derived vesicles to the Golgi membranes in yeast and in the homotypic tethering of ER-derived vesicles in mammals. Genetic defects of sedlin result in spondyloepiphyseal dysplasia tarda (SEDT), a condition characterised by short stature, flattening of the vertebrae, and premature osteoarthritis. Inherited epiphyseal dysplasias are genetically heterogenous disorders of chondrocytes, which include defects of secretory chondrocyte cargoes (matrilin, aggrecan, collagens II and IX), cargo processing apparatus (chondroitin sulfotransferase and sulfate transporter), and the trafficking machinery (sedlin). Prompted by the concept that defects in extracellular matrix components and in sedlin can cause similar phenotypes, and by the observation that chondrocytes from SEDT patients show indirect signs of impaired trafficking at the ER (i.e. dilated ER), we have analysed the role of sedlin and other TRAPP components in the trafficking of neosynthesised procollagen. We show that sedlin and the whole TRAPP complex are selectively required for procollagen to exit the ER, while they are not essential for ER exit of small soluble and membrane-associated cargoes. We have also identified the molecular mechanism underlying this role of TRAPP and sedlin in their ability to control the membrane–cytosol cycle of COPII and Sar1 at the ER exit sites.
Rac1 interaction with CaM. Importance of the activation state, the plasma membrane localization and the c-terminal polybasic domain

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Abstract:

The small GTPase Rac1 regulates different cellular processes like migration, intracellular trafficking or phagocytosis by activating actin polymerization. Rac1 switches between an active GTP-bound form and an inactive GDP-bound form. In the active form, Rac1 interacts with their effectors, including activators of the Arp2/3 complex, and induces actin polymerization at particular cellular sites. It has been described that calmodulin (CaM) interacts with several small GTPases and therefore regulates cellular signalling and actin cytoskeleton. For instance, it has been already demonstrated that CaM binds to the hypothetical amphipatic alpha-helix of Rac1, corresponding to amino acids:151-164, and this interaction could regulate its activity (Elsaraj SM and Bhullar RP, BBA 2008). In this study, we have also demonstrated, in COS-1 cells, that endogenous Rac1 interacts with CaM in a calcium dependent manner. In addition, a direct binding between both proteins was also demonstrated by GST-sepharose4B affinity chromatography using purified proteins. Moreover, the binding kinetics between GST-Rac1 and CaM were analyzed by in vitro surface plasmon resonance technology (Biacore T-100, BioRad). The GST-Rac1 exhibited a high affinity for CaM (KD = 173 nM). These results demonstrated a high affinity and direct binding between Rac1 and CaM. Furthermore, we have uncovered new additional binding sites for CaM. Pull down CaM-sepharose, expression of different Rac1 mutants and immunoprecipitation experiments have revealed that activation state, membrane localization and the polybasic domain in the c-terminal of Rac1 are also necessary for CaM interaction. Interestingly, the c-terminal region of Rac1 is important for interaction with its effectors. We have discussed that association of CaM to this region may regulate Rac signalling.
A structural analysis of the ER in budding yeast by high-resolution EM reveals how multiple ER domains are organized by membrane shaping proteins

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Abstract:

We have used electron microscopy and tomography of cryofixed cells to visualize at nanometer resolution the structural and spatial organization of yeast ER. These data allow the major domains of the ER to be characterized in fine detail. The peripheral ER in the mother cell consists of three continuous though structurally distinct domains that include the plasma membrane associated ER, central cisternal ER, and tubular ER. We show that these interconnected domains vary in location, shape, volume to surface area ratios, and ribosome density. We have compared the properties and organization of ER domains at high resolution during six sequential stages of ER budding to reveal a vivid picture of ER domain organization and inheritance. We then examined the ER structure in a yeast strain lacking the membrane-shaping proteins, reticulons and Yop1 to measure their contribution to ER domain organization. These data demonstrate that these membrane-shaping proteins regulate the dimensions, volume to surface area ratios, and ribosome density of all peripheral ER domains.
Ligand induced internalization and recycling of the human Y2 receptor is regulated by its C-terminal tail

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Abstract:

Agonist-induced internalization of G protein-coupled receptors plays an important role in signal regulation. The underlying mechanisms of the internalization of the human neuropeptide Y2 receptor (hY2R), as well as its desensitization, endocytosis and resensitization are mainly unknown. The Y2R is one of four human neuropeptide Y (NPY) receptor subtypes (Y1R, Y2R, Y4R and Y5R) and belongs to the rhodopsin-like superfamily (class A) of GPCRs. YRs together with their three native ligands, NPY, pancreatic polypeptide (PP) and peptide YY (PYY), form a multiligand/multireceptor system, which is involved in many important physiological processes, such as regulation of food intake, control of blood pressure, and regulation of pancreatic and gastric secretion. The Y2R is predominantly expressed in the central nervous system as well as in the periphery and is involved in the inhibition of neurotransmitter release, regulation of memory retention, circadian rhythm and angiogenesis, thus makes it an attractive target for drug development. Since it has been reported that Y2Rs are expressed in distinct tumors, e.g. renal cell carcinomas, ovarian cancers, adrenal gland and related tumors, the Y2R is furthermore a promising target for tumor diagnostics and therapy. In order to successfully treat Y2R related diseases, it is of fundamental interest to unravel the mechanisms and regulation modalities of receptor internalization and subsequent resensitization processes. To address this question, we generated a series of C-terminally truncated hY2R mutants in order to investigate the impact of C-terminal sequences on receptor internalization properties. We identified novel regulatory motifs within the hY2R C-terminal domain, which contribute to receptor internalization and arrestin3 association. Interestingly, our findings revealed arrestin3-dependent and -independent hY2R internalization, and also led to the identification of a sequence that modulates receptor recycling.
Cell cycle-regulated Golgi stack assembly and function

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Abstract:

The unique structure of the Golgi in almost all eukaryotic cells is a stack of flattened cisternal membranes, but how this structure is formed at the molecular level and why its formation is important for cellular functions remain elusive. We have developed an in vitro system to reconstitute the process of mitotic Golgi disassembly and post-mitotic reassembly in mammalian cells, which allowed us to reveal the molecular mechanism of the Golgi biogenesis during the cell division. Mitotic Golgi fragmentation involves membrane vesiculation coupled with cisternal unstacking; post-mitotic Golgi reassembly is mediated by membrane fusion to form single cisternae and by stack formation. Stack formation directly involves the Golgi stacking protein GRASP65 and GRASP55, which play complementary and essential roles in Golgi cisternal stacking by forming mitotically regulated trans-oligomers. By inhibition of GRASP65/55 oligomerization we are able to manipulate Golgi stack formation and thus determine the biological significance of stacking. We demonstrate that Golgi cisternal unstacking stimulates COPI vesicle budding and thus enhances protein transport. Golgi fragmentation, however, impairs glycosylation of cell surface proteins and reduces cell adhesion. Inhibition of Golgi disassembly at the onset of mitosis also affects cell cycle progression. We propose that Golgi stack formation is a flux regulator for protein trafficking and thereby maintain the quality of protein glycosylation. Structural and functional Golgi defects in disease models are explored in this study.
Membrane Fusion: 5 Lipids, 4 SNAREs, 3 Chaperones, 2 Nucleotides, and a Rab, All Dancing in a Ring

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Abstract:

Yeast vacuole fusion requires specific vacuolar lipids (PE, PA, DAG, Erg, and phosphoinositides), SNAREs, the disassembly chaperones Sec17p/Sec18p, the Rab effector/tethering/SM/lipid-recognition complex HOPS, and the Rab Ypt7p. Each of these requirements has been established in vivo by genetics, in vitro through studying fusion of the isolated organelle, and with proteoliposomes bearing the pure and functional components. The functions of each are coming into view: SNAREs are the driving force for bilayer destabilization that can lead to fusion or to lysis, Sec18p/Sec17p/ATP disassembles SNARE complexes, the Rab contributes crucial affinity for binding HOPS to the membrane, and HOPS first tethers membranes, then discriminates functional- from incorrect-SNARE complexes and protects functional ones from Sec18/17p-mediated disassembly. PE, PA, and DAG may contribute to selective bilayer desabilization, ergosterol to microdomain stability, and PI3P to the assembly of 3Q-SNARE subcomplexes and HOPS membrane affinity. These proteins and lipids are fully interdependent for their assembly into the fusion-competent, ring-shaped microdomain on the vacuole, perhaps requiring networks of modest-affinity interactions such as between each pair within the HOPS, PtdIns3P, and PX-Vam7p SNARE triad. We now seek quantitative understanding of these interactions, their ordering, an appreciation of the roles of lipid asymmetry and microdomain assembly, and to understand the regulation of fusion vs. lysis. See 2010 Ann. Rev. Cell Dev. Biol. for consideration at greater depth.
Rab 14 regulates tight junction integrity and epithelial polarity

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Abstract:

The establishment and maintenance of the epithelial tight junction is critical to the normal function of epithelial organs, both to serve as a selectively permeable barrier and for the maintenance of epithelial polarity. Membrane traffic plays an essential role in the establishment and maintenance of both tight junctions and polarity, but the molecular players in this process remain unclear. We have found that the small GTPase Rab14 regulates the establishment and maintenance of tight junctions and polarity in MDCK cells. We show that expression of an inactive mutant of Rab14 results in more rapid formation of tight junctions after calcium switch; this is likely due to decreased endocytosis of tight junction components, as endocytosis of occludin is inhibited in cell expressing inactive Rab14. Furthermore, confocal imaging indicates that Rab14 colocalizes with a subset of occludin- and claudin-1-positive vesicles. Furthermore, knockdown of Rab14 results in redistribution of claudin-1 to a perinuclear compartment. Finally, expression of inactive Rab14 prevents the cells from forming single lumen cysts in three-dimensional culture. These effects may be mediated by a previously unrecognized apical endosomal multi-protein complex, as Rab14 interacts with the apical endosomal protein endotubin and the polarity regulator aPKC. This complex may serve to organize and target tight junction proteins after endocytosis to maintain the epithelial barrier.
A novel Golgi-localized protein involved in the trafficking of dense core vesicle cargos

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Abstract:

Neuropeptides play significant roles in short-term and long-term regulation of excitability and remodeling of the neurons. Neuropeptides and peptide hormones are packaged and stored in a specialized intracellular organelle called dense core vesicle (DCV, also known as secretory granule, SG). The biological relevance and molecular mechanisms of the trafficking of DCV components before and after exocytosis are largely unknown. We have studied a highly conserved protein in both C. elegans and mammalian cells. Ablation of this protein reduced the storage of bioactive peptides intracellularly and hence the amount of release extracellularly. On the contrary, the level of DCV membrane proteins was significantly increased, which was caused by an accumulation of these membrane proteins in intracellular vesicles. This protein was localized to the Golgi complex. Our results identify a novel player in controlling the proper trafficking and recycle of DCV components and demonstrate its essential role in neuropeptide and hormone release.
Dissecting the roles of O-glycosylation and dimerization in the apical sorting of a model raft-independent protein

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Abstract:

Proteins sorted to the apical plasma membrane in polarized epithelial cells can be categorized into two classes based on their differential association with cholesterol and glycolipid-enriched membrane microdomains commonly termed “lipid rafts”. Our laboratory and others have previously demonstrated that lipid raft-associated and lipid raft-independent proteins take different routes to the apical surface (1, 2). Interestingly, raft-associated and raft-independent apical cargoes appear to be segregated into distinct subdomains even within the trans-Golgi network (TGN) (3). Moreover, TGN export requirements of the raft-independent protein p75 assessed using an in vitro assay are markedly different from that of a raft-associated protein. Self-association or “clustering” is important for the sorting of raft-associated proteins but the role of clustering in raft-independent sorting is unclear. The apical sorting determinant in p75 resides within its O-glycosylated stalk, and galectin-mediated crosslinking has been proposed as a sorting mechanism for this protein (4). Additionally, a novel dimerization motif (Cys257, A261XXXG266) has been identified in the transmembrane region of p75 that could contribute to the formation of oligomers (5). I have created a panel of p75 mutants to determine the roles of O-glycosylation and dimerization in the segregation and export characteristics of p75 staged in the TGN. In vitro TGN budding assays and immunofluorescence microscopy are being employed to examine segregation and TGN export of mutants compared with wild type p75. In addition the local cellular environment occupied by wild type and mutant proteins is being explored using biophysical approaches. 1) Cresawn, K. O. et al. (2007) EMBO J. 26, 3737-3748 2) Jacob, R., and Naim, H. Y. (2001) Curr. Biol. 11, 1444-1450 3) Guerriero, C. J. et al. (2008) JBC 283, 18040-7 4) Delacour et al. (2007) Traffic 8, 379-388 5) Vilar, M. et al. (2009) Neuron 62, 72-83.
From synthetic endosomes to a systems analysis of endocytosis

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Abstract:

Endocytosis is an essential process serving multiple key cellular functions, such as nutrient uptake, signal transduction, and defence against pathogens. Rab GTPases act as membrane organizers, specifying the structural and functional identity of endosomes. Rab proteins occupy distinct membrane territories or “Rab-domains” linked by a binary switch system consisting of divalent effectors, GEFs and GAPs that sequentially activate and repress Rab GTPases along the pathway. Using a biochemical approach, we reconstituted the Rab5 GTPase, its key regulators and effectors together with early endosomal SNAREs into proteo-liposomes using a set of 17 recombinant proteins. These vesicles behave like “synthetic” endosomes, fusing with purified early endosomes or with each other in vitro. Our results suggest that Rab effectors together with SNAREs constitute the core machinery of the membrane tethering and fusion process. To elucidate the machinery underlying endosome function and endocytosis further, we undertook a systems biology analysis. We systematically profiled the activity of human genes with respect to Transferrin and EGF endocytosis by performing an image-based RNAi screening of HeLa cells in cooperation with the HT-TDS, the screening facility of the MPI-CBG. The genes were identified on the basis of a multi-parametric analysis quantitatively measuring uptake and intracellular cargo distribution. We uncovered novel regulators of endocytosis and endosome trafficking, including many signalling pathways (e.g. Wnt, Integrin, TGF-β, and Notch). A systems analysis by Bayesian networks further uncovered design principles regulating the number, size, concentration of cargo and intracellular position of endosomes.
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Student-Centered Education in the Molecular Life Sciences II
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Kuan-Teh Jeang, National Institute of Allergy and Infectious Diseases, NIH
Douglas Lowy, National Cancer Institute, NIH
Guangzhou Baiyun International Convention Center

SEPT 27–Oct 2, Pacific Grove, CA
13th International ATPase Conference
Na, K-ATPase and Related P-ATPase: Structure, Biology, and Medicine
Kathleen J. Sweadner, Harvard Medical School and Massachusetts General Hospital
Co-Chairs: Svetlana Lutsenko, Johns Hopkins University School of Medicine; Jacob I. Sznajder, Northwestern University, Feinberg School of Medicine; Hiroshi Suzuki, Asahikawa Medical College, Japan; Zijian Xie, University of Toledo College of Medicine
Asilomar Conference Grounds

OCT 6–9, Snowbird, UT
Cellular Traffic of Lipids and Calcium at Membrane Contact Sites
Joint meeting with the Biochemical Society
Tim Levine, UCL Institute of Ophthalmology, London
Will Prinz, National Institute of Diabetes and Digestive and Kidney Diseases, NIH
Snowbird Ski and Summer Resort

OCT 12–16, Snowbird, UT
Chemical, Synthetic and Systems Biology: New Directions of Biochemistry in the 21st Century
Arcady Mushegian, Stowers Institute for Medical Research
Aled Edwards, University of Toronto, Canada
Snowbird Ski and Summer Resort

OCT 27–30, Tahoe City, CA
Gene Regulation by Non-Coding RNAs
Richard Carthew, Northwestern University
Jennifer Doudna, HHMI, University of California, Berkeley
Granlibakken Resort and Conference Center

NOV 17–20, Bethesda, MD
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