"Novel clathrin-independent endocytic routes: role of curvature sensing/inducing BAR domain proteins"

Renard, Henri-François

Abstract
Endocytosis is an essential cellular process required for uptake of nutrients from cell environment and turnover of plasma membrane components. Clathrin-mediated endocytosis is by far the best characterized endocytic process. Since the mid-90's, the existence of endocytic routes independent of clathrin emerged. Therefore, the most challenging question in membrane biology rose: how could the plasma membrane be deformed in the absence of an organized clathrin coat? Until today, this process is not fully understood. First attempts to shed light into this topic demonstrated the requirement of glycosphingolipids for membrane deformation in lectin-driven endocytosis. Recently, BAR domain proteins (BAR stands for Bin/Amphiphysin/Rvs) have been described to be crucial for clathrin-independent endocytic routes. BAR domain proteins interact with membranes and act as curvature sensors/inducers. The function of BAR domain proteins remains unclear in the landscape of clathrin-independent endocytos...

Document type: Communication à un colloque (Conference Paper)

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Co-organizer: Aurelien Roux, University of Geneva, Switzerland

Invited speakers

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Ivan Dikic, Goethe University Frankfurt, Germany
Arnaud Echard, Pasteur Institute, France
Adam Frost, University of California San Francisco, USA
Jennifer Gallop, The Gurdon Institute, UK
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Prisca Liberali, Friedrich Miescher Institute for Biomedical Research, Switzerland
Ya-Wen Liu, National Taiwan University, Taiwan
Michiyuki Matsuda, Kyoto University, Japan
Heidi McBride, McGill University, Canada
Rob Parton, University of Queensland, Australia
Francesca Peri, EMBL Heidelberg, Germany
Margaret (Scottie) Robinson, Cambridge Institute for Medical Research, UK
Mike Rosen, UT Southwestern Medical Center, USA
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PROGRAM

SUNDAY, 10 SEPTEMBER 2017

16.00-19.00 Arrival and registration
19.00-20.30 Dinner
20.30-21.30 Opening lecture
Margaret (Scottie) Robinson, Evolution of endocytic coats
21.30-23.00 Get-together drinks

MONDAY, 11 SEPTEMBER 2017

9.00-12.30 Session 1. Endocytic protein assemblies
Chair. Frances Brodsky

9.00-9.30 Ludger Johannes. Lectin-driven and glycosphingolipid-dependent construction of endocytic pits — The GL-Lect hypothesis
9.30-9.50 Ira Milosevic. Clathrin coat controls vesicle acidification by mechanical inhibition of vacuolar ATPase
9.50-10.10 Antoni Wrobel. Regulation of AP2 function in clathrin-mediated endocytosis

10.30-11.00 Coffee break

11.00-11.30 Tom Kirchhausen. Cellular dynamics visualized from molecules to organisms at increased spatio-temporal resolution
11.30-11.50 Delia Bucher. Transition from flat clathrin arrays to curved pits during clathrin-mediated endocytosis is coordinated by membrane tension
11.50-12.20 Jenny E. Hinshaw. Capturing the sequential steps of dynamin-mediated fission by cryo-EM

12.30-14.00 Lunch
16.00-19.30 Session 2. Endosomal trafficking
Chair. Kirsten Sandvig

16.00-16.30 Marino Zerial. Endosomes as a central hub in cell physiology
16.30-16.50 Ana Kojic. Minimal invasive fluorescent receptor labeling to study EGFR internalization
16.50-17.10 Merja Joensuu. Subdiffractional tracking of internalized molecules (sdTIM) is a single-molecule technique for dissecting heterogeneous motion states of endocytic compartments in live neurons
17.10-17.40 Jean Gruenberg. The many players of MVB formation

17.40-18.10 Coffee break

18.10-18.40 Harald Stenmark. Control of endocytic membrane traffic by phosphatidylinositol 3-phosphate
18.40-19.00 Kenneth Madsen. GPCRs shape their own destiny in constitutive endocytosis through surface activity of the amphipathic helix8
19.00-19.30 Adam Frost. Non-Canonical ESCRT Structures and Functions

19.30-21.00 Dinner

21.00-23.00 POSTER SESSION n°1

TUESDAY, 12 SEPTEMBER 2017

9.00-12.30 Session 3. Crosstalk between endosome traffic and other cell processes
Chair. Phil Robinson

9.00-9.30 Gillian Griffiths. Fine tuning lysosomal secretion
9.30-9.50 Virginie Stévenin. Communication between distinct infection-associated compartments drives the intracellular lifestyle of Salmonella
9.50-10.10 Léa Ripoll. Myosin VI and actin dynamics induce membrane constriction and fission at melanosome
10.10-10.40 Johanna Ivaska. Endosomal trafficking and signalling of integrins and receptor tyrosine kinases in cancer

10.40-11.10 Coffee break
11.10-11.40 Gia Voeltz. Unraveling the mechanism of ER-associated endosome fission
11.40-12.00 Raphaël Rodriguez. An iron hand over cancer stem cells
12.00-12.30 Heidi McBride. Mechanisms and implications of mitochondrial antigen presentation

12.30-14.00 Lunch

16.00-19.30 Session 4. Intracellular signaling and traffic
Chair. Patrick Caswell

16.00-16.30 Philippe Bastiaens. The interdependence of vesicular membrane dynamics and signal processing
16.30-16.50 Anja Zeigerer. Defining a novel role of distinct membrane trafficking regulators in blood glucose homeostasis
16.50-17.10 Kamil Jastrzębski. The role of endocytosis in NF-κB signaling mediated by lymphotoxin beta receptor (LTβR)
17.10-17.40 Prisca Liberali. Membrane trafficking and lipid signaling during intestinal organoid development

17.40-18.10 Coffee break

18.10-18.40 Roberto Zoncu. Roles of lysosomal cholesterol in mTORC1 signaling and growth regulation
18.40-19.00 Robbie Loewith. TORC1 Organised in Inhibited Domains (TOROIDs) regulate TORC1 activity
19.00-19.30 Michiyuki Matsuda. FRET mice, tools to visualize protein kinase activities in living organs

19.30-21.00 Dinner

21.00-23.00 POSTER SESSION n°2
9.00-12.30 Session 5. Endocytosis and disease
Chair: Stéphanie Kermorgant

9.00-9.30 Dafna Bar-Sagi. Macropinocytosis and Ras signaling in cancer
9.30-9.50 Yeesim Khew-Goodall. Control of endosomal maturation and receptor recycling by a tyrosine kinase signalling pathway: its role in cancer and in health
9.50-10.10 Mahel Zeghouf. A new inhibitor targeting BRAG2, an endocytic ArfGEF involved in cancer
10.10-10.40 Yosef Yarden. Potential Roles for Receptor Endocytosis and Degradation in Cancer Therapy

10.40-11.10 Coffee break

11.10-11.40 Ivan Dikic. Endoplasmatic reticulum remodelling via selective autophagy pathways
11.40-12.00 Swetha Gowrishankar. Impaired axonal lysosome transport contributes to Alzheimer’s pathology
12.00-12.30 Ya-Wen Liu. Functional role of dynamin in the muscle revealed by mutations causing Centro-Nuclear-Myopathies

12.30-14.00 Lunch

14.00-19.30 Excursion to Warsaw city center

19.30-21.00 Dinner

21.00-23.00 POSTER SESSION n°3
THURSDAY, 14 SEPTEMBER 2017

9.00-12.30 Session 6. Physical principles applied to endocytosis
Chair. Guillaume Montagnac

9.00-9.30 Aurelien Roux. Dynamical mechanics of the ESCRT-III complex
9.30-9.50 David Teis. Recruitment dynamics of ESCRT-III and Vps4 to endosomes and implications for the mechanism of ESCRT-mediated reverse membrane budding
9.50-10.10 Francesco Baschieri. Clathrin-coated membrane domains are new cellular mechanosensors
10.10-10.40 Mike Rosen. Molecular Mechanisms of Biological Phase Separation

10.40-11.10 Coffee break

11.10-11.40 Alf Honigmann. Dynamic interplays between lipid domains and actin polymerization
11.40-12.00 Ruchir Rastogi. Leishmania donovani modulates endo-lysosomal pathway by up regulating Rab5a for its successful survival in macrophages
12.00-12.30 Jennifer Gallop. Actin comets in Lowe syndrome and clathrin-mediated endocytosis are controlled by a coincidence of phosphoinositides and membrane curvature

12.30-14.00 Lunch

16.00-19.20 Session 7. Endocytic processes in development and cell organization
Chair. David Perrais

16.00-16.30 Marcos Gonzalez-Gaitan. Asymmetric endosomal trafficking in asymmetric division
16.30-16.50 Martin Baron. Misregulation at different regulatory nodes of an endocytic regulatory network underlies phenotypic diversity of a range of Notch disease-associated mutations
16.50-17.10 Paul Langridge. Epsin-dependent ligand endocytosis activates Notch by force
17.10-17.40 Arnaud Echard. Inversion of cell polarity controlled by Rab35

17.40-18.00 Coffee break
18.00-18.20 Maximilian Fürthauer. ESCRT-dependent exovesicles in the Zebrafish Left/Right organizer
18.20-18.50 Francesca Peri. Phagocytosis during neuron-microglia interactions in brain development

18.50-19.00 Break

19.00-20.00 Closing lecture sponsored by The Company of Biologists
Rob Parton. Cave exploration at the nanoscale. new insights into the structure and function of caveolae

20.00 Conference dinner and party

FRIDAY, 15 SEPTEMBER 2017
Departure
# LIST OF POSTERS

All posters should be displayed throughout the conference

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ABSTRACTS OF TALKS

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Evolution of endocytic coats

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The coats on clathrin-coated vesicles (CCVs) consist of two major components: clathrin itself and heterotetrameric adaptor protein (AP) complexes, with AP-2 acting at the plasma membrane and AP-1 at intracellular membranes. Part of the COPI coat is distantly related to the APs, and there are two additional AP complexes identified in the 1990s by sequence-based searches, AP-3 and AP-4. More recently, we have identified a fifth AP complex and a more distantly related coat, TSET. All of these coats must have been present in the last eukaryotic common ancestor, because they are found throughout the diversity of eukaryotes, but APs 1-3 are by far the best understood. Mutations in either AP-4 or AP-5 cause hereditary spastic paraplegia, and we have recently been studying the functions of these two coats by carrying out comparative proteomics on control and AP-4 or AP-5 knockout cells. Other coat components are more recent innovations. For instance, PTB domain-containing adaptors, which contribute to clathrin-mediated endocytosis, have so far been found only in metazoans. We are currently investigating whether PTB domain adaptors may also be present in other holozoans, in particular Capsaspora owczarzaki. These studies may help to shed light on the co-evolution of endocytosis and signalling in animals.
Lectin-driven and glycosphingolipid-dependent construction of endocytic pits — The GL-Lect hypothesis

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Several endocytic processes do not require the activity of clathrin, and it has been a major question in membrane biology to know how the plasma membrane is bent and cargo proteins are sorted in these cases. Our previous studies have allowed us to propose a novel hypothesis, termed GL-Lect hypothesis: nanodomain construction by GlycosphingoLipid-binding cellular or pathological Lectins induces membrane curvature changes and drives the formation of endocytic pits for the cellular uptake of glycosylated membrane proteins with critical roles in cell migration (CD44, alpha5beta1 integrin…), of pathogens (polyoma viruses, norovirus) or pathogenic factors (Shiga and cholera toxins). We are now analyzing how cortical actin dynamics contributes to the clustering of glycosphingolipid-lectin complexes on active membranes, thereby facilitating the nucleation of endocytic tubules exploiting fluctuation forces that had not been linked before to endocytosis. Furthermore, we are identifying mechanisms by which the GL-Lect mechanism is acutely controlled at the plasma membrane. Finally, we study how GL-Lect domain construction at the plasma membrane programs the intracellular distribution of cargo molecules, notably via the retrograde transport route.
Clathrin coat controls vesicle acidification by mechanical inhibition of vacuolar ATPase

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The vacuolar H+-dependent adenosine triphosphatases (vATPases) are highly conserved multi-subunit complexes that use the ATP hydrolysis energy to produce a proton electrochemical gradient across membranes and acidify numerous intracellular organelles, enabling their function. The vATPases are essential for various cellular processes, e.g. endocytosis and intracellular membrane traffic, including synaptic vesicle (SV) recycling. At the neuronal synapses, exocytosed membrane and proteins, including vATPases, are internalized mainly by clathrin-mediated endocytosis. Newly formed SVs are rapidly acidified by vATPases, generating an electrochemical gradient that is subsequently used by an antiporter to fill SVs with neurotransmitters. To date, it is unclear how the vATPase is regulated during endocytosis, in particular at which step endocytosed vesicles acidify. Here, we developed assays to monitor pH and electrical gradients at the single clathrin-coated vesicle (CCV) level. We observed that ATP-induced acidification of CCVs isolated from the mammalian brain is strongly reduced in comparison to SVs. When the clathrin coat is removed from CCVs, uncoated vesicles rapidly acidified. Based on biochemical, imaging and functional studies, we propose a model where formation of the clathrin coat around vATPase mechanically blocks its activity. Such regulation of vATPase activity likely applies in all clathrin-coated structures.
Regulation of AP2 function in clathrin-mediated endocytosis

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AP2 is the most abundant clathrin adaptor in endocytic clathrin coated pits (CCPs). Previous studies in our lab and others have indicated that AP2 is necessary for CCP nucleation and that AP2 assumes a number of different conformations during progression of CCP formation and undergoes regulatory phosphorylation. In order to further understand, how AP2 is activated during initiation of clathrin-mediated endocytosis (CME), we probed, how the interaction between the early endocytic proteins FCHO1/2 and Eps15/Eps15R concentrates AP2 on the plasma membrane at the sites of endocytosis.

We present a 2.4 Å crystal structure of a chimeric protein consisting of the mu-homology domain (MHD) of FCHO1 and the conserved DPF-rich region of the Eps15 unstructured domain, to which the MHD binds. Eps15 assumes a tight turn conformation in order to bind the conserved, deep trough in the subdomain A of the FCHO1 MHD. We support the structure with in vitro and in vivo analyses that indicate high strength of the interaction and show that simultaneous removal of FCHO1/2 and Eps15/Eps15R from the plasma membrane abolishes CME. We propose a model, in which the FCHO1/2-Eps15/Eps15R multimers concentrate AP2 at the site of CME initiation via an interaction between the AP2 alpha appendage and Eps15, while the interaction of the AP2 core with the FCHO1/2 interdomain linker helps to activate AP2 for cargo and clathrin binding.

To investigate the role of phosphorylation of Thr156 on the AP2 mu2 subunit we present crystal structures of AP2 core phosphorylated at the Thr156 residue of the mu2 subunit in closed and a new open conformation. The latter shows a large conformational movement of the Cmu2 domain, when compared with the previously known AP2 structures, and significant opening of the AP2 “bowl” formed by the remaining subunits of the core but is still competent for PIP2- and cargo-containing membrane binding. In agreement with this, we show by liposome-based SPR that the mu2 Thr156 phosphorylation has no significant effect on the recognition of cargo or plasma membrane lipids, as has been previously proposed, and must thus be involved in processes involved in CCP formation.
Novel motor-independent function of a dynein light chain in clathrin mediated endocytosis

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Clathrin- and actin-mediated endocytosis is essential in eukaryotic cells. Here we demonstrate Tda2 is a novel protein of the endocytic machinery necessary for normal internalization of native cargo in yeast. Tda2 has not been classified in any protein family. Unexpectedly, the crystal structure of Tda2 revealed it belongs to the dynein light chain family. However, Tda2 works independently of the dynein motor complex and microtubules. Tda2 forms a tight complex with polyproline motif-rich protein Aim21, which interacts physically with the SH3 domain of the Arp2/3 complex regulator Bbc1. The Tda2-Aim21 complex localizes to endocytic sites in a Bbc1 and filamentous actin-dependent manner. Importantly, the Tda2-Aim21 complex interacts directly with and facilitates the recruitment of actin-capping protein revealing barbed end filament capping at endocytic sites is a regulated event. Thus, we have uncovered a new layer of regulation of the actin cytoskeleton by a member of a conserved protein family that has not been previously associated with function in endocytosis.
Cellular dynamics visualized from molecules to organisms at increased spatio-temporal resolution

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Frontier optical-imaging modalities exemplified by the lattice light-sheet microscope invented by Eric Betzig sets new visualization standards for analyzing and understanding sub-cellular processes in the complex and dynamic three-dimensional environment of living-cells in isolation and within tissues of an organism. We believe this ability to image with minimal perturbations is ideally suited to support hypothesis-generating research geared towards new discoveries. This talk will describe our most recent efforts to link molecular structure and mechanisms of intracellular membrane traffic with the physiology of cells in isolation and within zebrafish embryos.
Transition from flat clathrin arrays to curved pits during clathrin-mediated endocytosis is coordinated by membrane tension

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During clathrin-mediated endocytosis (CME), clathrin and multiple adaptor and associated proteins assemble into a complex protein scaffold at the plasma membrane. This scaffold forms a clathrin-coated pit that grows in size to ultimately pinch off from the plasma membrane as a clathrin-coated vesicle. How assembly of the clathrin scaffold is regulated at the ultrastructural and spatio-temporal level is still under debate. There are currently two proposed models: 1) The constant curvature growth model which suggests curvature is acquired simultaneously as the clathrin-scaffold grows and 2) The constant area growth model where the clathrin structures initiate as a flat coat that acquire curvature only after reaching the full clathrin content. Using a combination of live fluorescent microscopy, correlative light and electron microscopy (CLEM) of metal replicas, statistical analysis and modeling of coat growth profile behaviors, we developed a comprehensive ultrastructural model of the dynamics of clathrin-coated structure formation. Methodical comparison of the predicted ultrastructural assembly of the clathrin scaffold calculated from the two previously reported growth models revealed that neither of the two models could fully explain the ultrastructural organization of clathrin structures observed in cells. We could show that clathrin structures initially grow flat. At around 50% of their final clathrin content, flat clathrin arrays acquire curvature and continue growing to form a fully formed clathrin-coated pit. The start of bending is associated with a change in the AP2/clathrin ratio. Importantly, we found that acquisition of curvature likely corroborates with the accumulation of enough energy in the flat clathrin array to bend the plasma membrane. Indeed, increasing plasma membrane tension resulted in a delay of the change in the AP2/clathrin ratio and an accumulation of flat clathrin arrays. Taken together our work reveals new insights into the ultrastructural rearrangements that proceed during clathrin-coated pit formation and helps to complete our understanding of the mechanisms underlying CME.
Capturing the sequential steps of dynamin-mediated fission by cryo-EM

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Our laboratory is interested in membrane fusion and fission mediated by dynamin and other dynamin-like proteins. We are currently focusing on dynamin and its role in endocytosis. The approach we are taking is to examine the structure and function of proteins involved in these processes by cryo-electron microscopy (cryo-EM), and biochemical methods. In addition to examining the gross morphology of protein complexes, we have solved the 3-dimensional structure of several dynamin family members by image processing methods. We currently have a 4.3 Å resolution cryo-EM structure of the constricted, membrane-associated helical polymer of human dynamin-1 in the GTP bound state. Compared to the soluble tetramer, the membrane-associated dynamin adopts a more extended conformation. In addition, our new model fits well into the cryo-EM map of a constricted, membrane-free spiral, suggesting membrane is dispensable for stabilizing the constricted conformation. The model also fits well into the map of a super-constricted polymer, confirming that the new interfaces are sufficient to bring the lipid bilayer close enough for spontaneous fission. The new reconstruction also allows unambiguous placement of dynamin’s membrane-binding pleckstrin homology domain, which has been poorly defined in previous models due to flexibility. Overall, our new structure, in combination with previous 3D reconstructions in different nucleotide states, capture conformational changes in the polymer that ultimately leads to membrane fission.
Endosomes are important organelles for the transport and sorting of endocytosed cargo but also for other functions, such as signal transduction, regulation of metabolism and stress response. The multi-functional activity of endosomes can be appreciated by manipulating its molecular machinery. Rab5 is necessary for the biogenesis of the entire endo-lysosomal pathway in vivo. It regulates the specificity and directionality of endosome fusion via the recruitment of tethering effectors that lead membranes to dock and fuse. EEA1 is a tethering factor that bridges a Rab5-positive early endosome with another vesicle harbouring Rab5. Upon binding, Rab5 induces an allosteric conformational change on EEA1, from extended to flexible, generating an entropic collapse force that helps pulling the membranes together. This means that the function of Rab proteins goes beyond effector recruitment. Besides transport, loss of Rab5 and of the endosomal pathway leads to alterations in sugar and lipid metabolism which are, in part, due to the sorting and activity of signalling receptors. Furthermore, endosome tethering and fusion responds to tyrosine kinase receptors to regulate their packaging in endosomes and determine the amplitude, lifetime and robustness of the signalling response. Finally, new data indicate a Rab5-dependent cross-talk between endosomes and mitochondria in response to stress.
Minimal invasive fluorescent receptor labeling to study EGFR internalization

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EGFR endocytosis has been extensively studied, often using imaging techniques involving EGFR tagged with fluorescent proteins. Although this method is a valuable tool for studying this process, advances in click chemistry and protein labeling have opened up the possibility for new methods of specifically labeling using small dyes. Genetic codon expansion allows the incorporation of non-canonical amino acids bearing ring-strained alkynes or alkenes at a specific site in a protein. The non-canonical amino acid is used to covalently bind to small fluorescent dyes via azide or tetrazine groups (1). By using hydrophilic dyes that do not pass the cell membrane, and by incorporating artificial amino acids in the extracellular domain of EGFR, it is possible to specifically label the portion of EGFR that is located at the plasma membrane at the time of labelling. Using this methodology, endocytosis can be observed by accumulation of the dye inside the cell (2). Previous work in our group has shown the influence of PI(3,4,5)P3 on EGFR endocytosis. Elevated levels of PIP3 induced EGFR endocytosis and recycling in the absence of a ligand and tyrosine phosphorylation. The mechanism by which this is achieved is not known (3). To elucidate the mechanism of PIP3 induced EGFR endocytosis, we produced different EGFR mutants that were checked for PIP3-induced internalization deficiency. For this purpose we used caged versions of PIP3 that are membrane-permeant. The caged PIP3 is not active because of a coumarin group masking the lipid head group that can be released by a flash of 405nm light. This controls the release of PIP3, both spatially and temporally, and allows us to precisely observe the effect on EGFR localization. These methods combined represent a potent platform for further studies of EGFR endocytosis that, despite years of research, is not yet fully understood.

Subdiffractional tracking of internalized molecules (sdTIM) is a single-molecule technique for dissecting heterogeneous motion states of endocytic compartments in live neurons

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Our understanding of endocytic pathway dynamics is severely restricted by the diffraction limit of light microscopy. To address this, we implemented a novel technique called the subdiffractional tracking of internalized molecules (sdTIM). This allowed us to track vesicle-associated membrane protein 2 (VAMP2)-pHluorin-bound anti-GFP Atto647N-tagged nanobodies trapped in synaptic vesicles (SVs) in the crowded presynaptic environment of live hippocampal neurons. With 36-nm localization precision, we revealed that, once internalized, VAMP2-pHluorin-bound Atto647N-tagged nanobodies exhibited a markedly lower mobility than on the plasma membrane, an effect that was reversed upon restimulation in presynapses but not in neighbouring axons. Using Bayesian model selection applied to hidden Markov modelling, we found that SVs oscillated between diffusive states or a combination of diffusive and transport states with opposite directionality. Importantly, SVs exhibiting low diffusive motion were relatively less likely to switch to the transport motion. The technique has also been adapted to track signalling endosomes containing cholera toxin subunit-B (CTB), and autophagosomes containing botulinum neurotoxin type A (BoNT/A), destined to undergo activity-dependent internalization and retrograde transport in hippocampal neurons cultured in microfluidic devices. The sdTIM method offers a unique and highly transferable approach to track a variety of subdiffractional endocytic structures in live neurons and other cell types.
During endocytosis, cell surface components including housekeeping receptors and activated signaling receptors are delivered to early endosomes, where they are sorted to be either reutilized via recycling pathways or transported towards late endosomes and then lysosomes for degradation. In the latter case, down-regulated receptors and other molecules destined for the lysosomes are incorporated into lumenal invaginations of the early endosome membrane, which are pinched off as free cargo-containing vesicles. These multivesicular regions detach and become multivesicular endosomes, which are transported to and fuse with late endosomes. Eventually, intraluminal vesicles (ILVs) and their cargo are delivered to lysosomes and degraded. Alternatively, ILVs can also be released extracellularly as exosomes, upon fusion of multivesicular endosomes with the plasma membrane. Low-density lipoproteins (LDL) that have been endocytosed are eventually delivered to multivesicular late endosomes. There, cholesteryl esters are de-esterified so that free cholesterol can be released and exported to other cellular destinations. The export mechanisms remain ill defined, but it is clear that disruption of the process has profound consequences for cellular sterol homeostasis and can result in the neurodegenerative disease Niemann-Pick C (NPC). Our previous studies suggest that the fate of LDL-derived cholesterol is linked to the unconventional phospholipid lysobisphosphatidic acid (LBPA) that is abundant in late endosome intraluminal membranes, since interfering with LBPA functions phenocopies NPC at the cellular level. Our approaches to study the mechanisms of ILV formation and endosomal cholesterol transport will be discussed, including biochemical strategies and unbiased high content image-based screens.
Control of endocytic membrane traffic by phosphatidylinositol 3-phosphate

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The membrane lipid, phosphatidylinositol 3-phosphate (PI3P), is enriched on endosome membranes to which it recruits various effector proteins that control several aspects of endosomal membrane dynamics. We have previously been characterising PI3P-binding proteins that mediate endosome fusion, endosomal sorting, endosome motility, cytokinesis and membrane contact site formation. Recently we identified PI3P-binding proteins that control cell signalling, endocytic recycling and macropinocytosis, and we are currently characterising the functional mechanisms of these proteins. We are also studying the involvement of their dysfunction in neurogenerative diseases and cancer.
GPCRs shape their own destiny in constitutive endocytosis through surface activity of the amphipathic helix8

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The existence of helix8 has been shown in a large number of class A GPCRs through functional and structural studies, and the motif has been accepted as a generic motif in this family. Helix8 has been implicated in localization, constitutive trafficking and agonist-induced signaling and trafficking, however, while a consistent protein machinery has been extensively mapped in regard to agonist induced signaling and internalization, no unifying pattern has emerged for the role of helix8 in the localization and constitutive internalization.

We here identify a pivotal role of helix8 in the constitutive trafficking properties of GPCRs. We show that helix8 confers the autonomous internalization properties to the full-length receptors, and demonstrate that chimera where only the helix8 has been switched also transform their constitutive internalization propensities to the full-length receptors. Amphipathic helices not associated with the endocytic machinery as well as an artificial amphipathic can likewise drive autonomous internalization. We further extract a bioinformatic parameter from an empirical combination of hydrophobicity and amphipathic moment. This parameter strongly predicts the extent of autonomous constitutive internalization and is used as a tool predictive tool and for evolutionary analyses. This novel pattern can be used to rationalize previous experimental observations and understand biological behaviors of GPCRs across biology.

Here we demonstrate that membrane-anchored helix8 stimulate constitutive internalization of the Transferrin Receptor in a manner that correlate with their calculated surface activity. This trans effect is regulated through membrane tension and is reproducible with an artificial helix that lack natural protein interaction partners in the cells. We apply the pulsed pH TIRF setup and discern a promiscuous mechanism that act through multiple known endocytic markers. We concomitantly use state-of-the-art three-dimensional STORM microscopy to identify and characterize the morphologies of the helix-induced internalization events both in conjunction with common endocytic markers and in what appears to be independent endocytic event of distinctive tubular morphologies. Altogether, we elucidate a mechanism in which the helix8-containing cargo regulates their own internalization rates through a combination of curvature sensitive recruitments and membrane deformation in several stages of endocytic trafficking.
Mitochondrial inheritance, genome maintenance, and adaptation to metabolic demands all depend on regulated organelle division events. Fission of mitochondrial tubules in human cells depends on the dynamin-family GTPase DRP1 and outer membrane-embedded receptor proteins like MiD49 or MiD51. The mechanisms by which these receptors recruit or modulate DRP1’s activities are unknown. Here we report the structural basis of nucleotide- and conformationally-selective binding of DRP1 by the MiD49/51 receptors. Using cryoEM, we show that upon GTP binding DRP1 adopts an extended conformation that is driven by major allosteric remodeling in the G-domain, BSE, and connecting hinges. The resulting conformation polymerizes into a linear filament stabilized by a network of multi-valent interactions with the MiD49/51 receptors. GTP hydrolysis and nucleotide exchange leads to receptor dissociation and, subsequently, curling of the Drp1 filaments into closed rings. The formation of these constricted rings is independent of, and inconsistent with, proposed mechanisms of G-domain dimerization in trans. The dimensions of the closed DRP1 rings are consistent with the pre-fission, constricted state of mitochondrial tubules observed in human cells. These structures are the first views of receptor- and nucleotide-bound Dynamin-family GTPase and, by comparison with the apo-state structures observed by crystallography, reveal the general principles of allosteric coupling that are responsible for the mechanical force these molecular machines generate.
Finely tuning lysosomal secretion

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Killer cells of the immune system recognize and destroy virally infected and cancer cells with remarkable precision. This is all controlled by polarized secretion of secretory lysosomes from the killer cell. The centrosome plays a key role, docking directly at membrane of the synapse. Exactly how the centrosome moves from one of the end of the cell to the other and focuses secretion at a very defined point is not fully understood. Our recent work has focused on changes in the membrane and how this controls successful centrosome docking and secretion. Strikingly, centrosome docking at the synapse bears remarkable similarities to centrosome docking during ciliogenesis, with contact made by distal appendages on the mother centriole. These parallels have pointed to a series of molecular mechanisms shared at the immunological synapse and sites of ciliogenesis and have provided ways to understand the mechanisms that regulate lysosomal secretion.
Communication between distinct infection associated compartments drives the intracellular lifestyle of Salmonella

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Salmonella Typhimurium is a Gram-negative bacterium that invades non-phagocytic intestinal epithelial cells. The bacteria induce membrane ruffles at the entry site and are engulfed into a membrane enclosure called “Salmonella-Containing Vacuole” (SCV). This SCV generally matures into a bacterial niche where Salmonella resides and replicates. However, in 10% of the infected epithelial cells the SCV ruptures and releases Salmonella into the cytosol where it replicates at high rate. The molecular factors leading either to the maturation or to the rupture of the SCV remains unknown. Salmonella entry is concomitant with the formation of newly formed macropinosomes, termed “Infection Associated Macropinosomes” (IAMs) that originate from the closure of the membrane ruffles. As these IAMs are found in close proximity to the SCV at the entry site, we investigated the potential interaction between both compartments. We found that in most cases IAMs fuse with the SCV within minutes after bacterial internalization, and the small percentage of SCVs that do not fuse with IAMs ruptured, which resulted in Salmonella vacuolar escape. Taken together, these data support a model where SCV-IAM fusion is needed to maintain the SCV membrane integrity, and determines the intracellular fate of Salmonella. In order to identify the host factors involved in SCV-IAMs fusion, we isolated IAMs to high purity and we analyzed their proteomic composition. Comparing the IAM proteome with the previously obtained SCV proteome we identified proteins that coordinate their interaction. Currently, we use cutting-edge dynamic imaging techniques including super-resolution to characterize the interaction between SCV and IAMs. Our approach elucidates new mechanisms of host-pathogen interactions, and more broadly in cell biology defines how distinct endomembrane compartments interact.
Myosin VI and actin dynamics induce membrane constriction and fission at melanosome

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Organelles have distinct and highly dynamic shapes, which are important for their function. By modifying membrane tension, the cytoskeleton and its motors are important regulators of membrane remodeling and organelle shaping. Here we show that myosin VI and actin dynamics function in remodeling the membrane of melanosome, a specialized organelle of skin melanocytes that synthesizes and stores melanin pigment. By constricting melanosomal tubules, myosin VI and WASH-dependent actin polymerization cooperate for the fission and release of those tubular intermediates. This fission process leads to the export of cargoes from melanosome, and importantly is functionally required for melanosome secretion and subsequent transfer of melanin to neighboring keratinocytes. Our data show that myosin VI, together with actin polymerization, controls the fission of transport intermediates. Such remodeling event contributes to the proper function of organelles.
Endosomal trafficking and signalling of integrins and receptor tyrosine kinases in cancer

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Endosomal trafficking of integrins is established as a key regulator of cell motility. Endocytosis and recycling of these major cell surface adhesion receptors facilitates turnover of focal adhesions and regulates formation of nascent cell protrusions. In particular, altered integrin endosomal traffic drives invasion and metastasis of cancer cells. Crosstalk between integrins and receptor tyrosine kinases (RTK) functions on multiple levels with adhesion enhancing RTK signalling on the plasma membrane and growth factor stimulus triggering enhanced integrin traffic. In addition, co-endocytosis of integrins and RTKs gives rise to additional endosomal signalling pathways providing cancer cells with anoikis resistance of prolonged RTK signalling. I will describe our recent findings on integrin traffic and their endosomal signalling in conjunction with RTKs in breast cancer.
Unraveling the mechanism of ER-associated endosome fission

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ER membrane contact sites (MCSs) define the position where early and late endosomes undergo constriction and fission. ER tubules are recruited to the divide between cargo sorting domains on endosomes that will then constrict and undergo fission. Our aim was to purify ER factors that regulate MCS formation and fission in animal cells. Based on the identity of the candidate proteins, we hope to be able to test hypotheses about the mechanisms used by the ER to drive organelle constriction and division at MCSs. Our strategy to identify MCS proteins was to target a promiscuous biotin ligase (BirA) to ER-associated fission sites. BirA has a modification reach to approximately 30nm, which is ideal for bridging membrane contacts (which have a typical spacer distance of ~5-15nm). Our hybrid construct localizes as predicted and biotinylates ER proteins at MCSs in mammalian cells that we could purify and then identify by mass spec. This strategy has identified ER proteins that regulate ER-associated endosome fission.
An iron hand over cancer stem cells

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Cancer stem cells (CSCs) represent a subset of cells within tumours that exhibit self-renewal and tumour seeding capacity. CSCs are typically refractory to conventional treatments and have been associated to metastasis and relapse. Salinomycin operates as a selective agent against CSCs through mechanisms that remain elusive. We have provided evidence that a synthetic derivative of salinomycin, which we named ironomycin (AM5), exhibits a more potent and selective activity against breast CSCs in vitro and in vivo, accumulates and sequesters iron in lysosomes. In response to the ensuing cytoplasmic depletion of iron, cells triggered the degradation of ferritin in lysosomes, leading to further iron loading in this organelle. Iron-mediated production of reactive oxygen species promoted lysosomal membrane permeabilization, activating a cell death pathway consistent with ferroptosis. These findings reveal the prevalence of iron homeostasis in breast CSCs, pointing towards iron and iron-mediated processes as potential targets against these cells.

Nature Chemistry DOI: 10.1038/NCHEM.2778
Mechanisms and implications of mitochondrial antigen presentation

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Parkinson's disease is caused by the degeneration and death of dopaminergic neurons, however the precise molecular mechanisms underlying this demise has remained unclear. Genetic studies in patients have identified a host of mutations linked to inherited forms of PD, including mutations in Parkin and PINK1, a ubiquitin ligase and serine/threonine kinase, respectively. These two proteins are known to mediate the degradation of depolarized or dysfunctional mitochondria within autophagosomes, a process termed mitophagy. However, they have also been linked to the generation and transport of mitochondrial derived vesicles, to proteolytic turnover of cytosolic cargoes, and to the activation of innate immune pathways. This has made the true contributory function of mutant PINK1 and Parkin to dopaminergic cell death difficult to determine. We recently characterized a novel cell biological pathway that drives the presentation of mitochondrial antigens to cytotoxic CD8$^+$ T-cells, and revealed a central function for PINK1 and Parkin in repressing this process to ensure mitochondrial content remains immunologically silent. Since this initial study microbiologist Samantha Gruenheid (McGill) and electrophysiologist/PD scientist Louis-Eric Trudeau (University of Montreal) have joined Michel Desjardins (U de M) and myself in examining the physiological contribution of these PD genes to MitAP and the degeneration of dopaminergic neurons in vivo. These results will be presented.

Supported by a Michael J Fox Foundation Validation Grant and CIHR MOP 133549
The interdependence of vesicular membrane dynamics and signal processing

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The proto-oncogenic epidermal growth factor receptor (EGFR) is a transmembrane receptor tyrosine kinase whose sensitivity to growth factors and signal duration determines cellular behavior. We resolved how these response dynamics originate from bidirectional interactions of EGFR with protein tyrosine phosphatases (PTPs) on distinct membranes. Quantitative imaging of EGFR phosphorylation in response to quantifiable genetic perturbations of PTPs, uncovered endoplasmic reticulum associated PTPN2 and the receptor-like PTPR-G at the plasma membrane as major EGFR dephosphorylating activities. Spatial-temporal PTP reactivity maps and EGFR response modulation in single cells revealed that recursive interactions of ligandless, autocatalytically activated EGFR with PTPRG and PTPN2 control responsiveness to EGF at the plasma membrane, whereas interactions of ligand-activated EGFR with PTPN2 throughout cytoplasmic space determine signal duration. Vesicular dynamics thereby couples EGFR activity to these PTPs on juxtaposed membranes, unifying their interactions into a network that dictates when and how to respond to growth factors.
Defining a novel role of distinct membrane trafficking regulators in blood glucose homeostasis

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The increase in the prevalence of type 2 diabetes has reached a qualified epidemic stage. The liver plays a central role in the development of type 2 diabetes due to its function in maintaining whole body glucose homeostasis. This is achieved by two cellular modules: glucose metabolism and endocytosis. Endocytosis is required for the uptake and signal transduction of growth factors, signaling receptors and hormones. The storage or release of glucose through a tight control of glucose metabolism allows maintaining whole body glucose homeostasis. These two modules need to be synchronized to collectively control glucose metabolism, where dysfunctions in this process promote the development of type 2 diabetes.

Recent evidences suggest that endocytosis participates in regulating glucose metabolism. First, overexpression of Rab25 in mammalian cells increased cellular glycogen stores to enhance cellular survival during energetic stress. Second, depletion of the endosomal PI-3-Kinase, Vps34 in mice in vivo led to hepatomegaly, hepatic steatosis and to a defect in glycogen storage. Third, knockdown of Rab5 in mouse liver caused hypoglycemia due to an inhibition of liver glucose production. Strikingly, loss of Rab5 in diabetic db/db mice rescued their elevated blood glucose levels highlighting a potential therapeutic application.

Here, we report a novel trafficking player in the control of liver metabolism, Rab24, which functions at the intersection between endoplasmic reticulum, endosomes and mitochondria and is required for autophagosome maturation. We find Rab24 to be upregulated in livers of high-fat diet mice and in diabetic patients with fatty liver disease. Depletion of Rab24 increases mitochondrial mass and function in primary mouse hepatocytes in vitro and improves glucose clearance and serum cholesterol levels in vivo, suggesting a regulatory role on glucose and lipid metabolism. Altogether, our data provide novel insights into the intracellular regulation of liver metabolism and pave the way for their exploitation in the control of hepatic glucose homeostasis and thus the treatment of type 2 diabetes.
The role of endocytosis in NF-κB signaling mediated by lymphotoxin beta receptor (LTβR)

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Cytokine receptors from the tumor necrosis factor receptor (TNFR) superfamily activate NF-κB signaling and undergo internalization upon ligand binding. We previously demonstrated that depletion of ESCRT-I components leads to endosomal accumulation of cytokine receptors (TNFRI, lymphotoxin beta receptor; LTβR) and their ligand-independent activation of NF-κB signaling. To further investigate a link between membrane transport and inflammatory signaling we performed microscopic high-content screen for regulators of endosomal trafficking of LTβR.

We found the HOPS complex and Rab7A, both required for late endosome function, as potential hits. Knockdown of HOPS components or Rab7A caused accumulation of ligand-free LTβR on endosomes, as observed upon ESCRT-I depletion. In addition, degradation of LTβR was perturbed after Rab7A knockdown. Moreover, we observed increased interactions between LTβR and its signaling adaptors TRAF2 and TRAF3 upon knockdown of HOPS components or Rab7A. However, unlike upon ESCRT-I depletion, NF-κB signaling was not induced under such conditions. We demonstrated that LTβR accumulated on the limiting membrane of endosomes after ESCRT-I depletion. In contrast, HOPS components or Rab7A knockdown caused LTβR accumulation inside endosomes, probably in intraluminal vesicles.

We propose that impairment of endocytic sorting of LTβR can cause its perturbed localization and accumulation. However, NF-κB signaling is induced only when LTβR accumulates on the limiting membrane of endosomes.
Collective behaviour is a complex behaviour in which understanding of the individual single cells does not necessarily explain the collective behaviour of the population of cells. The unexplainable behaviour is the self-organization of the system. Recently, model systems have been developed from stem cells that can self-organize into organoid structures in vitro. In particular, a model system of intestinal organoid structures in vitro, which recapitulates most of the processes of morphogenesis and patterning observed in intestinal tissue, is here used to unravel the determinants of pattern formation and the functional genetic interactions involved in self-organization and symmetry breaking events. To understand this process in a quantitative manner, advanced single-cell imaging and image analysis of intestinal stem cells during 3D organoid development are applied. Initially, a 3000 compounds perturbation screen targeting, amongst others, signalling kinases, membrane trafficking and lipid metabolism genes has been performed to map regulatory genetic interactions. Together, this revealed a unique genetic interaction map of symmetry breaking in pattern formation, which will find general applications beyond the specific model system of the intestinal organoid. One major finding from the image-based compound screen is the role of lipid metabolism in regulating stem cell proliferation. We then explored how basal lipid metabolism contributes to intestinal stem cells self-renewal and patterning. Using cutting-edge single-cell imaging, lipidomic and metabolomics analysis, combined with chemical and genetic tools our results show that different cells types have remarkably different lipid compositions. Preliminary results show that intracellular lipid processing is required to maintain a proper rate of stem cell proliferation. Our current hypothesis is that proliferation relies on metabolic cues, and to ensure robustness independently of nutrient level fluctuations, intestinal stem cells might have evolved an adaptive buffering mechanism to balance energy supply and signaling.
Roles of lysosomal cholesterol in mTORC1 signaling and growth regulation

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In order to grow, cells must tightly coordinate biosynthetic processes with catabolic programs. The master regulator, mechanistic target of rapamycin complex 1 (mTORC1) kinase, integrates signals from nutrients, growth factors and energy to drive cellular growth and homeostasis. Among the nutrient inputs that regulate mTORC1, lipids are currently the least understood. Using biochemical assays in vitro and in intact cells, we recently found that mTORC1 senses the lipid composition of the lysosomal membrane. Cholesterol carried by low-density lipoproteins activates mTORC1 via the heterodimeric Rag GTPases, which anchor mTORC1 to the lysosomal surface. Lipidomics analysis coupled with functional manipulations suggests that a local cholesterol pool modulates protein-protein between the Rag GTPase and the Ragulator complex, leading to mTORC1 recruitment. Together, these data identify lysosomal cholesterol as novel regulatory inputs to mTORC1 and implicate that cholesterol traffic through the endomembrane system may play an important role in mTORC1 signaling. We are currently exploring the functional relationship between mTORC1 and cholesterol transporting proteins such as Niemann-Pick C1, and how physical contacts between lysosomes and acceptor organelles may contribute to NPC1-mediated cholesterol unloading and mTORC1 regulation.
TORC1 Organised in Inhibited Domains (TOROIDs) regulate TORC1 activity

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The Target of Rapamycin complex 1 (TORC1) protein kinase is a master regulator of eukaryotic growth and metabolism and its dysregulation in humans has been implicated in cancer and metabolic syndrome. GTPases, responding to signals generated by abiotic stressors, nutrients, and, in metazoans, growth factors, play an important, but poorly understood role in TORC1 regulation. We found out that, in budding yeast, glucose withdrawal, which leads to an acute loss of TORC1 kinase activity, triggers a similarly rapid Rag GTPase-dependent reorganisation of TORC1 in the cell. We could identify, both by super-resolution optical microscopy and 3D reconstructions of cryo-electron micrograph (cryo-EM) images, that inhibited TORC1 molecules organise in a novel structure which we name a TOROID (TORC1 Organised in Inhibited Domain). Guided by this new structure, we could describe the first molecular mechanism of TORC1 inhibition in yeast.
FRET mice, tools to visualize protein kinase activities in living organs.

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Protein kinases play critical roles in most, if not all, signal transduction pathways including those regulating the endocytic pathways. Therefore, by knowing the activities of the critical protein kinases in each cell, we are able to estimate the activities of the signal transduction pathways of interest in each cell, tissue, and organ. Based on this concept, we have developed a series of transgenic mouse lines expressing FRET biosensors for PKA, ERK, JNK, Tak1, ROCK, AMPK, and S6K. These FRET biosensors have revealed heterogeneity and dynamics of protein kinase activities. We are currently focusing on the fluctuation of ERK activity. In epithelial basal layer cells, stochastic ERK activation in a few cells is propagated laterally up to ca. 50 µm in 30 min, which phenomenon was named stochastic propagation of radial ERK activity distribution (SPREAD). Frequency of SPREAD was increased by topical application of tumor promoter PMA. At the wound edge, the ERK activation wavelets were superimposed to generate large parallel waves, which reached up to several hundred micrometers. In contrast, SPREAD was not observed in the bladder, where urothelial cells form stratified layers as do epithelial cells. Moreover, ERK was not activated during the wound healing of urothelial cells. In agreement with this finding, the MEK inhibitor Trametinib perturbed the wound healing of epidermis, but not of urothelium, suggesting that urothelial cells and epidermal cells fix the wound by different molecular mechanisms. In the crypt of intestine, intestinal epithelial cells also exhibited stochastic ERK activation, and propagation of ERK activation to neighboring cells. By using in vitro crypt culture, we found that both the basal activity and stochastic activation of ERK contributed to cell cycle progression. In conclusion, stochastic ERK activation and ERK activity propagation are phenomena that are observed in not only epidermal cells but also intestinal epithelial cells. I will also show the contribution of EGF-family ligands to the stochastic activation and propagation of ERK activity.
Macropinocytosis and Ras signaling in cancer

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Cancer cells harboring oncogenic Ras undergo metabolic reprogramming that is characterized by the enhanced utilization of anabolic substrates such as glucose and glutamine for macromolecular synthesis. A ubiquitous consequence of oncogenic Ras activation is the stimulation of macropinocytosis - a non-selective endocytic mechanism that mediates the bulk uptake of extracellular fluids and their constituents. Tumor cells expressing oncogenic Ras take up albumin and other proteins via macropinocytosis and transport them into the lysosome where they are being degraded to produce free amino acids that in turn can be used as a source for TCA cycle anaplerosis. Inhibition of macropinocytosis has a pronounced effect on tumor development suggesting that the targeting of macropinocytosis might have therapeutic potential. In this context, efforts are underway to identify modulators of macropinocytosis. The results of these efforts will be discussed.
Control of endosomal maturation and receptor recycling by a tyrosine kinase signalling pathway: its role in cancer and in health.

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Elevated levels of growth-promoting receptors on the cell surface can contribute to oncogenesis and resistance to targeted therapy. We previously found that the tyrosine phosphatase PTPN14 (also called Pez) limits the recycling of endocytosed receptors back to the cell surface (Belle et al, 2015). We have now identified the key substrate of PTPN14 that regulates receptor recycling and the upstream kinase through which this regulation occurs. In this pathway, the protein kinase PKCδ when phosphorylated on Y374 limits trafficking to lysosomes and promotes recycling. PTPN14 limits this activity by dephosphorylating pY374-PCKδ. Phosphorylation of PKCδ leads to inhibition of the Rab5 to Rab7 exchange on nascent late endosomes, which is necessary for late endosome-lysosome fusion, thereby favouring the recycling path. The kinetics of recruitment of pY374-PKCδ to endosomes indicates that pY374-PKCδ is recruited early in the endosome trafficking itinerary and this is necessary for recycling to occur. Subsequent recruitment of PTPN14 results in dephosphorylation of pY374-PKCδ, to allow Rab5 shedding and continued maturation of late endosomes. This role of PTPN14 is consistent with the finding that PTPN14 is mutated in a proportion of breast and other cancers. Importantly, we find that in a subset of triple negative breast cancer (TNBC) cell lines and a cohort of TNBC patients, pY374-PKCδ levels are elevated and this corresponds to the increased presence of a stabilised hybrid Rab5-Rab7 endosomal compartment indicative of stalled endosomal maturation and the increased activation of multiple RTKs. This suggests that targeting the PTPN14-PKCδ-TK axis may lead to novel therapeutics for this cohort of TNBC patients. Targeting this axis may also overcome compensatory signalling by other RTKs in patients that are resistant to anti-EGFR therapy. We will also present data on a new PTPN14 knock-out mouse, which has elevated pY374-PKCδ levels. This mouse has defects in the epithelia of multiple organs, including hyperplasia and loss of polarity. The role of endosomal trafficking in the resultant phenotype will be presented.

A new inhibitor targeting BRAG2, an endocytic ArfGEF involved in cancer

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Small GTPases of the Arf family orchestrate many aspects of membrane traffic and organelle structure. Arf guanine nucleotide exchange factors (ArfGEFs) activate Arf small GTPases by stimulating their GDP/GTP exchange at the membrane-cytosol interface. Endocytic ArfGEFs, such as BRAG2/IQSec1, combine a conserved catalytic domain and a downstream Plekstrin Homology (PH) domain with distinct regulatory and membrane binding properties (Nawrotek et al. Small GTPases 2016). Among other functions, BRAG2 regulates cell surface levels of cadherin and integrin receptors and has been implicated in invasiveness and tumor metastasis in breast cancer, in melanoma and in pancreatic cancer cell lines. BRAG2 is thus an attractive therapeutic target, and there is a need to decipher its molecular mechanisms in both normal and disease-related signaling pathways. We present the characterization of Bragsin, a novel small molecule inhibitor that efficiently targets BRAG2-mediated activation of Arf when reconstituted on artificial membranes. We show that membranes are required for in vitro inhibition and that Bragsin affects Arf activation in cell. By combining our results with the crystallographic structure of Bragsin bound to BRAG2, we propose that Bragsin specifically inhibits BRAG2 by altering the geometry of the GEF/membrane interaction thus representing a novel mode of inhibition. These results reveal a novel concept for inhibiting GEF activation of small GTPases on a membrane.
Potential Roles for Receptor Endocytosis and Degradation in Cancer Therapy

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Tumor-specific combinations of oncogenic mutations often free cancer cells from their reliance on growth factors. One important example comprises the epidermal growth factor receptor (EGFR) and its kin, HER2. In tumors, both EGFR and HER2 frequently display overexpression, internal deletions and point mutations. Accordingly, monoclonal antibodies (mAbs) and tyrosine kinase inhibitors (TKIs) specific to these receptors have been approved for clinical applications. My lecture will introduce EGFR and HER2 in the context of a signaling network comprising two additional receptors, HER3 and HER4, and 11 growth factors, all sharing an EGF-like structure and binding to HER family members.

The principles of network biology, such as rewiring, robustness and pathway redundancy, translate to short-term responses to oncology drugs. In other words, patients treated with drugs intercepting EGFR or HER2 often develop resistance due to emergence of compensatory mechanisms. My lecture will exemplify these principles, especially potential roles played by receptor endocytosis and degradation, in context of advanced non-small cell lung tumors that acquire resistance to EGFR’s TKIs.
Regulation of endoplasmic reticulum turnover via selective autophagy

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The endoplasmic reticulum (ER) is the largest intracellular endomembrane system enabling synthesis and transport of cellular components. Constant ER turnover is needed to meet different cellular requirements and autophagy plays an important role in this process. ER sequestration in autophagosomes has been originally documented in yeast and it was suggested to regulate the cellular amounts of ER. However, the underlying regulatory mechanisms remain elusive. We have recently shown that FAM134B protein interacts with LC3, via conserved LC3-interacting region (LIR), and thereby serves as ER-specific receptor facilitating ER degradation by autophagy (ER-phagy). Moreover, FAM134B overexpression stimulates ER fragmentation and delivery to lysosomes via the autophagy pathway. Conversely, blockade of autophagy or depletion of FAM134B triggers a marked increase in the ER volume. Mutations of FAM134B in humans are unable to act as ER-phagy receptors and cause sensory neurodegeneration underscoring the physiological relevance of ER-phagy. The major question we are exploring at the moment deals with the regulatory mechanisms that co-ordinate the action of these receptors during the ER-phagy. I will describe the identification and characterization of novel ER-phagy receptors for tubular ER in mammalian cells.
Impaired axonal lysosome transport contributes to Alzheimer’s pathology

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Through a comprehensive analysis of organellar markers in mouse models of Alzheimer’s disease (AD), we documented a massive and robust accumulation of lysosome-like organelles at amyloid plaques and establish that the majority of these organelles reside within swollen axons that contact the amyloid deposits. Notably, we discovered that lysosomes that accumulate in such axons are lacking in multiple soluble luminal proteases and thus predicted to be unable to efficiently degrade proteinaceous cargos. Of relevance to AD, β-secretase (BACE1), the protein that initiates amyloidogenic processing of the amyloid precursor protein (APP), is a substrate for these proteases and builds up at these sites. Furthermore, through a comparison between the axonal lysosome accumulations at amyloid plaques and neuronal lysosomes of the wildtype brain, we identified a similar, naturally occurring, population of lysosome-like organelles in neuronal processes that is also defined by its low luminal protease content. In conjunction with emerging evidence that the lysosomal maturation of endosomes and autophagosomes is coupled to their retrograde transport, our results suggested that extracellular β-amyloid deposits cause a local impairment in retrograde axonal transport, leading to the accumulation of lysosome precursors and a blockade in their further maturation.

In testing this hypothesis, we have identified JNK interacting protein 3 (JIP3) as an important regulator of both axonal lysosome abundance and maturation state. JIP3 KO neurons accumulate lysosomes within focal axonal swellings that closely resemble the dystrophic axons at amyloid plaques- including high levels of APP processing enzymes (BACE1 and presenilin 2) and are accompanied by elevated Aβ peptide levels. We tested our hypothesis that these traffic jams of axonal lysosomes could thus potentially serve as disease-relevant sites of APP processing by depleting JIP3 in a mouse model of AD. We observed that JIP3 haploinsufficiency strongly increased both the abundance and size of amyloid plaques. These results establish a critical role for efficient axonal lysosome transport and maturation in protecting the brain from amyloid plaque pathology.
Skeletal muscle comprises approximately 40% of our body weight and has received significant scientific attention given its important contribution to mobility, exercise capacity, glucose regulation, longevity and healthy aging. Muscle is equipped with elaborated membrane and cytoskeleton structure designed for excitation–contraction coupling and force generation; however, how its membrane and cytoskeleton organization is built and maintained remains unclear. Dynamin-2 is one of the best-studied mechanochemical GTPases which convert the chemical energy into mechanical force to catalyze membrane fission. Therefore, dynamin-2 is critical for regulating endocytosis and is essential for cell signaling, survival and growth. Recently, many research groups have found that mutations of the ubiquitously expressed dynamin-2 would lead to a tissue-specific human congenital disease, centronuclear myopathy (CNM). To understand the cellular functions of dynamin-2 in muscle and its pathogenic mechanisms of CNM-associated mutations, we utilize biochemistry, cell biology and model organism to explore its effects. We find that dynamin-2 regulates multiple membrane remodeling events during myogenesis. Importantly, the CNM-associated dynamin-2 mutations lose its auto-inhibition ability thus become hyperactive, and their irregular membrane fission activity result in disruption of the unique membrane domain of muscle plasma membrane, transverse-tubule. We further find a negative regulation mechanism for dynamin-2 activity in muscle which is mediated by its interaction with Bin1, another CNM-causing protein. Intriguingly, defect in dynamin-2 and Bin1 interaction underpins certain Bin1-associated CNM. Together, our findings demonstrate that skeletal muscle is highly sensitive to the membrane fission ability of dynamin-2 thus its activity must be tightly regulated in this tissue. Mutations lead to the hyperactivity of dynamin-2 would cause transverse-tubule fragmentation and defective excitation–contraction coupling thereby muscle disorder.
Dynamical mechanics of the ESCRT-III complex

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Cells and organelles are delimited by lipid bilayers. Since these membranes are impermeable to most solutes, in order to exchange material with their environment, organelles and cells have developed a large protein family involved in budding membranes to form membrane carriers. These carriers transport material between organelles. Proteins involved in intracellular membrane traffic can remodel the membrane by several ways. Clathrin, for example, polymerizes into a spherical cage onto the membrane, forcing it to curve. Here we describe a recently discovered protein complex called ESCRT-III, which has the property of forming spirals at the surface of the lipid bilayer. This unique structural feature did not suggest any known mechanism by which it could deform the membrane. It was theoretically proposed that, while growing into a spiral, it accumulates stress energy which can be released by buckling of the central part of the spiral1. By using high-speed AFM and biophysical tools to measure membrane elasticity we show how the elastic and polymerization properties of the ESCRT-III filament are compatible with such model2. We further investigated the dynamics of the complex when the Vps4 - an ATPase that causes the ESCRT complexes to disassemble - is present. We found that Vps4 promotes a dynamic instability within the ESCRT polymers in a similar way than for actin or microtubules. We propose that this instability is necessary for assembly in presence of growth inhibiting subunits Vps2 and Vps24, and to allow constriction by relaxation of elastic stress within large ESCRT assemblies3.

Recruitment dynamics of ESCRT-III and Vps4 to endosomes and implications for the mechanism of ESCRT-mediated reverse membrane budding

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The ESCRT machinery mediates reverse membrane budding on endosomes, on the nuclear envelope, and at the plasma membrane. By quantitative fluorescence lattice light-sheet microscopy, we show that ESCRT-III subunits polymerize rapidly on yeast endosomes, together with recruitment of at least two Vps4 hexamers. During their 5-45 second lifetimes, the yeast ESCRT-III assemblies accumulate 75-200 Snf7 and 15-50 Vps24 molecules and recruited additional Vps4 hexamers. Membrane budding required continuous, stochastic exchange of Vps4 and ESCRT-III components, rather than steady growth of fixed assemblies, and depended on Vps4 ATPase activity. Acute disruption of Vps4 recruitment stalled membrane budding. An all-or-none step led to concomitant release of ESCRT-III and Vps4. From these quantitative observations we propose a model in which multiple Vps4 hexamers stochastically constrict several ESCRT-III filaments to generate the neck of a nascent bud, leading to membrane constriction and ultimately to vesicle fission.
Clathrin-coated membrane domains are new cellular mechanosensors

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The extracellular matrix (ECM) can influence many cellular processes such as gene expression, proliferation, and differentiation. This implies that cells need to sense physical properties of the surrounding extracellular matrix. Integrins are among the most important proteins with regards to the communication with the extracellular environment and it is necessary for cells to finely tune the levels of integrins and receptors exposed to the ECM in order to properly answer to the needs imposed by the environment. To do so, cells utilize Clathrin-Mediated Endocytosis, their most prominent endocytic route. Endocytosis is therefore needed not only to internalize nutrients, but also to sense the environment surrounding the cell. Clathrin-Coated Pits (CCPs) represent the endocytic units of cells, but they are not the only existing clathrin-coated structures. In fact, Clathrin-coated membrane domains (CCMDs) have also been observed. Those domain are flat and bigger than classical CCPs; in addition they are extremely stable at the plasma membrane and their function is not clear.

Given that CCMDs only form upon adhesion, we reasoned that they could be extracellular matrix sensors. We observed that CCMDs only form when cells adhere on a stiff surface and they are completely lost when cells grow on soft substrates. We showed that activated signaling receptors (EGFR, C-Met) accumulate in CCMDs and signal from this location. In particular, signaling downstream of EGFR from CCMDs leads to an increased ERK phosphorylation ultimately resulting in increased proliferation. We conclude therefore that CCMDs are mechanosensors of the cell. CCMDs formation, however, does not depend on the actin cytoskeleton. Instead, the integrin dimer AlphaV-Beta5 is fundamental to form CCMDs and removal of integrin Beta5 results in loss of CCMDs without affecting other mechanosensitive structures such as focal adhesions. We propose that a stiff extracellular matrix can drive increased proliferation via CCMDs formation and given that one characteristic common to most tumors is increased stiffness, the study of CCMDs could open the road to new cancer therapies.
Physical Mechanisms of Cell Organization on Micron Length Scales

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Cells are organized on length scales from Angstroms to microns. But the mechanisms by which Angstrom-scale molecular properties are translated to micron-scale macroscopic properties are not well understood. We have shown that interactions between multivalent proteins and multivalent ligands can cause oligomerization and concomitant liquid-liquid phase transitions, resulting in formation of micron-sized liquid droplets in aqueous solution and micron-sized puncta on membranes. Through this idea of multivalency-driven phase transitions we have explained behaviors of multidomain proteins, intrinsically disordered proteins and nucleic acids. I will discuss how such transitions may control the spatial organization and biochemical activity of actin regulatory signaling pathways, and contribute to formation of membraneless organelles, including PML nuclear bodies and P bodies. Our data suggest a general mechanism by which cells may achieve micron-scale organization based on interactions between multivalent macromolecules.
The tight junction is a complex lipid-protein assembly that is located at the interface between the apical and lateral membrane of epithelial cells. While the main function of the tight junction, the control of para-cellular transport, is achieved by adhesion/polymerization of the claudin protein family, a scaffold on the cytoplasmic side is required for tight junction assembly, localization and signaling. This highly dynamic scaffold is formed by proteins of the MAGUK family (ZO1, ZO2, ZO3) and it sequesters cytoskeleton, signaling and key polarity proteins. In this work, we discovered the molecular basis of scaffold formation and sequestering of interaction partners to the tight junction. We found that the ZO proteins can undergo condensation into liquid domains in vitro and in vivo. Condensation results from low affinity interactions of the intrinsically disordered C-termini of the ZO-homologs, with different self-assembly affinities for each homolog. Only the condensed scaffold efficiently recruits interaction partners via its multiple protein-protein interaction modules (PDZ, SH3, GUK). This interaction/recruitment switch is the result of the low affinity binding of ligands to PDZ domains in combination with the high concentration of binding sites within the condensed scaffold. Our findings provide a new molecular function for the complex structure of ZO-proteins. The low complexity C-terminus provides ability to self-organize into a condensed scaffold. The N-terminal protein-protein interaction cassette provides specificity for recruitment of client proteins with an affinity that is tuned to be effective only within the condensed scaffold. Overall, this work explains the supra-molecular structure, dynamics and function of the tight junction and it has implications for the role of the tight junction in polarity and transcriptional regulation.
Leishmania donovani modulates endo-lysosomal pathway by up regulating Rab5a for its successful survival in macrophages

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Successful intracellular pathogens avoid their lysosomal transport by modulating the intracellular trafficking pathways for their successful survival in the host cells. However Leishmania is suggested to successfully carve its niche and reside in a phagolysosomal compartment called Leishmania Parasitophorous vacuole (PV) by virtue of acquiring Lamp1, vacuolar ATPase, and CathepsinD. However, in the present study we have found that Leishmania donovani infection specifically upregulates the expression of Rab5a in THP-1 differentiated human macrophages and human PBMCs. Subsequently, L. donovani recruits and retains Rab5a and its effector EEA1 on its PV to reside in modified early endosomes in THP-1 differentiated human macrophages and human PBMCs. Consequently, we also have observed that recruitment of higher amounts of Rab5a by fusing with Lamp1 and Pro-CathepsinD containing vesicles. Thus, Leishmania PV contain Lamp1 and Pro-CathepsinD which results into reduce proteolytic capacity of the PV in infected cells. Furthermore, siRNA mediated knockdown of Rab5a in human macrophages significantly inhibits the survival of the parasite. These results provide the first mechanistic insights how Leishmania remodeling of endo-lysosomal trafficking to reside in a specialized early endocytic compartment. Furthermore this study also raises the possibility of modulating endo-lysosomal pathway in parasite infected cells by small molecules to divert trafficking of Leishmania probably to lysosome which might be useful for developing future therapeutic intervention.
Actin comets in Lowe syndrome and clathrin-mediated endocytosis are controlled by a coincidence of phosphoinositides and membrane curvature

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Actin polymerization accompanies the invagination and scission of a subset of clathrin-coated vesicles. Despite long-standing models of Rho GTPase activation dictating localised actin dynamics, it is unknown how the endocytic cycle prompts actin reorganization. We have found that the coincidence of phosphatidyinositol 4,5-bisphosphate (PI(4,5)P2), phosphatidylinositol 3-phosphate (PI(3)P) and high membrane curvature during clathrin-mediated endocytosis triggers actin polymerization. This occurs through BAR domain protein SNX9, N-WASP/WIP, Cdc42 and the Arp2/3 complex. The structure of SNX9 determines binding for PI(4,5)P2, PI(3)P and curvature, while Cdc42 activation is responsive to PI(4,5)P2 alone. PI(3)P arises from curvature-stimulated INPP4A-mediated hydrolysis of phosphatidyinositol (3,4)-bisphosphate prior to scission. The hierarchy of curvature-sensitive PI(3)P production and binding steps increases the actin response to highly-curved membranes in a process we name "curvature cascade amplification". Furthermore the actin comets observed in cells lacking oculocerebrorenal syndrome of Lowe (OCRL) are reduced by PI 3-kinase inhibitors. This shows a role for PI(4,5)P2/PI(3)P-triggered actin polymerization in human disease and suggests a possible therapeutic strategy in Lowe syndrome.
Asymmetric endosomal trafficking in asymmetric division

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Asymmetric cell division is used for asymmetric cell fate assignation and self renewal of stem cells. We have previously shown that trafficking of endosomes is asymmetric during the asymmetric division of sensory organ precursors in Drosophila. We have now shown the same phenomenon in asymmetric neural stem cells in fish and flies as well as in intestinal stem cells. Our data show that the FYVE domain protein Sara is required for asymmetric targeting of endosomes. Interestingly, the signaling cargo, the receptor Notch, also tags the endosomes to move asymmetrically. Finally we studied the mechanism of asymmetric motility, which involves the asymmetric organization of the spindle and implicates machineries involved on the dynamics of the minus end of microtubules. We finally explore the physics of the process.
Misregulation at different regulatory nodes of an endocytic regulatory network underlies phenotypic diversity of a range of Notch disease-associated mutations

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The endo-lysosomal pathway plays an important role in modulating the activity of membrane signalling receptors, directing them into intraluminal vesicles of the endosomal lumen, ultimately for their degradation. However the endocytic pathway can also have a positive effect by bringing together components into an appropriate signal-competent environment or "signalosome". Hence endocytic flux and residency time of signalling components in different endosomal locations can shape the dynamics of signalling activity in different ways. Notch receptor signalling is one such pathway whose signalling levels are tuned by endocytic flux and sorting between different endosomal pathway domains. Using the Drosophila model system we have found that Notch can traffic through network of competing endocytic routes, both clathrin-dependent and independent, with respectively positive or negative consequences on signalling output. We found that the choice between alternative means of entry of Notch into the cell depends on Deltex and Suppressor of deltex, which are two ubiquitin ligase proteins respectively of the ring finger and HECT domain families. Deltex promotes clathrin-dependent endocytosis of Notch and further acts to retain Notch on a specific clathrin enriched platform of the endosomal outer membrane. This means that the Notch intracellular domain is exposed cytoplasmically and can be proteolytically released in order to traffic to the nucleus and regulate gene transcription. Suppressor of deltex promotes Notch entry into a clathrin-independent, cholesterol-dependent endocytic route that directs Notch to endosomal domains that can be marked with gpi-GFP and has a negative effect on Notch signalling through transfer of Notch off the endosomal surface membrane into intraluminal vesicles (ILVs). Ubiquitination of Notch by Suppressor of deltex is not required for Notch endocytic entry but is required for transfer of Notch into ILVs. The highly conserved Notch gene has complex genetics with a variety of differently located missense mutations linked to different loss and gain of function developmental and disease associated phenotypes. We have mapped the consequences of several of these mutations to misregulation at different regulatory nodes in the regulatory endocytic network suggesting underlying differences in cell biology of Notch misregulation underlie diverse phenotypic effects at the tissue and organismal level.
Epsin-dependent ligand endocytosis activates Notch by force

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DSL ligands activate Notch by inducing proteolytic cleavage and shedding of the receptor ectodomain — an event that requires ligand to be endocytosed in signal sending cells by the adaptor protein Epsin. Two classes of explanation for this unusual requirement are: (i) recycling models, in which ligand must be endocytosed to be modified or repositioned before it can bind Notch, and (ii) pulling models, in which ligand must be endocytosed after it binds Notch to exert force that exposes an otherwise buried site for cleavage. We demonstrate in vivo that ligands that cannot enter the Epsin pathway nevertheless bind Notch but fail to activate the receptor because they cannot exert sufficient force. This argues against recycling models and in favor of pulling models. Our results also suggest that activation depends on competition between ligand internalized via Epsin, which signals, and uptake of ligand by receptor, which aborts the incipient signal.
Phosphoinositide homeostasis requires a tight spatial and temporal regulation during the endocytic process. Indeed, PtdIns(4,5)P2 plays a crucial role in endocytosis by controlling clathrin-coated pit formation, whereas its conversion into PtdIns4P right after scission of clathrin-coated vesicles (CCVs) is essential for successful uncoating and cargo sorting. In non-neuronal cells, endosomal PtdIns(4,5)P2 hydrolysis critically relies on the lipid phosphatase OCRL, the inactivation of which causes the Oculo-Cerebro-Renal syndrome of Lowe. A key issue is thus to unravel the mechanism by which OCRL is recruited on CCVs precisely after their scission from the plasma membrane. We will show that the Rab35 GTPase directly recruits the OCRL phosphatase immediately after scission of the CCVs, and that Rab35 act together with OCRL in post-endocytic steps. We propose that the precise spatial and temporal activation of Rab35 acts as a major switch for OCRL recruitment on newborn endosomes, post-scission PtdIns(4,5)P2 hydrolysis and subsequent endosomal trafficking. We will further show that manipulating PtdIns(4,5)P2 levels using small molecules rescues the trafficking defects observed in OCRL-depleted Drosophila cells, Lowe patient renal cells and in a recently developed zebrafish model of the disease. We will discuss how these findings pave the way for potential therapeutic strategies.
ESCRT-dependent exovesicles in the Zebrafish Left/Right organizer

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In addition to their endocytic functions, ESCRT proteins have been implicated in the release of extracellular vesicles that arise through exosomal secretion from multivesicular endosomes or ectosomal cell surface budding. The biological functions of these vesicles remain however poorly understood. Through our previous work we have shown that, in Drosophila, the ESCRT complex is essential for the release of ectosomes that ensure the secretion and long-range transport of Hedgehog. Here, we provide evidence that the formation and directional transport of ESCRT-dependent extracellular vesicles is important for the establishment of zebrafish Left/Right (L/R) asymmetry.

L/R asymmetry is essential for organ development during embryonic morphogenesis and laterality defects have been linked to a number of human pathologies. Zebrafish L/R asymmetry is controlled by a specific L/R organizer, Kupffer's vesicle (KV). KV is an epithelial vesicle in which motile cilia generate a circular fluid flow. While it is well established that KV flow is essential for L/R asymmetry, the mechanism through which the flow triggers symmetry breaking remains to be elucidated. We show that ESCRT-positive exovesicles are present in the KV lumen. Based on results obtained using different loss of function approaches, including the creation of genetic mutants, we propose that ESCRT function is specifically required at the level of the L/R organizer. Interfering with ESCRT function reduces the number of KV exovesicles and causes organ laterality defects. These data raise the question whether ESCRT-positive exovesicles could promote the directional transport of symmetry-breaking signaling molecules?

To address this issue, we have performed a quantitative analysis of exovesicle transport dynamics in the L/R organizer. These measurements have allowed us to show that in spite of its overall circular geometry, the L/R organizer fluid flow can ensure the directional transport of exovesicles in the organ lumen. Altogether, our findings are supportive of a mechanism according to which ESCRT-positive exovesicles may ensure the directional transport of symmetry-breaking signals in the fish L/R organizer.
Phagocytosis during neuron-microglia interactions in brain development

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In recent years it has become clear that most aspects of brain development, function and repair are not mediated by neurons alone but emerge from the interaction with other cell types. Of particular interest are microglia, the tissue resident macrophages of the brain. Work from many labs has shown, for example, that these cells remove unwanted neurons and synapses and that these activities can influence animal behavior by shaping neuronal connectivity. Beside their importance in brain development and homeostasis, microglia offer a number of key features—including their long-range migration into the brain, their adaptation to the local environment, and their interactions with neurons—that make them a great resource for addressing key questions in biology.

We take an in vivo approach that combines quantitative live imaging and cutting-edge perturbation in zebrafish. Using this system we have defined for the first time how microglia interact and remove neurons at single cell resolution in vivo and we have developed tools to visualize and manipulate signals that control microglial responses. Recently, the have developed a new microscope that combines dual-view imaging in an upright SPIM setup with electronic confocal slit detection (eCSD) on modern sCMOS sensors. This multi-position SPIM allows long-term imaging and we are using this instrument to investigate how microglia, digest, process and recycle dead neurons.
Cave exploration at the nanoscale: new insights into the structure and function of caveolae

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Caveolae are the most abundant features of the plasma membrane of many vertebrate cells. The surface of adipocytes, skeletal muscle, and many other cell types is densely covered by these small invaginations. The major membrane proteins of caveolae are small integral membrane proteins called caveolins. Three caveolins are present in mammalian cells with caveolin-1 (CAV1) and caveolin-3 (CAV3) essential for caveolar formation in non-muscle and muscle cells respectively. We have investigated the conservation of caveolins and caveola formation through evolution and we have combined these studies with caveolin-induced caveola formation in caveolin-null fibroblasts, in a model bacterial system, and more recently in a cell-free system to derive a model for caveolin-induced membrane remodelling. In vertebrate cells, caveolins work together with lipid-binding peripheral membrane proteins termed cavins. We have shown that Cavin1/PTRF is essential for caveola formation. Structural studies are starting to reveal how cavin1 works together with CAV1 and membrane lipids to sculpt the membrane to control the formation of caveolae and regulate their disassembly.

Studies of the cavins and their dynamics of caveola association/dissociation provide new insights into the role of caveolae in cells and tissues. In response to membrane stress caveolae flatten with release of cavins into the cytoplasm. In cells with abundant caveolae, such as skeletal muscle and the zebrafish notochord, flattening of caveolae can play a mechanoprotective role. In parallel the release of cavins and their interaction with intracellular targets forms a signaling mechanism. In response to a number of stimuli the released cavins can interact with intracellular targets to trigger cellular responses. Cytosolic levels of Cavin1 are kept low under steady state conditions by ubiquitination and proteasomal degradation of a lipid-binding site which is exposed when Cavin1 is released from the membrane. These results highlight a critical role for caveolae and the caveolin-cavin system as protective and stress signaling elements of the plasma membrane.
POSTER ABSTRACTS
Modulation of human Lemur Tyrosine kinase 2 expression by PKC; implications in CFTR trafficking

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Lemur Tyrosine kinase 2 (LMTK2) is a membrane-anchored Serine/Threonine kinase involved in endocytic trafficking in mammalian cells. It has been specifically shown to be associated with cystic fibrosis (CF), a lethal genetic disease caused due to mutations in cftr gene, which leads to defective CFTR (Cystic Fibrosis Transmembrane conductance Regulator) trafficking, resulting in overall osmotic imbalance in the body. Our previous studies have shown that lmtk2 knockdown or overexpression affects CFTR-mediated chloride conductance at the plasma membrane of polarized epithelial cells. Moreover, CFTR has been shown to be a substrate for LMTK2 kinase activity. Based on these observations, we propose LMTK2 to be a novel therapeutic target for cystic fibrosis. However, the precise mechanism to modulate the expression or activity of endogenous LMTK2 is not understood. In our present study, we have established LMTK2 as a novel TPA-response element containing gene, which is upregulated downstream of protein kinase C (PKC) activation by phorbol ester TPA. Using a combination of genomic and molecular approaches, we have shown that the TPA response on endogenous LMTK2 expression is mediated by transactivation of the Activator Protein-1 (AP-1) transcription factor complex and requires binding of AP-1 to the gene promoter. The effect is competitively inhibited by AP-1 binding inhibition, and completely abrogated by mutating the putative TPA response element on lmtk2 promoter.

Since LMTK2 is associated with cystic fibrosis, our current studies are aimed at elucidating the effect of modulating endogenous LMTK2 expression on intracellular trafficking and hence function of CFTR, especially in airway and intestinal epithelial cells, as they express CFTR maximally. Our study will provide a deeper understanding on the role of PKC on CFTR transport and function, and the involvement of LMTK2 in the process. Moreover, the outcome will assess the potential of LMTK2 as a therapeutic target for defective CFTR trafficking, as seen in cystic fibrosis.
Trastuzumab treatment unravels a novel ERK-dependent ERBB2 signalling pathway involved in AKT downregulation

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Approximately 20-30\% of breast cancers display ERBB2 (HER2) protein overexpression. Targeting HER2 with the humanized therapeutic antibody Trastuzumab (TZ), represents the front-line treatment for these tumours, and leads to the downregulation of the AKT survival pathway. Primary and acquired resistance to TZ are frequent events, both characterized by AKT hyperactivation.

Using human breast cancer cell lines, and patient’s primary tumour cells, we found that: TZ binding to ERBB2 triggers an ERK-dependent signalling cascade culminating with AKT dephosphorylation via Ser/Thr phosphatases; the immunophilin Cyclophilin A is a key modulator of this process and its silencing bypasses resistance to TZ treatment. In particular, we showed that Cyclophilin A competes with the Ser/Thr phosphatase PP2A for the binding to phosphorylated AKT (p-AKT), thus preventing phosphatase activity; TZ promotes Cyclophilin A redistribution from p-AKT to ERBB2 (allowing the binding of PP2A to p-AKT) in an ERK-dependent manner.

In summary, we identified and characterized for the first time the ERBB2 signalling pathway leading to AKT dephosphorylation, recognizing Cyclophilin A as a druggable key regulator of this process and a putative target for breast cancer therapy. Moreover, these data shed new light on ERBB2 activity as a negative modulator of other ERBBs activity.
Characterising the endocytosis of nanomedicines

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Nanomedicine promises to revolutionise the way we currently deliver drugs to their site of action. Nano-sized materials in fact can enter cells easily by active processes, using cellular pathways rather than freely diffusing according to their solubility and partition coefficient, as many drugs we use. Nanotechnology nowadays allows us to engineer materials of many different properties such as size, charge, shape etc. and to screen them for their efficacy, while different targeting strategies can be used to try to achieve recognition of the nanocarrier on the cells of interest and increase the specificity of the delivery. However, even for successful materials, the fraction that arrives to the target is in most cases very small and a clear understanding of the reasons why a certain design seems to be successful is often missing. This has led to a growing realization within the drug delivery community that in order to fully exploit nanomedicine potential, the molecular details of the mechanism of nanoparticle uptake and intracellular trafficking need to be characterised.

To this aim, we have combined different methods such as colocalisation with endocytic markers, the use of transport inhibitors and RNA interference, together with newer methods not yet used for addressing the question of nanoparticle uptake. The results obtained so far clearly illustrate the complexity of the pathways involved and advantages and limits of the different methods tested.

Furthermore we show how the uptake mechanisms is affected by the modification nanoparticles encounter once in contact with a biological environment, where they typically absorb a layer of molecules forming the so called nanoparticle corona (1). We previously showed that the formation of this layer can lead to loss of nanoparticle targeting (2) and that nanoparticle uptake can depend on the recognition of corona proteins by cell receptors (3).

The basic process of clathrin coat formation has been refined during evolution to perform specialized functions in differentiated cells and tissues. A gene duplication that occurred when vertebrates emerged generated two types of clathrin; CHC17, which binds clathrin light chain (CLC) subunits, and CHC22, which does not. CHC17 clathrin is highly conserved from yeast to humans and controls the classic clathrin pathways of receptor-mediated endocytosis, lysosome biogenesis and trans-Golgi network sorting in all tissues. CHC22 clathrin shares 85% identity with CHC17, but is mainly expressed in muscle and adipose tissue. CHC22 is involved in targeting the GLUT4 glucose transporter to the intracellular storage compartment where GLUT4 is held until released by insulin to clear blood glucose after a meal. CHC22 accumulates in muscle of insulin-resistant type 2 diabetic patients, suggesting a potential role in disease, as well as a role in human glucose homeostasis. The biochemistry and cell biology of CHC22 indicate that its regulation and membrane traffic functions differ from conventional clathrin, and that it mediates a novel secretory pathway emerging from the ERGIC. The gene encoding CHC22 has been lost in some vertebrate species and two major variants are found in human populations, suggesting this gene is still undergoing selection. We hypothesize that because of its properties and intracellular location, CHC22 could contribute to pathways of human insulin resistance.
COUPLING OF ACTIN TO CLATHRIN DURING ENDOCYTOSIS AT SITES OF CELL-CELL INTERACTIONS

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We previously showed that membrane tension determines the actin dependence of clathrin-coat assembly, providing a unifying explanation for the role of actin dynamics in coated-pit budding1. Actin must engage to complete membrane deformation on apical surfaces of polarized cells and, more generally, on the surface of any cell in which the plasma membrane is under tension from osmotic swelling or mechanical stretching.

To explore the influence of cell-cell interactions in the dynamics and organization of clathrin coat formation we asked the following questions: (1) What is the level of (canonical) coated-pit formation? (2) Is actin activity required for coated-vesicle budding in regions of tight cell contact (at which membrane tension is presumably high)? (3) How does HIP1R links clathrin assembly with local actin dynamics? We have generated clonal cell lines homozygously edited with fluorescently tagged AP2, HIP1R, epsin1 and visualized in real time coat dynamics at free and attached surfaces by combined used of single-fluorophore sensitivity two-color TIRF (total internal reflection fluorescence) microscopy and 3D LLSM (lattice light sheet microscopy). We reached the following conclusions. (1) Canonical coated pits actively formed in free surfaces and at the interphase between two WT cells. (2) Knockout of HIP1R stalled the assembly of coated pits at membrane interfaces but had no effect on free membranes. (3) Deletion of THATCH (the actin/cortactin binding domain in HIP1R had no detectable effect in the assembly dynamics of coated pits in the free or contact surfaces. (4) Knockout of HIP1R or of its THATCH domain increased cortical F-actin structures, as previously shown2. These results suggest that in the context of coated pit formation, the linkage of HIP1R with actin is indirect, presumably by modulating the interaction of epsin with actin3.

2. Engqvist-Goldstein et al. RNAi-mediated Hip1R silencing results in stable association between the endocytic machinery and the actin assembly machinery. MBoC 15, 1666–1679 (2004).
Antigen uptake and processing in B cells

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B cells are an essential part of the adaptive immune system. They are activated by a long signaling cascade including antigen recognition, antigen processing onto MHCII complexes and subsequent peptide antigen presentation for T cells. This antigen processing cascade lays the base for an efficient immune response by licensing subsequent high affinity antibody production by B cells. Despite the importance of antigen processing, intracellular compartments involved and the molecular mechanisms that guide the events are not known.

We aim to reveal the antigen processing pathway in B cells, and the molecular mechanisms in the process. Our studies, characterizing various endosomal vesicle markers, have revealed both classical and atypical features of the antigen trafficking in B cells. We found that shortly after internalization, B cells transport antigen to specialized late endosomes that possess both late- and early endosomal characters. Late endosomal marker, Rab9, colocalizes with antigen in peripheral vesicles already within 10–15min after antigen internalization. Some of them are also positive for early endosomal markers Rab5 and EEA1, as well as for MHCII and exosomal tetraspanins. Part of the antigen, on the other hand, is directed to a more classical pathway where antigen is gathered to the perinuclear area in 1–2 hours after the activation. In the perinuclear cluster, antigen colocalizes with late endosomal/lysosomal markers Rab7 and LAMP1, where also the late recycling Rab11 is recruited. To study the functionality of these two different pathways and the associated endosomal compartments, we have set up an antigen degradation reporter system using antigen linked to DQ fluorogenic protease substrate. Also, different inhibitors and dominant negative or constitutively active mutants of Rab-proteins are utilized to dissect the role of these two vesicular pathways in antigen processing.

Successful B cell activation and antigen processing are critical not only for mounting of the normal immune response, but also for effective vaccination strategies based on generation of specific, high-affinity antibodies. Malfunctions in antigen processing cause immunodeficiencies and autoimmunity disorders. Thus, detailed molecular studies to understand antigen processing and trafficking in B cells, are necessary for healthier tomorrow.
Clathrin coat controls vesicle acidification by mechanical inhibition of vacuolar ATPase

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The vacuolar H\textsuperscript{+}-dependent adenosine triphosphatases (vATPases) are highly conserved multi-subunit complexes that use the ATP hydrolysis energy to produce a proton electrochemical gradient across membranes and acidify numerous intracellular organelles, enabling their function. The vATPases are essential for various cellular processes, e.g. endocytosis and intracellular membrane traffic, including synaptic vesicle (SV) recycling. At the neuronal synapses, exocytosed membrane and proteins, including vATPases, are internalized mainly by clathrin-mediated endocytosis. Newly formed SVs are rapidly acidified by vATPases, generating an electrochemical gradient that is subsequently used by an antiporter to fill SVs with neurotransmitters. To date, it is unclear how the vATPase is regulated during endocytosis, in particular at which step endocytosed vesicles acidify. Here, we developed assays to monitor pH and electrical gradients at the single clathrin-coated vesicle (CCV) level. We observed that ATP-induced acidification of CCVs isolated from the mammalian brain is strongly reduced in comparison to SVs. When the clathrin coat is removed from CCVs, uncoated vesicles rapidly acidified. Based on biochemical, imaging and functional studies, we propose a model where formation of the clathrin coat around vATPase mechanically blocks its activity. Such regulation of vATPase activity likely applies in all clathrin-coated structures.
Role of multivesicular bodies in the formation of exosomes

Alexander Mironov, et al.

The FIRC Institute of Molecular Oncology
European Institute of Oncology

Now analysis of extracellular micro vesicles (EMVs or EVS) and their role in different physiological and pathological events became extremely fashionable. Several names were proposed for these structures, namely, exosomes, exovesicles, ectosome, oncosomes et al. Until now, the consensus says: "exosomes are small EVs (<150 nm) release through multivesicular bodies (ILV; see Maas & 2017. Trends Cell Biol. 27(3):172-188). However, there are no convincing evidences that intra-luminal vesicles (ILVs) of MVBs could be secreted. Moreover in most papers, EMVs have diameter more than 100 nm and very rarely less than 50 nm. Meanwhile, 90% of ILVs have diameter below 50 nm. For instance, only 3-5% of EMVs generated by cells of glioblastoma cells in vitro have diameter below 50 nm (Setti & 2015. Oncotarget. 6(31):31413-27). Here, we show at electron microscopic level that in cell culture, stripped gold particles (Lehn & 2013. Nano Lett. 13(9):4060-7) with the diameter of 4-5 nm were accumulated in MBVs and in particular in ILVs and then aggregates of EMVs with the diameter of 50 nm and labelled with these gold particles were observed outside cells. In vitro (glioblastoma and STEM cells) and in situ (samples from patients with glioblastomas and EMVs in their blood), we found the significant correlation between the number of MBVs in cells and the percentage of EMVs with diameter of 50 nm or lower. Thus, ILVs are not the main source of EMVs. I thank Drs. G. Beznoussenko, D. Osti, G. Pelicci, C. Martinelli, S. Krol, and F. Stellacci for their help in experiments.
Peroxidase Enzymes as Tools to Analyse the Trafficking Mechanism of Endothelial Junctional Adhesion Molecule C

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The vascular endothelium is located at the interface of blood and tissue and serves a multitude of purposes such as regulation of blood flow, control of vessel-wall permeability as well as interaction with circulating leukocytes. Under inflammatory conditions these functions are imperative as a rapid recruitment of leukocytes and their transmigration into inflamed tissue is a prerequisite for an efficient immune response. The regulation of these processes is mainly governed by interendothelial junctions which are dynamic structures consisting of a multitude of junctional proteins. These proteins control the opening and closing of the junction as well as the transmigration of leukocytes by a process requiring regulated endocytic trafficking between cell surface and intracellular pools.

The junctional adhesion molecule C (JAM-C) is pivotal during junctional remodelling and faulty expression/function has been associated with multiple inflammatory diseases including atherosclerosis and heart disease. Following an inflammatory stimulus dynamic changes of JAM-C levels can be observed at endothelial junctions and in intracellular vesicular pools. This might serve to regulate JAM-C functions, however, little is known regarding the mechanism of JAM-C internalisation and redistribution.

To gain deeper insight into the intracellular trafficking of JAM-C we developed a novel biotinylation-based pull-down and mass spectrometry approach using genetically targetable peroxidase enzymes. Inserting a peroxidase tag in the extracellular domain of JAM-C enabled a spatially and temporally resolved mapping of the proteomic inventory surrounding surface as well as vesicular JAM-C. This allowed the analysis of its trafficking route and intracellular storage relative to other proteins including junctional proteins such as PECAM-1, JAM-A and CD99. Furthermore, it has been investigated how newly discovered co-trafficked proteins such as VE-Cadherin and Neuropilins regulate JAM-C trafficking. In order to determine the exact endocytic trafficking pathway of JAM-C the peroxidase tag was inserted in its intracellular domain allowing for the identification of trafficking machinery localised at the intracellular site of the membrane.

This use of peroxidase tags provides an excellent means to elucidate the mechanism of dynamic JAM-C trafficking and may also serve as a novel approach to identify key
players involved in the trafficking of other up to now uncharacterised receptor proteins.
Regulation of AP2 function in clathrin-mediated endocytosis

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AP2 is the most abundant clathrin adaptor in endocytic clathrin coated pits (CCPs). Previous studies in our lab and others have indicated that AP2 is necessary for CCP nucleation and that AP2 assumes a number of different conformations during progression of CCP formation and undergoes regulatory phosphorylation. In order to further understand, how AP2 is activated during initiation of clathrin-mediated endocytosis (CME), we probed, how the interaction between the early endocytic proteins FCHO1/2 and Eps15/Eps15R concentrates AP2 on the plasma membrane at the sites of endocytosis.

We present a 2.4 Å crystal structure of a chimeric protein consisting of the mu-homology domain (MHD) of FCHO1 and the conserved DPF-rich region of the Eps15 unstructured domain, to which the MHD binds. Eps15 assumes a tight turn conformation in order to bind the conserved, deep trough in the subdomain A of the FCHO1 MHD. We support the structure with in vitro and in vivo analyses that indicate high strength of the interaction and show that simultaneous removal of FCHO1/2 and Eps15/Eps15R from the plasma membrane abolishes CME. We propose a model, in which the FCHO1/2-Eps15/Eps15R multimers concentrate AP2 at the site of CME initiation via an interaction between the AP2 alpha appendage and Eps15, while the interaction of the AP2 core with the FCHO1/2 interdomain linker helps to activate AP2 for cargo and clathrin binding.

To investigate the role of phosphorylation of Thr156 on the AP2 mu2 subunit we present crystal structures of AP2 core phosphorylated at the Thr156 residue of the mu2 subunit in closed and a new open conformation. The latter shows a large conformational movement of the Cmu2 domain, when compared with the previously known AP2 structures, and significant opening of the AP2 “bowl” formed by the remaining subunits of the core but is still competent for PIP2- and cargo-containing membrane binding. In agreement with this, we show by liposome-based SPR that the mu2 Thr156 phosphorylation has no significant effect on the recognition of cargo or plasma membrane lipids, as has been previously proposed, and must thus be involved in processes involved in CCP formation.
Loss of the AP-5 Adaptor Protein Complex Results in Defects of Endosomal to Golgi Retrieval

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Adaptor protein complexes (AP1-5) play fundamental roles in facilitating selective transport of biologically important proteins from one cellular compartment to another. AP-5 was the last complex to be identified, and as such the one we know the least about. One of the key research goals is to determine the cargoes that AP-5 may sort, and on which sorting pathway it might function. To this end we have ablated AP-5 function using CRISPR Cas9 knockout, and undertaken several proteomic approaches to identify proteins whose trafficking or steady state distribution is affected by the loss of AP-5. We have used a novel proteomic technique termed ‘Dynamic Organeller Mapping’, which is a fractionation method that achieves resolution of major organelles (Itzhak et al., 2016); we find that the majority of proteins whose localisation is affected by loss of AP-5 are components of the endosomal machineries retromer and the HOPS complex. These results are consistent with the observed defects in endolysosomes in AP-5 patient lines (Hirst et al., 2015). We have further investigated the relationship between AP-5 and retromer and find that AP-5 facilitates the retrieval of a number of known retromer cargo proteins. We have also identified a number of other proteins whose trafficking is affected by the loss of AP-5 (and therefore possible cargoes) using a second proteomic method that isolates a cellular fraction that is enriched in transport vesicles; we find that the top affected proteins are a cluster of Golgi-associated proteins. We further characterise the relationship of AP-5 and these Golgi proteins and find that their retrieval from endosomes to the Golgi is affected when AP-5 is ablated. These results lead us to propose a model for AP-5 function in late endosomal to Golgi retrieval.


Primary cilia are sensory organelles present on almost all vertebrate cells. Several studies have shown that cilium length and morphology are modulated by environmental signals. We study this process in the amphid channel cilia of the nematode C. elegans. We focus on gpa-3QL mutant animals that carry a dominant active mutation in a sensory heterotrimeric G protein alpha subunit, gpa-3, and have short cilia.

To find out how sensory G protein signalling modulates cilium length, we screened for suppressors of the gpa-3QL short cilia phenotype. This screen identified three components of the dual leucine zipper-bearing DLK/p38 MAP kinase pathway, the MAP3K dlk-1, the p38 MAPK pmk-3 and the E2 ubiquitin-conjugating enzyme variant uev-3. The DLK/p38 MAP kinase pathway regulates several processes, including endocytosis. Endocytosis at the base of the cilium is important for proper cilium function. We found that reducing endocytosis by mutating rabx-5 or rme-6, GEFs for RAB-5, or chc-1, the clathrin heavy chain, suppressed the gpa-3QL induced short cillum phenotype. In addition, gpa-3QL animals showed reduced levels of two GFP-tagged proteins involved in endocytosis, RAB-5 and DPY-23, whereas pmk-3 and pmk-3; gpa-3QL mutant animals showed accumulation of GFP-tagged RAB-5. These results suggest that dominant activation of GPA-3 leads to excessive RAB-5 mediated endocytosis, resulting in shorter cilia.

We previously found that inactivation of sql-1 (suppressor of gpa-3QL #1), which encodes the homologue of the mammalian Golgi protein GMAP210, also suppresses the short cillum phenotype of gpa-3QL. Interestingly, sql-1 loss-of-function also resulted in accumulation of GFP::RAB-5 at the base of the cilium, suggesting that also SQL-1 regulates RAB-5 mediated endocytosis. sql-1; pmk-3 double mutants showed very low GFP::RAB-5 levels, suggesting that the effects of mutation of sql-1 and pmk-3 are mediated by different mechanisms, e.g. by changes in supply of membrane and protein on the one hand and changes in removal on the other. Together our suggest that the regulation of cilium length by G protein signalling involves modulation of protein and/or lipid supply from the Golgi and retrieval by endocytosis.
Nutrient sensing in real time: how dynamic membrane recruitment regulates mTORC1

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The mechanistic Target of Rapamycin Complex 1 (mTORC1) kinase is a master regulator of mammalian cell growth that senses nutrients through incompletely understood mechanisms. In response to amino acids both in the cytoplasm and in the lysosomal lumen, mTORC1 relocalizes from a soluble cytoplasmic pool to the surface of lysosomes, where it becomes activated and enabled to phosphorylate downstream targets. The Rag Guanosine Triphosphatases (GTPases) act downstream of amino acids in order to recruit mTORC1 to the lysosomal surface. While it is known that the nucleotide binding states of the Rag GTPases impact mTORC1 localization and activity, it is unclear how Rag GTPases physically recruit mTORC1 to the lysosome, as well as how mTORC1 residence time and activity are controlled following binding to the Rag GTPases. I will present ongoing studies based on the combined use of live imaging and in vitro biochemistry to visualize the dynamic behavior of the Rag-mTORC1 complex. Our results shed light on how the Rag GTPases provide precise control over mTORC1 activation status at the lysosome, and explain how misregulation of the Rag GTPases in cancer settings disrupts fine-tuning of mTORC1 signaling output.
Structural insights into Legionella RidL-Vps29 retromer subunit interaction reveal displacement of the regulator TBC1D5

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The environmental bacterium Legionella pneumophila naturally replicates in amoebae. By utilizing a similar mechanism, the pathogen also parasitizes macrophages in the lung, possibly leading to a severe pneumonia called “Legionnaires' disease”. Hereby, the formation of a replication-permissive compartment, the Legionella-containing vacuole (LCV), is a crucial process. LCV formation is dependent on the Icm/Dot type IV secretion system (T4SS), which translocates approximately 300 different "effector" proteins into the host cell, where they modulate cellular processes. The retrograde vesicle trafficking pathway recycles cargo receptors along the endosomal route back to the Golgi apparatus and the ER. A key mediator of retrograde trafficking is the retromer complex, consisting of the heterotrimeric cargo-selective subcomplex (Vps26, Vps29, Vps35) and heterodimeric membrane-deforming sorting nexins.

A functional retrograde pathway restricts intracellular bacterial replication. Recently, we have shown that the L. pneumophila T4SS substrate RidL (Retromer interactor decorating LCVs) binds to Vps29, interferes with the retrograde vesicle trafficking pathway and is required for efficient intracellular replication of the pathogen. Recent data from our lab reveal that the 29 kDa N-terminal domain of RidL adopts an unprecedented foot-like fold comprising a protruding β-hairpin at its heel. The deletion of the hydrophobic β-hairpin, and the mutation I170E in the β-hairpin or L152E in Vps29 abolishes the RidL-Vps29 interaction in eukaryotic cells and in vitro. RidL-2-258 or RidL displace the Rab7 GTPase activating protein (GAP) TBC1D5 from the retromer and LCVs, respectively, and TBC1D5 promotes intracellular growth of L. pneumophila. Thus, the β-hairpin of RidL is critical for binding of the L. pneumophila effector to the Vps29 retromer subunit and displacement of the regulator TBC1D5.
INTERACTION BETWEEN EXO- AND ENDOCYTOSIS

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Intracellular membrane transport includes two main components, namely, exocytosis or secretory pathway and endocytosis or endocytic pathways. Several lines of evidence suggest that these two pathways are mutually dependent. Here, we tested this hypothesis using synchronized exocytosis and endocytosis and found that when endocytosis is stimulated speed of exocytosis increased. In turn, when exocytosis is synchronized and a bolus of membrane moved along the endocytic pathway, endocytosis became enhanced. Similarly, when exit of cargo from the ER is blocked the speed of endocytosis was decreased, whereas the blockage of endocytosis using different methods led to the decrease of the speed of exocytosis. Morphology of the Golgi complex (GC) is affected by the level of its trafficking activity and endocytosis. Resting stacks contained more COPI-dependent vesicles, less intercisternal connections, and no the cis-most cisterna and did not form ribbon. Here, we demonstrated that only transporting stacks contained the trans-most cisterna. Attachment of TMC to the medial-cisterna of the GC was Golgin97-dependent whereas clathrin coat inhibited TMC attachment. These data indicate that endocytosis and exocytosis influence each other.
Proteomic Analysis of Fibroblast Growth Factor Receptor (FGFR) Trafficking-dependent Signaling

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Fibroblast Growth Factor Receptors (FGFRs) regulate several cellular processes, including proliferation, migration, and differentiation, during embryonic development and in adult tissues. The activation of specific signaling pathways may depend on the engaged FGFR ligand, which controls receptor endocytic route after internalization. For instance, Fibroblast Growth Factor 10 (FGF10) induces FGFR2b internalization and recycling to the plasma membrane, which results in sustained signaling activation and cell migration. Conversely, FGF7 promotes FGFR2b degradation, transient signaling activation, and cell proliferation. However, the molecular bases of FGFR trafficking-dependent signaling remain largely unclear.

FGFR aberrant signaling has been associated to several human diseases, including breast cancer, where over expression of FGFR1 and 2 correlates with poor prognosis. We investigated 1) alterations in FGFR signaling and 2) the role of FGFR trafficking in regulating signaling specificity in epithelial breast cancer cell lines of distinct molecular subtypes. We stimulated cells for different time points with FGFs inducing either receptor degradation or recycling, respectively, and we used a system biology approach to determine changes in signaling activation. We combined shotgun mass spectrometry (MS)-based quantitative phosphoproteomics and proteomics, bioinformatics, with functional cellular assays to validate our findings.

Preliminary results of label-free phosphoproteomics showed that signaling cascades were modulated in a ligand/FGFR pair-, receptor trafficking route-, time-, and cellular context-dependent manner. We also confirmed this idea at global proteomic level upon depletion of signaling adaptor proteins or kinases. Finally, we analyzed how FGFs regulate long-term responses of breast cancer cells depending on cellular context.

Our results suggest that the specificity of FGFR response depends on the engaged ligand and on spatio-temporal regulation of signaling. Therefore, identifying signaling events associated to a specific ligand and receptor endocytic route may have relevant pathological implications in breast cancer.
Salmonella targets the host endo-lysosomal fusion machinery to promote its intracellular survival.

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Salmonella (Salmonella enterica serovar typhimurium) extensively manipulates the host endocytic machinery as it strives to establish its replicative vacuolar niche (Salmonella containing vacuole, SCV) within host cells. To maintain the integrity of this compartment Salmonella translocates several effector proteins (including SifA) into the cytoplasm that interfere and initiate dynamic fusion events with the endosomes and lysosomes of the host cells. By maintaining a prolonged interaction with these compartments Salmonella instigates remodeling of these endocytic membranes into an interconnected tubular network originating from the SCVs (known as Salmonella induced filaments, Sifs) which in turn facilitates the acquisition of membrane and nutrients essential for intracellular survival and replication of this pathogen. However, the mechanism by which Salmonella acquires the host membranes has been poorly characterized.

An essential pre-requisite for fusion of two vesicular compartments is the bridging of the opposing membranes into close proximity which is carried out by a dedicated class of proteins known as tethering factors operating at different stages of membrane trafficking. Here in this study, we have identified that a lysosomal tether, HOPS (HOMotypic fusion and Protein Sorting complex) temporally localizes to these bacteria-containing vacuoles, thereby acting as a molecular bridge linking the SCV membranes to the late endocytic compartments, essential for SCV maturation, Sif formation and acquisition of nutrients for intravacuolar proliferation. Notably, upon depletion of HOPS subunits we observed a significantly reduced bacterial load in various cell types as well as in mice primarily due to reduced interaction between the SCVs and lysosomes. We also found that the bacterial effector SifA, secreted by type III secretion system, recruits HOPS complex onto SCVs and Sifs while another host protein SKIP (also a well known binding partner of SifA) serves as an adaptor for this critical interaction. Our findings elucidate that Salmonella modifies its vacuole by recruiting host vesicle fusion machinery including HOPS complex to establish its replicative habitat inside the host.
MICAL-L1 associates with PACSIN3, shapes endosomal membrane tubules required for the recycling to the plasma membrane

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The recycling of receptors and lipids is tightly regulated and is essential for plasma membrane homeostasis, adhesion, cell migration and phagocytosis. It requires small GTPase Rab proteins and their effectors. Although MICAL-L1, an effector of several Rabs, associates with membrane tubular endosomes, its role in membrane remodeling is still unclear. Here, we investigate the role of MICAL-L1 and PACSIN3 in HeLa cells and human monocyte-derived macrophages. We report a novel interaction between MICAL-L1 and the F-BAR protein, PACSIN3. We find that MICAL-L1 plays a key role in PACSIN3 localization. MICAL-L1 and PACSIN3 localize to recycling endosomes and newly formed phagosomes. We identify two hydrophobic residues at the C-terminus of MICAL-L1 involved in phosphatidic acid (PA) binding. Mutations within the RBD domain (L742A and L743) affect PA interaction and impair both MICAL-L1 tubules formation and targeting to the recycling tubular endosomes. We monitor in vitro the process of membrane tubulation, and surprisingly find that MICAL-L1 alone is able to nucleate/shape membranes whereas PACSIN3 promotes the elongation/extension of MICAL-L1-induced membrane tubules. Depletion of either MICAL-L1 or PACSIN3 inhibits transferrin receptor recycling and results in a defect in phagosome formation. Our results reveal that MICAL-L1/PACSIN3 act as a critical regulator of membrane remodeling and endosomes recycling. They also highlight a novel role for MICAL-L1 in phagocytosis in primary macrophages.
Rab40b coordinates actin dynamics and extracellular matrix remodeling during invadopodia formation and MMP secretion in cancer cells

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Remodeling of the extracellular matrix (ECM) is a key process during cancer cell migration and metastasis and is mediated via formation of structures, known as invadopodia, as well as targeted transport and secretion of matrix metalloproteases (MMPs). However, at this point we know little about the machinery mediating targeted MMP secretion at the tips of the invadopodia. Recently we identified Rab40b as a key regulator of MMP secretion and invadopodia formation during cancer metastasis in vitro and in vivo. We also have shown that Rab40b is targeted to the invadopodia by binding to its tethering factor Tks5. Finally, we have determined that in addition to mediating MMP secretion Rab40b also regulates localized actin polymerization by binding to SGEF, a known GEF for RhoJ. Rab40b is a unique Rab since it contains SOCS box domain at its C-terminus. Since no other Rab has SOCS domain it is tempting to speculate that Rab40b-SOCS box impart a unique regulation of Rab40b function. In this study we identified Cullin-5 as a Rab40b-SOCS binding protein and have shown that Cullion-5 binding regulates Rab40b activity by mediating Rab40b mono-ubiquitination. Taken together, we propose that Rab40b stimulates cell migration by integrating actin polymerization and MMP secretion at the leading from of invadopodia and that Rab40b is regulated by Cullon-5-dependent mono-ubiquitination.
Impact of R465W dynamin-2 mutation on its structure and dimer interactions

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Dynamin-2 is a cytosolic GTPase that catalyzes membrane fission during endocytosis. This protein has five different domains: a GTPase domain, a middle domain, a pleckstrin homology domain, a GTPase effector domain, and a proline-arginine rich domain. Mutations in this enzyme produce centronuclear myopathy. The most common mutation is R465W. In vitro, this mutation forms more stable oligomers and hydrolyzes more GTP than the wild-type protein. In the absence of crystallographic structure data of dynamin-2, we built a structural model of this protein using crystallographic data of dynamin-1, and homology modeling techniques. Then, using Molecular Dynamics Simulations, we elucidated structural features produced by the R465W mutation in dynamin-2 dimers. We created three different dimer systems. One wild type dimer; an heterodimer composed of one wild type dynamin and a mutated dynamin; and a mutant dimer composed of two mutated dynamins. We found that exchanging an arginine for a tryptophan in the position 465 in the middle domain decreases both dimer length and the BSE-stalk angle. In addition, the mutation increases both free binding energy in the mutated dimer and the number of hydrogen bonds and salt bridges between dynamin monomers in the heterodimer and mutant dimer. Moreover, we found the W465 in both mutated dimers increase the curvature of the four alpha-helices of the stalk. These conformational changes could be related to the reported altered functions in centronuclear myopathy.
Screening towards the mechanism of Invariant chain-mediated endosomal fusion

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Professional antigen presenting cells present exogenous peptides on MHC class II to stimulate the adaptive immune response. The processing and loading of these peptides onto MHC II occurs within the endosomal pathway. Invariant chain (Ii) is a multifunctional subunit of the MHC II complex, essential for proper MHC II formation, trafficking to endosomes and protection from binding the wrong peptides. In addition, Ii enhances MHC II half-life by slowing down the endosomal pathway, presumably to optimize the conditions for maximum efficiency in peptide processing and loading. This property of Ii has been linked to its ability to enlarge endosomes in many cell types, independent of MHC II expression. This enlargement effect has been ascribed to Ii’s fusogenic properties, which is independent of PI3K activity, but is inhibited by NEM. We therefore performed an siRNA-mediated image-based screen of all the human SNARE and Sec1/Munc18-family proteins for a role in Ii-mediated endosome enlargement. Several of the candidates identified by the screen were found on endosomes and in complex with Ii, suggesting that Ii plays a role in recruitment or recycling of SNAREs to endosomes to enhance fusion. Investigation of this mechanism improves our understanding of processes underlying antigen presentation and will help in the development of novel treatments, such as cell-based anti-cancer therapy.
Transition from flat clathrin arrays to curved pits during clathrin-mediated endocytosis is coordinated by membrane tension

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During clathrin-mediated endocytosis (CME), clathrin and multiple adaptor and associated proteins assemble into a complex protein scaffold at the plasma membrane. This scaffold forms a clathrin-coated pit that grows in size to ultimately pinch off from the plasma membrane as a clathrin-coated vesicle. How assembly of the clathrin scaffold is regulated at the ultrastructural and spatio-temporal level is still under debate. There are currently two proposed models: 1) The constant curvature growth model which suggests curvature is acquired simultaneously as the clathrin-scaffold grows and 2) The constant area growth model where the clathrin structures initiate as a flat coat that acquire curvature only after reaching the full clathrin content. Using a combination of live fluorescent microscopy, correlative light and electron microscopy (CLEM) of metal replicas, statistical analysis and modeling of coat growth profile behaviors, we developed a comprehensive ultrastructural model of the dynamics of clathrin-coated structure formation. Methodical comparison of the predicted ultrastructural assembly of the clathrin scaffold calculated from the two previously reported growth models revealed that neither of the two models could fully explain the ultrastructural organization of clathrin structures observed in cells. We could show that clathrin structures initially grow flat. At around 50\% of their final clathrin content, flat clathrin arrays acquire curvature and continue growing to form a fully formed clathrin-coated pit. The start of bending is associated with a change in the AP2/clathrin ratio. Importantly, we found that acquisition of curvature likely corroborates with the accumulation of enough energy in the flat clathrin array to bend the plasma membrane. Indeed, increasing plasma membrane tension resulted in a delay of the change in the AP2/clathrin ratio and an accumulation of flat clathrin arrays. Taken together our work reveals new insights into the ultrastructural rearrangements that proceed during clathrin-coated pit formation and helps to complete our understanding of the mechanisms underlying CME.
The uptake of fluorescein-labelled hyaluronan is independent of CD44 on HT-29 cells

Barbora Šafranková, Vladimír Velebny
Contipro a.s.

The CD44 is commonly considered to be one of the most important hyaluronan (HA) recognized molecules and it plays pivotal role in HA effects including its endocytosis in cells. For that reason, HA and its binding receptor CD44 are used as promising drug delivery system, which has a potential, for example, in anticancer therapy. However, the mechanism of HA uptake is dependent on cells type. We used the model of human colorectal adenocarcinoma cell line HT-29 with normal and decreased CD44 expression. Surprisingly, the presence of surface receptor CD44 have not an influence on 100kDa fluorescein-labelled HA (fl-HA, degree of substitution 5%) cellular uptake. The uptake was observed after 3 hours or longer incubation of cells in different temperature conditions (4°C and 37°C) and the fluorescence signal was detected by flow cytometry and confocal microscopy. Finally, we tested the effect of different endocytosis inhibitors (rotterlin, chlorpromazine, cytochalasin D, methyl-β-cyclodextrin, genistein, amiloride and pitstop 2). Rottlerin, which is considered to be an inhibitor of macropinocytosis, showed the greatest ability to arrest of fl-HA uptake in case of HT-29 cell line.
**Membrane scission driven by the PROPPIN Atg18**

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Sorting, transport, and autophagic degradation of proteins in endosomes and lysosomes, as well as the division of these organelles, depend on scission of membrane-bound tubulo-vesicular carriers. How scission of endosomal compartments occurs is poorly understood, but PROPPIN family proteins bind these membranes. Here, we show that the yeast PROPPIN Atg18 carries membrane scission activity. Purified Atg18 drives tubulation and scission of giant unilamellar liposomes. Upon membrane contact, Atg18 folds its unstructured CD loop into an amphipathic α-helix that inserts into the bilayer. This allows the protein to engage its two lipid binding sites for PI(3)P and PI(3,5)P2. PI(3,5)P2 induces Atg18 oligomerization, which should concentrate lipid-inserted α-helices in the outer membrane leaflet and drive membrane tubulation and scission. The scission activity of Atg18 is compatible with its known roles in endo-lysosomal protein trafficking, autophagosome biogenesis and vacuole fission. Key features required for membrane tubulation and scission by Atg18 are shared by other PROPPINs, suggesting that membrane scission may be a generic function of this protein family. Intriguingly, Atg18 self assembles into 25 nm tubes in solution. We have solved the structure of these tubes at 3.9 Å by cryo-EM and are now investigating the in vivo relevance of Atg18 self-assembly.
The role of AP180 and CALM in the neuronal generation of beta-amyloid

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During late-onset Alzheimer’s disease (AD), the mechanisms driving pathological beta-amyloid (Aβ) generation remain still under debate. The neuronal assembly protein 180 (AP180) and its ubiquitously expressed cousin clathrin assembly lymphoid myeloid leukaemia (CALM), endocytic adaptors for VAMP2, have been identified as new risk genes for different neurological diseases such as psychotic bipolar disorder and AD, respectively. An endothelial role of CALM in Aβ clearance was previously suggested, however, the involvement of CALM and AP180 in neuronal Aβ generation and Amyloid Precursor Protein (APP) trafficking is still highly controversial.

In order to investigate the in vivo impact of these endocytic adaptors, we generated AP180−/− and neuron-specific CALM−/− (KO) mice. AP180−/− mice display severe neurological symptoms and drastically reduced postnatal viability; contrarily, neuron-specific CALM−/− mice are viable without obvious signs of neuronal impairment. To assess the potential impact of AP180 and CALM absence on AD progression, we will measure both the Aβ ratio as well as the mRNA and expression levels of major synaptic proteins found to be perturbed in the brain of AD patients. Furthermore, live-imaging in hippocampal neurons derived from our KO mice combined with immunoelectron microscopy experiments will help us to decipher the contribution of AP180 and CALM regarding APP and BACE-1 localization, their intracellular trafficking pathways as well as Aβ formation. Finally, employing SILAC-based labeling and high-resolution mass spectrometry, we will quantify the surface proteome of AP180 and CALM depleted neurons, providing an unbiased global view of membrane sorting mediated by these endocytic proteins.

Our ongoing investigation supports the previously proposed role of endocytic adaptors in AD pathogenesis; however, given the discrepancies in the literature about the mechanisms by which AP180 and CALM might control Aβ species production, further in vivo experiments are required to dissect the molecular mechanism and the specific contribution(s) of AP180 and CALM in the neuronal generation of Aβ. Unravelling the possible link between AP180, CALM, APP trafficking and Aβ generation will have an important impact on our understanding of the disease and will foster the development of targeted therapies for AD.
The first comprehensive time-resolved investigation of glycosphingolipid turnover: Measured by stable-isotope tracer lipidomics

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Synthesis and degradation of lipids have previously been studied using radioactively labelled isotopes, fluorescent lipid analogues or siRNAs against enzymes involved in lipid metabolism. These methods have clear disadvantages and such studies have mostly been restricted to lipid classes, leaving the complexity of lipid species within each class unexplored. In this study water soluble stable mass isotopes were used to measure the turnover of endogenous glycosphingolipid (GSL) species in Hep-2 cells. The use of [13C-U]glucose results in a mass increase of 6 Da for each hexose unit added to ceramide, and addition of [13C-U]serine gives a mass increase of 2 Da for all substances synthesized via de novo pathway from serine.

The data obtained from experiments with [13C-U]glucose demonstrate different lipid pools with approx. 70% of GlcCer and 40-50% of LacCer and Gb3 showing a much faster turnover than the rest of these classes. These data also demonstrate remarkable differences in the turnover of different species within each class; the long-chain GlcCer species (e.g. 16:0) showed a much more rapid turnover than the very-long-chain species (e.g. 24:0 and 24:1). When measuring de novo synthesis of ceramide (using [13C-U]serine), the turnover was fastest for the very-long-chain species, i.e. the opposite as that observed for the glycosylation.

All hexose units added after addition of [13C-U]glucose will have an increased mass of 6 Da when formed via de novo synthesis or via the salvage pathway (i.e. after partial degradation of GSLs in lysosomes and thereafter reuse of sphingosine). On the other hand GSLs that have been transported retrogradely from the plasma membrane to the Golgi apparatus can contain an increased mass for only some of the hexose residues. The data revealed various ratios of labelling of 1, 2 or 3 hexose units for the different GSL species. This made it possible to use our data to estimate both the total turnover of the different GSL species, as well as the amount of turnover being due to de novo synthesis, reuse via the salvage pathway or reuse after retrograde transport from the plasma membrane to the Golgi apparatus.
Retrograde transport of protein toxins – modulation by changes in lipid species

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A number of proteins and protein complexes are known to be important for endocytosis and intracellular transport. However, also the lipid composition of cells, both lipid classes and certain lipid species, are important for uptake and retrograde transport of protein toxins such as ricin and Shiga toxin (1-3). With the improved mass spectrometry analysis of lipids one can get an increasing understanding of the dynamics of lipids (4) and their role in intracellular transport. Furthermore, toxins themselves may by crosslinking of lipids at the cell surface induce signaling and change the lipid composition, thereby mediating an increase in their own transport (5).

We are currently studying how one by changing the balance between different lipids such as phosphatidic acid and diacylglycerol may affect retrograde transport, and we are correlating such findings with the lipid composition. The retrograde transport is studied both by microscopy and by using genetically modified toxins such as ricin that can be sulfated in the Golgi and mannosylated in the ER to quantify these transport steps. Moreover, toxicity is used as a readout for transport to the cytosol. Our recent data reveal that one can increase retrograde transport of ricin by manipulating the lipid composition. The characteristics of this transport will be discussed.

Reference List
Rho GTPases regulate cytoskeleton remodeling for ATP-dependent GLUT4 translocation in skeletal muscle cells

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Skeletal muscle glucose uptake during exercise involves the active recruitment of GLUT4 to the muscle cell surface. Key to this process is Rac-dependent reorganization of actin beneath the plasma membrane both in response to insulin and to contraction, but the intracellular mechanisms that mediate these effects are not well understood. Using L6 rat skeletal myoblasts stably expressing myc-tagged GLUT4, we found that exogenous ATP increased GLUT4 translocation to the cell surface and glucose uptake, both process requires actin filament remodeling. Members of the Rho GTPases family, like Rac1 and Cdc42 are responsible for the cortical actin polymerization evoked by exogenous ATP, ATP-dependent Rac1 and Cdc42 activation was also inhibited by the phosphatidylinositol 3-kinase (PI3K) inhibitor LY-294002, suggesting that both Rac1 and Cdc42 are effectors downstream of PI3K. ATP-dependent GLUT4 translocation was inhibited by Arp2/3 inhibitor (CK-869) or by siRNA-mediated silencing of Arp3 subunit of the Arp2/3 complex. ATP also led to dephosphorylation of the actin-severing protein cofilin on Ser3. Cofilin knockdown via siRNA partially inhibited GLUT4 translocation. We propose that ATP-dependent actin remodeling mediated by Rac1 and Cdc42 effectors downstream of PI3K, modulates both Arp2/3 and cofilin in a coordinate a dynamic cycle of actin branching and severing at the cell cortex, essential for ATP-mediated GLUT4 translocation in muscle cells.

Funding Proyecto Fondecyt 11130424, Proyecto anillo ACT1411
Identification of endosomal regulators with a novel function in the fasting-feeding transition in hepatocytes

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Hepatocytes are highly complex and multifunctional cells in the liver, which are able to adapt their metabolic activities depending on the environmental cues. This is particularly evident during the fasting-feeding transition, where many different metabolic processes are modulated, e.g. fasting turns on gluconeogenesis and fatty acid oxidation but represses glycolysis and fatty acid synthesis. To achieve this metabolic switch, hepatocytes need to sense the extracellular metabolic cues and integrate this information intracellularly in order to adapt their glucose and lipid metabolism. This is generally obtained by the action of several hormones (insulin, glucagon....) that control the fasting-feeding transition by modulating enzymatic activity and a complex network of transcription factors. Recently, new data highlighted the endocytic system as a new cellular module involved in the regulation of glucose and lipid metabolism in hepatocytes in vivo. In addition, several transcriptome analyses revealed a differential expression of many endosomal genes, such as Rab4a, Vps37a and Vps25, after 24h fasting and 24h refeeding. These data suggest a bi-directional control between endocytosis and metabolism in hepatocytes that might be necessary to regulate the fasting feeding response. In agreement with that, we find an increase of Rab4a, Vps37a and Vps25 gene and protein levels in the liver after 24h fasting and 6h refeeding, suggesting its regulation on a transcriptional level. To investigate whether the fasting feeding transition also influences endosomal activity, we performed subcellular fractionation using a continuous sucrose gradient followed by organelle proteomic analysis from liver tissues of 12h fasted and 2h re-fed mice. Surprisingly, we found a strong relocalization of several endosomal proteins, such as Rab4a, Vps37a, Vps25, Vps33b, Tsg101 and Rab11fip1, from the cytosolic compartments to the endosomal fractions upon refeeding. Interestingly, these candidates are modified in expression in metabolic liver diseases with an altered fasting-feeding switch. These data suggest a preferential recruitment of endosomal components to the endosomal membrane that might have functional metabolic consequences during the fasting-feeding transition.
FTY720-induced endocytosis of amino acid transporters in yeast and human cells

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FTY720 is a sphingosine-like drug selectively inhibiting growth of various cancer cells (1, 2). Its antitumor activity is linked to its ability to promote endocytosis of several nutrient transporters thereby causing nutrient limitation (3). However, the molecular mechanism underlying FTY720-induced endocytosis of transporters remains elusive. As FTY720 also inhibits growth of yeast cells, and because this effect is dependent on the Rsp5 ubiquitin ligase known to catalyse permease ubiquitylation and endocytosis (4), we sought to further investigate the influence of FTY720 using the general amino acid permease, Gap1, as a model system. We found that FTY720 triggers Gap1 ubiquitylation via Rsp5 acting together with four possible adaptors of the alpha-arrestin family. In keeping with our previous observations that these adaptors promote Gap1 ubiquitylation when the TORC1 kinase is inhibited (5), we found that FTY720 also rapidly inhibits TORC1. Addition of FTY720 to human HeLa cells promoted endocytosis of the large neutral amino acid transporter, LAT1, as well as inhibition of mTORC1. Further investigation of FTY720-elicited endocytosis of yeast Gap1 and human LAT1 suggest that similar mechanisms are involved in both systems.

References

The novel antibody mixture Sym004 inhibits EGFR downstream signalling and modulates trafficking

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Epidermal growth factor receptor (EGFR) is overexpressed in several cancers. There are a limited number of targeted therapies in colon and head and neck cancers (HNC), with anti-EGFR antibodies cetuximab and panitumumab being some of the few options currently available. There are no positive predictive biomarkers for the response to EGFR-targeting antibodies, although lack of response is associated with mutations in the RAS/PI3-kinase pathways.

In this study, we investigated whether Sym004, a mixture of two antibodies which recognise non-overlapping epitopes within EGFR, modulates EGFR signalling/trafficking in combination with a chemotherapeutic cisplatin. Sym004 has been shown to be superior to cetuximab in treatment of HNC human xenografts. Currently two clinical trials employing Sym004 are ongoing in colorectal cancer and GBM.

We show that both Sym004 and cetuximab promote a 1.3±0.03 and 1.33±0.05 times increase, respectively, in release of apoptotic caspases 3/7 and a similar increase when combined with cisplatin (1.35±0.08 and 1.29±0.19, respectively, compared to cisplatin). Cisplatin induces phosphorylation of Erk, which is abrogated by cetuximab and further reduced by Sym004 (0.32±0.15 compared to control). Sym004, unlike cetuximab, promotes EGFR internalisation and intracellular trafficking. We previously showed that p38 is required for cisplatin-mediated EGFR endocytosis (Tomas Nat. Commun. 2015). In contrast, p38 inhibitor SB203580 does not affect EGFR internalisation following Sym004 treatment. Sym004 promotes increased phosphorylation of EGFR on Y1068, indicating activation of the EGFR kinase, but causes significant downmodulation of total EGFR. Decreased EGFR levels following Sym004 treatment are not affected by proteasomal (MG132) or lysosomal (Bafilomycin) inhibitors, indicating that EGFR is not degraded by those pathways. Instead, we recover increased amounts of EGFR from a detergent-insoluble fraction following lysis. This indicates that EGFR may localise within the plasma membrane or subcellular compartments inaccessible with standard lysis conditions. Finally, we performed mass spectrometric analysis of changes in phosphopeptides and preliminary data indicates that Sym004 may modulate phosphorylation of PKC substrate MARCKs and thus affect cell motility.

In conclusion, Sym004 differs substantially from cetuximab in processing of EGFR. We propose that crosslinking of EGFR moieties with Sym004 may render EGFR inaccessible to signalling molecules, thus contributing to inhibition of downstream
signalling.
CMTM6 maintains the expression of PD-L1 and regulates anti-tumour immunity

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Cancer cells exploit the expression of the programmed death-1 (PD-1) ligand 1 (PD-L1) to subvert T-cell mediated immunosurveillance. Therapies that disrupt PD-L1 mediated tumour immune evasion have achieved considerable clinical success, highlighting the need to understand the molecular regulation of PD-L1 expression. Using a genome-wide CRISPR/Cas9 loss of function screen we have identified the uncharacterised protein CMTM6 to be a key regulator of both constitutive and cytokine-induced PD-L1 expression in a broad range of cancer cells. CMTM6 is a ubiquitously expressed MARVEL-domain containing protein that associates with PD-L1 in a membrane-anchored complex. CMTM6 is not required for export of PD-L1 from the endoplasmic reticulum or trafficking via the Golgi but is essential to maintain PD-L1 expression at the plasma membrane. In the absence of CMTM6, recycling of endocytosed PD-L1 back to the cell surface is markedly impaired and PD-L1 is instead rerouted for lysosome-dependent degradation. Using a quantitative global proteomics approach to identify proteins with altered plasma membrane expression following loss of CMTM6, we find that CMTM6 displays functional specificity and regulates a very select group of proteins in addition to PD-L1. Importantly, loss of CMTM6 decreases PD-L1 without compromising the cell surface expression of MHC class I. CMTM6 depletion in tumour cell lines alleviates PD-L1-mediated suppression of tumour specific cytotoxic T-cell activity, highlighting the functional importance of CMTM6 in maintaining the PD-L1/PD-1 immune checkpoint. Together these findings provide novel insights into the biology of PD-L1 regulation and reveal a potential therapeutic target to overcome immune evasion by cancer cells.
Role of WASH-mediated, actin-dependent recycling of Her2 in Her2+ve cancer and drug resistance

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Amplification of tyrosine kinase receptor expression Her2 is seen in 20-30% of breast cancer patients and correlates with poor prognosis. While Trastuzumab based combination therapies are an effective treatment strategy, a high proportion of patients, particular those with metastatic disease, display acquired drug resistance over time. An imbalance of RTK trafficking is known to contribute to a number of diseases including cancer, this study set out to investigate whether targeting endosomal recycling affects Her2-dependent invasion and drug resistance.

WASH complex mediated endosomal actin polymerisation is required for endosomal receptor recycling of selected cargo such as α5β1-integrin, β2AR, EGFR and CIMPR. Here we show that the overexpression of Her2 correlates with an increase in WASH expression in Her2 positive breast and lung cancer patients and we can demonstrate an increase in Her2 cell surface expression upon induced WASH overexpression in Her2 positive cell lines. Depletion of WASH leads to a decrease in Her2 from the cell surface and inhibition of Her2-dependent cell invasion, suggesting WASH plays a significant role in the trafficking and activation of Her2. We have identified a novel actin binding domain (ABD) in Her2 that regulates its endocytic recycling and activation status via WASH-mediated endosomal actin polymerisation. We demonstrate that Her2 traffics in a WASH-dependent manner with the egress rate of Her2 from endosomal membrane reduced upon expression of a dominant negative WASH construct (ΔVCA) or removal of this putative ABD (Her2AAAA).

Finally, we report elevated expression of WASH and other proteins involved in endosomal recycling in Trastuzumab resistance. This introduces WASH-dependent endocytic recycling as a novel mechanism of acquired drug resistance. We propose that in steady state, active WASH/actin- recycling of Her2 enables Her2 positive breast tumour progression and that this mechanism is reinforced in Trastuzumab resistance to overcome the effects of the drug on receptor trafficking.
BMP2K kinase regulates endocytosis and differentiation in erythroid cells

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Profound exploration of intracellular mechanisms regulating endocytosis has revealed the complexity of endocytic pathways. Most studies so far were performed in model cellular systems and focused on describing universal aspects of vesicular trafficking, however recent reports begun to draw more attention to cell type-specific functions of endocytic machineries. It is conceivable that among putative endocytic regulators there are still proteins of ill-defined functions, which could play important roles in particular tissues but are dispensable in commonly used cell lines. We found that an example of such protein is bone morphogenetic protein 2-inducible kinase (BMP2K), which is a homologue of an established endocytic regulator AAK1, but its involvement in vesicular trafficking has not been described. We performed proximity biotinylation (Bio-ID) in HEK293 cells, followed by mass spectrometry (MS) analysis which revealed that BMP2K primarily interacts with adaptors involved in clathrin-mediated endocytosis (CME) and with several components of ER export sites (ERES). In parallel, we discovered that this serine-threonine kinase is highly expressed primarily in erythroid cells where it phosphorylates the \(\mu\) subunit of the AP2 complex. Moreover, knock-down of BMP2K elevates endocytosis rate in erythroleukemia K562 cells and induces their erythroid differentiation. Our results suggest that this kinase regulates endocytosis to support proper maturation of the erythroid lineage. Ongoing experiments aim at dissecting which membrane proteins and downstream signaling pathways depend on the BMP2K function and whether BMP2K-\(\mu\)2-mediated regulation of endocytosis contributes to cell differentiation.
Studying consequences of vascular dementia CADASIL Notch3 mutations in cell culture and iPS derived in vitro differentiated tissue models

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Notch signalling is highly important to develop and maintain vasculature. CADASIL, an adult-onset autosomal dominant vascular dementia disease, is caused by human Notch3 mutations. A likely pathological mechanism is the misregulation of Notch3 trafficking because the CADASIL is associated with an excessive accumulation of the Notch extracellular domain. Here we aim to develop an in vitro model using patient-derived induced pluripotent stem cells (iPSCs) to study the role of Notch trafficking in CADASIL pathogenesis. Initially, signalling and protein localisation assays using expressed protein will be established to identify locations in Notch where a fluorescent tag can be inserted for live image analysis, without compromising Notch function, and to compare wild type and CADASIL mutant Notch. For more physiological studies using endogenous Notch3 we will also use patient derived iPS cell and CRISPR approaches and establish in vitro models of vascular differentiation to investigate mutant Notch mislocalisation and CADASIL phenotypes.
Stimulation of EGF-mediated endocytosis induces changes in the mobility of the early endosomal tethering factor EEA1 on endosomes

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Early endosomal antigen-1 (EEA1) is a well-characterized protein known to mediate tethering of early endosomes. EEA1 is associated with endosomal membrane by its FIVE domain which binds phosphatidylinositol-3-phosphate (PI3P) and by Rab5-binding RBD-domain both localized at the C-terminus. Its additional N-terminal Rab5-binding domain allows EEA1 to serve as the «tether» between two vesicles.

According to the current model, EGF-endocytosis stimulation leads to the Rab5 activation and PI3P generation at the early endosome that should induce EEA1 recruitment to the early endosomal membrane. Unexpectedly, EEA1 shows preferentially vesicular with very minor cytosolic localization not only in EGF-stimulated but also in serum-deprived cells, in which receptor-mediated endocytosis is minimized. To explore if the presence of cargo influences the characteristics of EEA1 membrane association we have compared EEA1 binding dynamics in serum-deprived (control) and EGF-stimulated cells using fluorescence recovery after photobleaching (FRAP) analysis.

HeLa cells were transfected with the plasmid encoding N-terminal EGFP-tagged EEA1 protein and serum-starved for 12 hours. EGF-receptor endocytosis was stimulated by addition of 2 nM EGF. Circular region of the cell of 4 μM in diameter was photobleached and a single-vesicle fluorescence recovery was monitored.

We have revealed that the bulk of membrane-associated EEA1 protein is represented by the mobile fraction both in control and EGF-stimulated cells. Fitting of the single-organelle fluorescence recovery curves with one- and two-state binding models showed that the curves can be better described by the two-state model. Thus, EEA1 recovery is represented by two kinetic components with different lifetimes onto the endosomal membrane (fast and slow). Although the recovery half-time of fast kinetic component of EEA1 did not changed upon EGF-stimulation, the recovery half-time of slowly cycled EEA1 fraction significantly increased (from 21 to 99 s). These data could be interpreted in terms of additional Rab5 activation. However, EEA1-bearing vesicles’ clusterization in EGF-stimulated cells clearly seen in the images can as well stabilize a portion of EEA1 molecules between opposing membranes.

The work was supported by a grant from the Russian Science Foundation (project № 14-50-00068) and with financial support from the Federal Agency of Scientific
Organizations (Russia).
A knock-sideways mouse system for acute protein inactivation in primary cells and tissues

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Whilst undoubtedly powerful, fast and relatively straightforward, genetic perturbation by gene knock-down or knock-out in model cell systems can become experimentally limiting in two important ways. Firstly, the long timescales involved, days for knock-down to weeks for knock-out studies, can lead to secondary effects or mechanistic compensation complicating phenotypic analyses. Secondly, there are specialised cell types and cellular processes for which genetically tractable model cell systems either do not exist or do not fully replicate the in vivo situation.

To address the first of these problems we previously developed the knock-sideways approach combining the speed of small molecule inhibition with the specificity of genetic manipulation. Through rapamycin inducible hetero-dimerisation between a protein of interest and a mitochondrially localised anchor, the system allows sequestration of the target protein away from its site of action, leading to a fast (minute time-scale) inactivation. This allows extremely early phenotypic analyses, greatly reducing the opportunity for secondary effects and compensation to arise.

Traditionally, to examine the function of a protein of interest in specialised cell types for which no suitable model system exists people have turned to mouse genetics. Whilst arguably more faithful than model systems, mouse mutants are also susceptible to compensation and secondary defects as well as the potentially severe limitation of developmental defects and embryonic lethality. We have therefore developed a mouse system using our knock-sideways approach for the acute inactivation of proteins of interest in primary cells and tissues from otherwise developmentally normal animals.

We are initially applying this technology to the study of the AP-1 adaptor complex for which a number of cell type specific functions have been suggested but that has previously proven largely intractable in mouse systems due to embryonic lethality of gene knock-outs of common complex subunits.
Studies on infectious entry pathway and uncoating of Enteroviruses

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We are focused on understanding the mechanisms and cellular factors behind enterovirus infection. Our previous results have suggested that enteroviruses follow a macropinocytic entry to host cells. While inside endosomes, a novel uncoating intermediate particle is formed of echovirus 1: a highly infectious virus particle that is permeable to small molecules such as ions and SYBR Green. It is still unknown what are the cues inside endosomes that finally cause the virus to finally release its genome. In order to follow virus uncoating and other details in the infectious pathway, we have produced covalently conjugated probes for virus capsid or genome. We have developed a protocol for site-specific covalent conjugation of atomically monodisperse gold clusters with 1.5 nm metal core to viral surfaces to target cysteines on viral outer capsid (PNAS 2014). Another site-specific probe was developed for the hydrophobic pocket of enteroviruses (Nanoscale 2015). A derivative of Pleconaril was conjugated to fluorescent labels and Au102. The probe mildly stabilized the virus particle and caused a delay in the virus uncoating, but could not however inhibit the receptor binding, cellular entry or infectivity of the virus. The hydrophobic pocket binding was proven by STD and tr-NOESY NMR methods and TEM. The virus-fluorescent probe accumulated in endosomes but was seen to leak from the virus-positive endosomes from the capsid proteins suggesting that, like the physiological hydrophobic content, the probe may be released upon virus uncoating. Our recent studies using real-time spectroscopy and radioactive gradients have revealed ionic factors that are promoting RNA release from virus capsid in relevant physiological conditions.
Echovirus 1 internalization negatively regulates epidermal growth factor receptor downregulation

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We have demonstrated previously that the human picornavirus Echovirus 1 (EV1) triggers an infectious internalization pathway that follows closely, but seems to stay separate, from the epidermal growth factor receptor (EGFR) pathway triggered by epidermal growth factor (EGF). Here, we confirmed by using live and confocal microscopy that EGFR and EV1 vesicles are following intimately each other but are distinct entities with different degradation kinetics. We show here that despite being sorted to different pathways and located in distinct endosomes, EV1 inhibits EGFR downregulation. Simultaneous treatment with EV1 and EGF led to an accumulation of EGFR in cytoplasmic endosomes, which was evident already 15 min p.i. and more pronounced after 2 hr p.i. EV1 treatment led to reduced downregulation, which was proven by increased total cellular amount of EGFR. Confocal microscopy studies revealed that EGFR accumulated in large endosomes, presumably macropinosomes, which were not positive for markers of the early, recycling, or late endosomes/lysosomes. Interestingly, EV1 did not have a similar blocking effect on bulk endosomal trafficking or transferrin recycling along the clathrin pathway suggesting that EV1 did not have a general effect on cellular trafficking pathways. Importantly, EGF treatment increased EV1 infection and increased cell viability during infection. Simultaneous EV1 and EGF treatment seemed to moderately enhance phosphorylation of protein kinase C α. Furthermore, similar phenotype of EGFR trafficking could be produced by phorbol 12-myristate 13-acetate treatment, further suggesting that activated protein kinase C α could be contributing to EGFR phenotype. These results altogether demonstrate that EV1 specifically affects EGFR trafficking, leading to EGFR downregulation, which is beneficial to EV1 infection.
Endosomes are dynamic and multi-functional organelles involved in events ranging from trafficking to signaling. Among them early endosomes consist in a pleiomorphic structures composed of tubular and vacuolar domains which progressively mature into late/ multivesicular endosomes and lysosomes. In specialized cell types such as skin melanocytes, early endosomes give rise to the conventional lysosome, but also to a specialized lysosome-related organelle, the melanosome. Indeed, melanosomes serve specific function by synthesizing and storing the melanin pigment. Yet segregating two morphologically and functionally distinct compartments originating from a single endosomal population is not well explored. Interestingly, a rather novel sub-population of early endosomes, named signaling endosomes has emerged. Signaling endosomes have specific adaptor and multi-domains proteins called APPL that possibly control various functions like signaling, endocytic trafficking, transcription and epigenetic processes, thanks to interaction with diverse molecules. We hypothesize that APPL-signaling and EEA1-conventional early endosomes co-exist in melanocytes to guide the concomitant biogenesis and maintenance of lysosome and lysosome-related organelle.

In melanocytes, APPL1 interacts and co-distributes with melanin-synthesizing enzymes in undefined intracellular vesicles, and silencing its expression decreases pigmentation. However, whether signaling endosomes exist as distinct entities that define particular trafficking routes, components and steps during the biogenesis of melanosomes are not known. Extinguishing APPL expression in melanocytes impacted modestly the pigmentation and ultrastructural morphology of melanosomes, but dramatically increased lysosome-associated degradative structures. Then, specific steps of the formation of melanosomes that rely on lysosomal activity were affected.

Our data lead us to propose that APPL-endosomes contribute to functioning of lysosomes by controlling its homeostasis with other organelles, while conventional early endosomes generate melanosomes. Together, this study highlights how specialized cell types can cope the formation of two closely related yet functionally
related organelles.
Minimal invasive fluorescent receptor labeling to study EGFR internalization

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EGFR endocytosis has been extensively studied, often using imaging techniques involving EGFR tagged with fluorescent proteins. Although this method is a valuable tool for studying this process, advances in click chemistry and protein labeling have opened up the possibility for new methods of specifically labeling using small dyes. Genetic codon expansion allows the incorporation of non-canonical amino acids bearing ring-strained alkynes or alkenes at a specific site in a protein. The non-canonical amino acid is used to covalently bind to small fluorescent dyes via azide or tetrazine groups (1). By using hydrophilic dyes that do not pass the cell membrane, and by incorporating artificial amino acids in the extracellular domain of EGFR, it is possible to specifically label the portion of EGFR that is located at the plasma membrane at the time of labelling. Using this methodology, endocytosis can be observed by accumulation of the dye inside the cell (2). Previous work in our group has shown the influence of PI(3,4,5)P3 on EGFR endocytosis. Elevated levels of PIP3 induced EGFR endocytosis and recycling in the absence of a ligand and tyrosine phosphorylation. The mechanism by which this is achieved is not known (3). To elucidate the mechanism of PIP3 induced EGFR endocytosis, we produced different EGFR mutants that were checked for PIP3-induced internalization deficiency. For this purpose we used caged versions of PIP3 that are membrane-permeant. The caged PIP3 is not active because of a coumarin group masking the lipid head group that can be released by a flash of 405nm light. This controls the release of PIP3, both spatially and temporally, and allows us to precisely observe the effect on EGFR localization. These methods combined represent a potent platform for further studies of EGFR endocytosis that, despite years of research, is not yet fully understood.

Structural insights into Rabs and MyosinV interactions

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MyosinV is an unconventional actin-based molecular motor that acts as a transporter or a tether for a variety of membrane cargoes. In the resting state MyosinV adopts an autoinhibited conformation, forming a cytosolic pool of inactive motors. Several Rab GTPases and their effectors directly bind and cooperate with MyosinV to regulate membrane trafficking. Structural studies of MyosinV motor and its complexes with Rabs and Rab effectors provide insights into the mechanism of the motor's membrane recruitment and how that is coupled with myosin activation. Discovery and characterization of the direct interaction between the actin assembly regulator Spir and MyosinV, and Spir/MyosinV/Rab11 complex provide evidence for a synergic recruitment to promote both actin track generation and myosin motor activity in vesicle transport processes.
Myosin VI and actin dynamics induce membrane constriction and fission at melanosome

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Organelles have distinct and highly dynamic shapes, which are important for their function. By modifying membrane tension, the cytoskeleton and its motors are important regulators of membrane remodeling and organelle shaping. Here we show that myosin VI and actin dynamics function in remodeling the membrane of melanosome, a specialized organelle of skin melanocytes that synthesizes and stores melanin pigment. By constricting melanosomal tubules, myosin VI and WASH-dependent actin polymerization cooperate for the fission and release of those tubular intermediates. This fission process leads to the export of cargoes from melanosome, and importantly is functionally required for melanosome secretion and subsequent transfer of melanin to neighboring keratinocytes. Our data show that myosin VI, together with actin polymerization, controls the fission of transport intermediates. Such remodeling event contributes to the proper function of organelles.
Functional recruitment of dynamin requires multimeric interactions with SH3 domain containing proteins for efficient endocytosis

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Clathrin mediated endocytosis is controlled by a number of cytosolic parameters as well as extensive protein-protein interactions. An essential interaction occurs between the proline/arginine rich domain of dynamin and the Src homology domain 3 (SH3) of various proteins, including amphiphysin, endophilin, intersectin and syndapin. Indeed, disruption of these interactions by isolated SH3 domains or a 15-mer peptide named D15, bearing a PxRPxR motif mainly corresponding to amphiphysin binding, blocks endocytosis in living cells. D15 has become a popular tool to rapidly disrupt dynamin mediated endocytosis when injected in cells. It is however used at high concentrations (1-2 mM) and its specificity for various SH3 domain containing proteins has not been assessed. Moreover, dynamin and associated proteins are probably concentrated in ring-like structures around the neck of nascent vesicles, bringing multiple interaction sites in close proximity, a mechanism still poorly understood in living cells. To gain insight into the parameters of such interactions, we took a multidisciplinary approach using peptide design and characterization of binding with pull-down and surface plasmon resonance assays, quantitative live cell imaging assays of endocytosis (Merrifield et al. Cell 2005) and rescue of endocytosis with dynamin mutants in dynamin triple knock-out (TKO) cells (Park et al. J Cell Science 2013). We found that elongated and divalent D15-derived peptides bind more strongly to multimers of SH3 domains and are more effective at blocking endocytosis than D15. Moreover dynamin mutated in the D15 motif could partially rescue dynamin activity, and an additional mutation in flanking PxxPxR made dynamin completely inactive in TKO cells. From this data we conclude that dynamin interacts with multiple SH3 domain containing proteins, most likely amphiphysin, to be recruited efficiently to the neck of nascent vesicles and mediate vesicle scission in cells.
Novel function of an old enzyme, iPLA2, in the regulation of endocytic trafficking of MT1-MMP

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Calcium-independent phospholipase A2 (Group VI phospholipase A2; iPLA2) catalyzes the hydrolysis of glycerophospholipids at the sn-2 ester bond, producing unesterified fatty acids and 2-lysophospholipids. iPLA2 contains seven to eight ankyrin repeats at N-terminus, a linker region in the middle, and a catalytic domain at the c-terminus. Our previous studies indicated that iPLA2 play important roles in the regulation of endocytic recycling of c-Src and integrin alpha6. Also, we recently found that ankyrin repeats of iPLA2 binds to Rab5, also suggesting that iPLA2 might have a regulatory role in endocytic trafficking. Studies have demonstrated that upregulation of iPLA2 contributes to tumourigenesis and majority of human melanoma cell lines expressed, at mRNA and protein levels, high level of iPLA2. Several converging studies establish that MT1-MMP (also known as MMP14) comprises an essential arm of the tissue-invasive program of tumor cells. Based on these findings, we hypothesized that iPLA2 activity might be required for the regulation of MT1-MMP recycling, which would have a direct impact on the tumor invasion. Immunofluorescence staining and MT1-MMP endocytosis assays revealed that internalized MT1-MMP remains in the perinuclear area when iPLA2 activity is inhibited by pharmacological inhibitors, indicating that iPLA2 activity is not required for the endocytic internalization of MT1-MMP, but required for the recycling from endocytic recycling compartment to the cell surface. Inhibition of the iPLA2 activity of knockdown with shRNA significantly inhibits the formation of invadopodia and invasion of melanoma cells in collagen or Matri-gel invasion assays. These results suggest that high level of iPLA2 contributes to the invasiveness of melanoma via the regulation of MT1-MMP recycling.
Podosomes as endocytosis sites of activated integrin receptors at the cell-matrix interface

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Podosomes are adhesion structures at the cell-matrix interface. Each podosome comprises localized F-actin polymerization at the core surrounded by ring-shaped integrin-mediated adhesions. Invasive migrations, such as macrophage chemotaxis and cancer cell metastasis are mediated by the dynamic assembly of podosomes and closely-related invadopodia. We previously revealed that clustering of integrin-beta3 recruited endocytic proteins Dab2 and clathrin, and developed podosomes on viscous RGD membranes. However, the functional link between podosome and integrin endocytosis remains unclear. Here, we use labeled RGD peptides as integrin ligands to study molecular events of integrin-beta3 endocytosis at the podosome on RGD membranes. We find that the level of internalized RGD is elevated in podosome-forming cells. When podosome formation is inhibited by Src inhibitor PP2, the level of internalized RGD significantly decreases. By live-cell TIRF microscopy, we monitor the fine steps of podosome formation and associated endocytic events at individual RGD-integrin-beta3 adhesion clusters (RGD clusters). The intensity decrease of RGD clusters takes place in the initial phase of podosome formation (phase 1) and indicates the occurrence of RGD endocytosis. During the phase 1, there is a distinct increase of Dyn2 recruitment at RGD clusters. The intensity decrease of RGD clusters is synchronized with elevated recruitment of Dyn2 in prior to the maturation of F-actin podosome core. The intensity of Dyn2 then decreases before F-actin reaches to its peak intensity and subsequently forms matured podosome (phase 2). When GTPase-deficient mutant Dyn2-K44A is introduced into cells, the intensity of RGD clusters at the adhesion remains stable, and the level of internalized RGD is reduced during the podosome formation. Taking together, our observations suggest that Dyn2 facilitates the endocytosis of activated RGD-integrin clusters in the initial phase of podosome formation. The functional role of podosomes in integrin endocytosis can shed new light on dynamic regulation of cell motility and cancer cell invasive migration.
The differential regulation of clathrin-dependent and -independent endocytic pathways during proliferative quiescence and their implication on quiescence maintenance

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Under certain conditions, such as contact inhibition and withdrawal of growth factors, cells can exit from the cell cycle during a short time frame in G1 phase and enter a quiescent, non-proliferative (G0), state. The G0 cell population accounts for a considerable proportion of cells in the adult human body. Naive lymphocytes, hepatocytes, endothelial, heart, epidermal, and dormant stem cells primarily reside in proliferative quiescence. They can re-enter the cell cycle upon appropriate stimuli, and start proliferation to maintain tissue homeostasis. This feature distinguishes them from senescent or apoptotic cells, which have irreversibly left the cell cycle.

Quiescent cells exhibit very reduced activity of clathrin-mediated endocytosis (CME) of transferrin, low density lipoprotein (LDL) and epithelial growth factor. Clathrin-independent pathways, however, are differentially regulated during G0. Quiescent cells show active macropinocytosis of dextrans, and internalisation of specific cargoes is either down-regulated (integrin beta 1), unchanged (integrin alpha 3) or significantly up-regulated (oxidised LDL) when compared to cycling cells.

Integrin-mediated internalisation of extracellular matrix components has been shown to enhance cell survival by promoting mechanistic target of rapamycin complex 1 (mTORC1) activity. Using h-TERT immortalised RPE1 cells as a model for cellular quiescence, we are interested in the implication of differentially regulated endocytic activity of specific cargoes on mTORC1 signalling and its role in maintaining the quiescent state.
MACC1, a prognostic biomarker for colorectal cancer is involved in clathrin-mediated endocytosis

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Colorectal cancer (CRC) progression and metastasis formation is directly linked to poor overall patient survival. The recently identified gene MACC1 (Metastasis Associated in Colon Cancer 1) is a prognostic biomarker for metastasis and survival in CRC. MACC1 regulates the expression of the hepatocyte growth factor (HGF) receptor MET and plays a key role in the HGF/MET-signaling, which is often deregulated in metastatic cancer. Interestingly MACC1 shows a 40\% homology with SH3BP4 also known as transferrin trafficking protein (TTP). SH3BP4 has been characterized as an important protein involved in CME and in particular its perturbation selectively affects the transferrin receptor (TfR) trafficking from the plasma membrane to endosomes. MACC1 and SH3BP4 show at their N-Terminus some CME cassettes, involved in binding proteins such as a clathrin box, NPF- and DPF-sites, and an SH3 domain for potential binding of clathrin,epsin15 homology (EH) domain proteins, adaptor protein 2 (AP2), and dynamin (DNM2), respectively. Here we aim to understand the involvement of the metastasis inducer MACC1 in CME. To dissect the functional relevance of MACC1 in cellular processes we performed a mass spectrometry analysis of interaction partners. This approach identified 1103 proteins associated with MACC1. By analyzing the interaction partners with DAVID (a Data base for Annotation Discovery) tool, we found interesting GOTERMs, and focused on a set of CME related proteins. We validated the interaction of MACC1 and factors of CME (clathrin, DNM2 and AP2) and two receptors (MET, TfR) via co-IP. We confirmed an increase of colocalization of MACC1 within the cytoplasm with both clathrin and TfR, upon holotransferrin stimulation. MACC1 involvement in CME might be directly dependent on both its N-terminus -with NPF and DPF binding motifs, and the clathrin box - and the SH3 domain. In SW480 cells MACC1 overexpression interferes with the internalization of TfR, its surface distribution, and defines a faster recycling of TfR. Our next step is to determine whether the deletion of potential CME binding sites influences MACC1-dependent changes of endocytosis in CRC cell lines. In summary, protein interactions, colocalization and surface and uptake distribution of the TfR indicate a role of MACC1 in receptor endocytosis.
Intrinsic phosphorylation and ubiquitination properties of EGFR regulate endosomal maturation.

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Specific interactions between the epidermal growth factor receptor (EGFR) and downstream effectors regulate the receptor signaling and trafficking. Activated receptors become autophosphorylated and recruit Cbl and Grb2 to specific phosphorylation sites. These two adaptor proteins have been shown to be important for endocytosis and degradation. However, the specific regulatory roles of the phosphorylation and its binding partners, Cbl and Grb, in endosomal receptor progression have not been fully investigated. We have generated a panel of live cell imaging experiments to investigate the trafficking of receptors with impaired binding sites for Cbl and Grb2. Receptors with mutations in tyrosine phosphorylation sites 1045 (Y1), 1068 and 1086 (2Y) and 1045, 1068 and 1086 (3Y) were imaged live to decipher their progression in the endocytic pathway. Through an extensive quantitative live cell imaging analysis we have shown that the mutated receptors induce alternative ubiquitylation patterns that affect internalization, endosomal progression and degradation. EGFR can through the intrinsic phosphorylation properties and the binding of Cbl and Grb2 control the endosomal progression by tuning the switch between Rab5 and Rab7 positive endosomes. We here suggest a regulatory mechanism where EGFR inherently can regulate endosomal maturation to modify its recycling and degradation.
New roles of the PROPPIN Atg18 in retrograde traffic.

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Protein sorting in the endocytic and secretory pathway of cells is a highly important mechanism. Endocytosed receptors need to be sorted either for recycling back to the plasma membrane or to be targeted for degradation. This recycling is fundamental to maintain a high availability of transmembrane receptors at the plasma membrane. The yeast lysosome-like vacuole is an essential organelle where membrane sorting takes place in response to changes in environmental conditions. These membrane sorting events depend on proteins that are capable of deforming and cutting tubulo-vesicular intermediates to generate transport vesicles. Previous studies have already highlighted the role of proteins as the dynamin-like protein Vps1 during endocytosis and retrograde traffic in yeast. Recent works in our laboratory have highlighted the essential role of PROPPIN proteins, and in particular Atg18 (WIP11/2 in mammals), for membrane scission activity during vacuole fragmentation. Based on these new observations, we are now trying to better understand the molecular mechanism and regulation of PROPPINs in-vivo.

To this end, we performed an affinity purification of the PROPPIN Atg18 coupled to mass spectrometry to reveal new putative partners. Interestingly, this screen has revealed several partners implicated in retrograde trafficking. We are now deciphering those interactions to better characterized how PROPPINS can contribute to the recycling of proteins cargo from the vacuolar membrane.
Correct plasma membrane expression of SV2A is critical for synaptotagmin-1 trafficking at the synapse

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Synaptic vesicle protein 2A (SV2A) is a large 12 transmembrane domain protein present on synaptic vesicles (SVs). Whilst the exact function of SV2A is unknown, it is linked to epilepsy, with knockout mice showing seizures and dying early after birth. SV2A is necessary for the correct trafficking of the vesicular Ca2+ sensor, synaptotagmin-1 (syt-1) via a phosphorylation-dependent interaction of the N-terminus of SV2A and the C2B domain of syt-1. Since the mechanism through which SV2A controls syt-1 trafficking is undefined, we investigated whether mutations identified in human disease could reveal potential modes of action. One such mutation was identified by Serajee and Huq (2015) in the 3rd cytosolic loop of SV2A (Arg383Gln) in a patient with intractable epilepsy. The effect of the R383Q mutation was determined in primary neuronal cultures using a molecular replacement strategy, since the patient was homozygous for the mutation. The R383Q mutation had no effect on activity-dependent SV recycling, which was evaluated by the pH sensitive reporter, synaptophysin-pHluorin. However the R383Q mutation did result in increased plasma membrane stranding and accelerated retrieval of syt-1-pHluorin, a phenotype identical to that displayed upon SV2A depletion with shRNA. Thus the R383Q mutation is a loss of function mutation. When the effect of the R383Q mutation on SV2A trafficking itself was investigated, it resulted in increased plasma membrane stranding of SV2A-pHluorin with no discernable effect on its activity-dependent trafficking. These results suggest that control of syt-1 trafficking by SV2A is manifested at the plasma membrane and not during SV recycling itself. A determination of how the increased plasma membrane residency of SV2A causes dysfunctional syt-1 trafficking will be critical for determining its molecular role at the synapse.
Rab5-to-Rab7 conversion during EGF receptor endocytosis occurs on separated domains of endosome

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Small GTPases Rab5 and Rab7 are the key regulators of endocytic degradative pathway. During the early-to-late endosome progression so-called “Rab5-to-Rab7 conversion” occurs during which Rab5 is switched to Rab7 on endosomal membrane. A mechanism that implies dissociation of Rab5 from the endosomal membrane and its replacement by recruiting of Rab7 to the same membrane from the cytoplasm was described for endocytosis of LDL and BSA. However, it is not clear yet whether this mechanism is common for all cargoes. No data available for endocytosed signaling tyrosin kinase receptors which TK domain is known to be essential for the arrangement of its own endocytic pathway.

Using live-cell confocal microscopy we followed endocytosis of EGF receptor in HeLa cells transiently co-transfected with plasmids encoding early endosomal antigen 1(EEA1)-EGFP and Rab5-mCherry or Rab7-DsRed. Soon after endocytosis stimulation by EGF conjugated with quantum dots (EGF-QDs) the ligand and EEA1 were expectedly found on Rab5-positive vesicles. Rab5 was relatively uniformly distributed over the surface of the vesicles, however, EGF-QD and EEA1 occupied distinct non-overlapping domains. At about 20—30 min during segregation process started that resulted in Rab5/EEA1-positive vesicles’ formation and its pinching off multidomain endosome. Using dynasore and CK666, we have showed that dynamin and dynamic actin networks polymerized by the Arp2/3 complex and cortactin, are involved in segregation of Rab5- and EEA1-positive domain from the multidomain endosome. Moreover, Rab7 was recruited onto EGF-containing domain shortly before EEA1-positive domain pinched off multidomain endosome.

Thus, the mechanism of Rab switch during the EGF receptor endocytosis is different from the that described for LDL and BSA but similar to Rab5-to-Rab7 conversion reported for endocytosis of viruses. Thus, conversion mechanisms may depend on the cargo.

The research was supported by research resource center «Molecular and cell technologies» of St. Petersburg State University, by the grant from the Russian Science Foundation (project №14-50-00068) and with financial support from the Federal Agency of Scientific Organizations (Russia).
Genome-wide CRISPR/Cas9 screen identifies components of the B cell antigen internalisation pathway

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B cells interact with antigen presenting cells to acquire antigen for processing and presentation to T cells. Upon antigen recognition, the B cell receptor (BCR) generates activating signals leading to myosin- and clathrin-dependent endocytosis. Endocytosed antigens are trafficked to lysosomes for processing.

Endocytosis impacts on several B cell activation steps important for antibody responses as well as control of B cell autoimmunity and malignant transformation. However, the mechanisms linking BCR signalling, antigen uptake, and antigen processing pathways remain unclear. A better understanding of the machinery regulating BCR-dependent endocytosis will enable the design of antigens that promote acquisition and presentation by B cells, with direct implication for vaccine design. In addition, internalisation of surface BCR is an important regulator of cell anergy and thus plays a role in limiting cell activation and malignant transformation.

We aim to investigate the mechanisms regulating B cell antigen uptake likely involve a very large and incompletely understood network of genes. To understand the complexity of B cell activation and endocytosis on a genome-wide scale, we have established a pooled library approach using the lentiviral CRISPR/Cas9 system. We created Ramos cells stably expressing Cas9 nuclease and then introduced targeting single guide RNAs. The effects of genetic disruptions were measured in terms of B cell survival and ability to internalise antigen using flow cytometry and next-generation sequencing. This assay can easily be adapted to compare the recycling of distinct receptors and the signaling pathways controlling them.

A screen for antigen internalisation defects has identified components of clathrin coated pits (EPN1, PICALM), intracellular trafficking (RAB7A, VPS13), regulators of the actin cytoskeleton (ACTR2, CCZ1B) and BCR signalling components (GRB2, LYN, CBL). Further, I have reproducibly detected depletion of sgRNAs targeting essential genes, such as Syk and CD19, over time. These results support the use of pooled CRISPR screens to detect essential components of BCR effector pathways. Using antigen-presentation assays and imaging protein localisation, a number of candidates have been validated as individual knockouts in B cell lines and primary mouse cells, including a recently described component of fast endocytosis, not previously seen in B cells.
UDP-galactose transporter as a novel regulator of endocytosis

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Nucleotide sugar transporters (NSTs) are Golgi- and/or ER-resident multitransmembrane proteins which transport active forms of carbohydrates across the organelle membranes to supply glycosylation enzymes with their substrates. UDP-galactose transporter (UGT) is one of the best-characterized NSTs. It translocates UDP-galactose from the cytosol into the Golgi lumen, thereby feeding a series of galactosylation reactions. A ricin-resistant MDCK-derived cell line (MDCK-RCAr) bears an inactivating mutation within the UGT-encoding gene. Carbohydrate parts of glycoconjugates synthesized by these cells are markedly truncated and significantly enriched in terminal N-acetylglucosamine and N-acetylgalactosamine residues, which are barely exposed in wild type cells due to capping with galactose. This phenomenon reflects the indispensability of UGT for proper galactosylation.

We recently observed that UGT deficiency causes noticeable alterations in endocytosis. Namely, in MDCK-RCAr cells EEA-1-positive early endosomes were substantially depleted and reduced in size when compared with their wild type counterparts. Importantly, UGT-deficient human cell lines generated via CRISPR/Cas9 approach displayed similar characteristics. On the other hand, overexpression of epitope-tagged UGT increased the number of EEA-1-positive early endosomes and forced their accumulation in perinuclear areas. Moreover, in the majority of the transfected cells a subset of EEA-1-positive early endosomes clearly colocalized with UGT-rich membranes, suggesting the existence of a UGT-dependent endosome to Golgi trafficking pathway. However, the biological significance of this phenomenon is yet to be determined. All these findings encouraged us to comparatively examine the uptake of biotinylated dextran by UGT-deficient and wild type cells. The results of this functional assay were consistent with the data obtained from the immunofluorescence experiments, i.e. UGT-deficient cells displayed lower dextran internalization and accumulated it in smaller vesicles than wild type cells.

Our findings reveal the existence of a novel, previously unrecognized functional link between a nucleotide sugar transporter and endocytosis. The study here presented firmly demonstrates that galactose-bearing macromolecules are important players in the formation and maturation of early endosomes, which is in line with the GL-Lect hypothesis recently addressed by L. Johannes and his colleagues.
Systems biology approach: dependencies of cell adhesion and endocytosis

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Functional genomics provides a massive amount of data reflecting the organization of cellular mechanisms at the system level. However, a lack of analytical approaches and tools limits our ability to go beyond a mere catalogue of genes and infer the functional organization of cellular processes. Here, we present an integrated experimental and computational approach aimed at learning the hierarchy of cellular modules from functional genomic datasets. We applied newly developed network reconstruction algorithms to a genome-wide multi-parametric image-based RNAi screen on endocytosis [Collinet et al., 2010] and found unexpected relationships between cell spreading and endocytic cargo distribution. Because signalling cargo distribution over endosomes plays a role in the tuning of cellular signal processing mechanisms [Villaseñor et al., 2015], we formulated hypotheses on the regulation of endocytosis by cell adhesion. We experimentally tested these hypotheses by plating cells on substrates with controlled stiffness to mechanically modulate cell spreading. The increased cell spreading predictably shifted the endosome distribution towards a higher number of small and peripheral endosomes, leading to slower cargo processing and increased cell proliferation. Our results revealed a hierarchy of functional connections between cell adhesion, actin organization, endosomal distribution and signal transduction.
High resolution 3D cryo-electron microscopy of multiple clathrin cage geometries

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Clathrin lattices in cells are highly dynamic and adaptable in shape and this supports their function in clathrin-mediated endocytosis. When purified, individual clathrin triskelions can also assemble to form multiple cage architectures. This intrinsic ability of clathrin to form stable cages with such a variety of geometries is important for its biological role yet we still do not understand the structural origin of this assembly process. We have determined cryoEM structures of five different clathrin cage arrangements, that form in vitro, at 9 – 24 Å resolution. Local reconstruction methods enabled us to further improve the resolution of hub structures to 4.2 Å resolution. Our highest resolution map reveals points of contact at key positions in the clathrin cage structure and new structural features within the clathrin trimerisation domain. Fitting triskelion legs into four different cage arrangements showed that a discrete set of heavy chain conformations are capable of creating all of the geometries we have observed. These structures explain how triskelion leg conformations determine variable cage geometries and suggest a molecular basis for adaptable cage assembly during endocytosis.
INPP5B: A new player in the regulation of Akt signaling

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Type II inositol polyphosphate 5-phosphatase B (INPP5B) is known to hydrolyze the 5-phosphate group from water- and lipid-soluble signaling molecules including Ins(1,4,5)P3, Ins(1,3,4,5)P4, PtdIns(4,5)P2, and PtdIns(3,4,5)P3. INPP5B localizes to the early endocytic pathway, and has been shown to bind specific Rab GTPases including Rab5 as well as the endosomal signaling adaptor protein APPL1. However, the cellular function of INPP5B remains poorly defined, and whether it can participate in cellular signaling pathways has yet to be determined.

Here, we used genome editing to create a cell line in which endogenous INPP5B can be acutely removed (within 30 minutes) by tagging the endogenous protein with the auxin degron. By using this strategy, we found that acute removal of INPP5B enhances insulin-induced Akt phosphorylation, and the effect is most evident at 15 minutes post-stimulation. Moreover, rescue experiments revealed that the effect of INPP5B on Akt signaling is dependent on both its phosphatase activity and its ability to bind APPL1. At this stage, it is unclear how and where INPP5B exerts its effect on the Akt pathway, and clarification requires additional work. Current experiments using a combination of genome editing, imaging and mass spectrometry are aimed at dissecting the site of action of INPP5B and the mechanism by which it modulates signaling via the Akt pathway, as well as the cellular consequences of this altered signaling.
ENDOCYTIC REGULATION OF THE JAK/STAT PATHWAY

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The JAK/STAT pathway is an evolutionarily conserved pathway involved in a variety of cellular processes, from proliferation to apoptosis. The pathway is crucial during development and immune responses, with its importance being highlighted through its association with multiple diseases, such as leukemia and gigantism. Due to the range of cellular events influenced by the JAK/STAT pathway, tight regulation is required to produce a defined response. Increasingly, endocytosis is understood to not only regulate signaling by altering the number of active cell-surface receptors, but also to have a significant role in the specific outcome of individual signals. Therefore, endocytosis is a potential mechanism by which, a signal with a broad range of outcomes, can be tightly monitored. However, the role(s) of endocytosis in specifying the outcome of the JAK/STAT pathway are only beginning to be elucidated.

The genetically tractable Drosophila has a conserved JAK/STAT pathway, which is considerably simpler than the complex networks seen in mammals. Utilising Drosophila cell lines we demonstrated that endocytosis is capable of differentially regulating the transcriptional outcome of JAK/STAT pathway activation. Our data suggests that, as the signaling-complex undergoes endocytic trafficking, it reaches specific signalosomes, whereby a distinct subset of pathway targets are expressed. This demonstrates the importance of endocytosis in defining a specific signal. We now focus on identifying novel, trafficking-dependent, modifications and/or binding partners of the JAK/STAT transcription factor, STAT92E, whilst defining the signalosomes which enable this regulation. This data will not only enhance our understanding of JAK/STAT pathway regulation, but may also highlight novel endocytic proteins important in the regulation of multiple signaling pathways.
Investigation into the mechanisms of endocytosis of Targeted Secreted Inhibitors (TSI), derivates of Botulinum neurotoxins, in non-neuronal mammalian cell lines

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Botulinum neurotoxins (BoNTs), the most potent toxins known to man are widely used in cosmetic and medical treatments. They are the agents of botulism and exert their action by inhibiting neurotransmitter release at nerve terminals. BoNTs are single chain peptides formed of The N-terminal light chain (LC) domain, responsible for cleavage of soluble N-ethylmaleimidesensitive factor attachment protein receptor (SNARE) proteins in intoxicated neurons, an N-terminal domain (HN) responsible for translocation and a C-terminal domain (HC) responsible for neuronal binding.

The modular arrangement of BoNTs has enabled a number of engineering approaches aimed at extending their therapeutic application. In particular, the BoNT derivates Targeted Secreted Inhibitors (TSI), in which the HC binding domain is replaced by an alternative cell-binding domain or receptor ligand, are of great interest because they can target non-neuronal cell types beyond the neuromuscular junction. However, the mechanism of plasma membrane internalization, endocytosis and translocation of TSIs following binding to their cell surface receptor targets are not yet known. A better understanding of such mechanisms may help with the design of new TSI molecules which will enable specific targeting of non-neuronal cell types with SNARE cleavage potencies similar to the natural toxin. In this study, we investigate these mechanisms using a microscopic approach. Confocal imaging of pulse-chase experiments using a TSI targeted to the Epidermal Growth Factor Receptor (EGFR) showed binding to the cell surface EGFR when internalisation was blocked by low temperature. Subsequently, 5 minutes after raising the temperature to induce TSI internalisation, the TSI is shown to co-localise with both the early endosomal marker EEA1 and EGFR, forming protein aggregates characteristic of EGFR endocytosis. Endocytosis and protein aggregation reached a peak after 30 minutes with translocation of the LC starting to appear after 60 minutes and continuing beyond 4 hours. Further studies are now being performed in which a fluorescent tagged TSI is used to determine the endocytic mechanisms of these BoNT derivates by confocal live imaging.
A proteomic interaction screen identifies mutation-induced gains of endocytosis motifs as a cause of disease

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Mutations in intrinsically disordered regions (IDRs) of proteins can cause a wide spectrum of diseases. Since IDRs lack a fixed three-dimensional structure, the mechanism how such mutations cause disease remains unknown. Here, we employ a proteomic screen to investigate the impact of mutations in IDRs on protein-protein interactions. We find that mutations in the cytosolic tails of three transmembrane proteins (GLUT1, ITPR1 and CACNAH1) lead to increased binding of clathrins. In all three cases, the mutation creates a dileucine motif which is known to mediate clathrin-dependent endocytosis. Follow-up experiments on GLUT1, a glucose transporter involved in GLUT1 deficiency syndrome, revealed that mutated GLUT1 mislocalizes to intracellular compartments. A systematic analysis of known disease-causing variants revealed a significant overrepresentation of dileucine motifs in cytosolic tails of transmembrane proteins. Based on their putatively shared mechanism of pathogenesis we propose to classify these diseases as “dileucineopathies”.
Phosphorylation of clathrin light chains regulates the cargo specific maturation of clathrin coated pits

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Clathrin light chains (CLCs) are part of every clathrin triskelia but their role in clathrin mediated endocytosis is still poorly understood. Although apparently not required for the uptake of transferrin or EGF, in vitro (Ferreira et al., Current Biol, 2014) and in vivo studies (Wu et al., PNAS, 2016) have shown that they are required for the internalisation of a subset of G protein-coupled receptors (GPCRs). This uptake is regulated through phosphorylation of the CLCs at specific serine residues which differ for different GPCRs. CLCs also modulate the mechanical properties of the clathrin lattice and are required for the deformation of membranes with an increased bending stiffness but are dispensable for the deformation of softer membranes (Dannhauser et al., Traffic, 2015).

Here we investigate further the mechanism by which the CLCs control the uptake of a specific subset of cargo receptors. We demonstrate that the phosphorylation of the CLCs is required for the stability of the clathrin lattice and are able to link this phenotype to the recruitment of the clathrin rearranging protein (GAK) to short lived, abortive clathrin coated pits (CCP). Furthermore, we can demonstrate that site specific phosphorylation of the CLCs is required for the rapid turnover of clathrin in a cargo dependent manner. Our working hypothesis is that a high local concentration of GPCRs in a CCP increases the local bending stiffness of the membrane and therefore requires the contribution of the CLCs for its deformation. This deformation depends on the rapid exchange of clathrin, mediated by GAK and is controlled by the phosphorylation status of the CLCs.
The second polar body is internalized and degraded by LC3-associated phagocytosis in C. elegans

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To understand how early embryonic cells deal with cell corpses or released organelles containing potentially harmful cargos, we examined the fate of C. elegans polar bodies. Polar body extrusion is an extreme asymmetric cell division resulting in a tiny polar body containing maternal DNA and a large oocyte. In human and mouse embryos, polar bodies are thought to undergo apoptosis, but the final fate of the polar body is not well studied.

Surprisingly, we found that the second polar body is internalized in a stereotyped manner at the 2- or 4-cell stage in C. elegans. Prior to internalization, phosphatidylserine is externalized on the polar body and the cell responds by recruiting the phagocytic receptor CED-1/MEGF11 to the plasma membrane neighboring the polar body. CED-1 and other CED-10/Rac-dependent engulfment pathways are required for uptake, and actin is enriched around the polar body, consistent with phagocytic cup formation. Thus, the polar body is internalized by receptor-mediated phagocytosis, similar to cell corpse engulfment.

As the second polar body sends signals to embryonic cells, the polar body may have a function after extrusion, which could indicate that polar body removal for genetic testing may not be non-invasive. We examined the pattern of inheritance of the worm polar body and did not discover a link between polar body inheritance and cell fate. Thus, the polar body is unlikely to be a cell fate determinant. We are currently testing whether polar body removal alters embryonic development or whether the polar body is phagocytosed to avoid auto-inflammatory signaling.

After polar body phagocytosis, the double membrane phagosome recruits RAB-5, RAB-7, and autophagy-associated Atg8/LC3 proteins. Polar body degradation is delayed when Atg8/LC3 lipidation is disrupted by atg-7 knockdown, but not when the macroautophagy-specific PI3K subunit EPG-8 is deleted, suggesting that the polar body is degraded via LC3-associated phagocytosis. Before degradation, the polar body phagosome fragments, a rare example of double membrane budding. This work establishes a new model system for studying the mechanisms of receptor-mediated phagocytosis, double membrane budding, degradation via LC3-associated phagocytosis, and also reveals novel insights into the mechanisms of cell corpse clearance.
Integrin Endocytic Trafficking in miR-30b Mediated Epithelial-to-Mesenchymal Transition

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Ubiquitously expressed miR-30b is playing an important role in epithelial-mesenchymal transition (EMT), cell migration, survival, autoimmune response and some other. Here, we attribute for the first time the novel function of miR-30b in regulation of membrane trafficking. Specifically, Golgi complex to PM transport and endocytosis of diverse cargoes is regulated via simultaneous targeting of AP3S1, golgin-97 and SCAMP1. Among other, down-regulation of these proteins prevents efficient internalization of integrin alpha2beta1 and, consequently, increases epithelial-mesenchymal transition (EMT). As integrin alpha2beta1 expression is little changed under these conditions, we suggest the essential role of the membrane trafficking in aggravating miR-30b-mediated EMT.
Simulations of clathrin coated pit formation

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We present simulations of the assembly process of clathrin coated pits during endocytosis, using coarse-grained models of clathrin, adaptor protein and membrane. Coated pits form spontaneously in nucleation events at the membrane and subsequently steadily grow in size by binding triskelia and adaptor proteins from the cytosol, thereby curving the membrane. Curiously, the growth becomes arrested when a coat has evolved into a hemi-spherical dome. By performing free energy calculations, we find that the hemi-spherical dome forms a local free energy minimum along the reaction path. The coated pit has to surmount an activation barrier of about 30 kBT before the continued growth of this pit once more becomes a spontaneous process and the coat matures into a nearly completed cage. This barrier might be related to a 'checkpoint' suggested by experimental studies, continuing those pits that are likely to be productive while discontinuing those that are not.

We also present a statistical-mechanical theory explaining the role of adaptor proteins in in vitro cages and their impact on the cage size, revealing the prominence of the mechanical properties of the adaptor protein's disordered linker. This linker also plays a pivotal role in vivo by ensuring that coated pits bend the membrane into the cell, rather than into the exterior of the cell.

M. Giani et al., Biophys. J. 111, 222 (2016).
M. Giani et al., manuscripts in preparation.
Communication between distinct infection associated compartments drives the intracellular lifestyle of Salmonella

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Salmonella Typhimurium is a Gram-negative bacterium that invades non-phagocytic intestinal epithelial cells. The bacteria induce membrane ruffles at the entry site and are engulfed into a membrane enclosure called “Salmonella-Containing Vacuole” (SCV). This SCV generally matures into a bacterial niche where Salmonella resides and replicates. However, in 10\% of the infected epithelial cells the SCV ruptures and releases Salmonella into the cytosol where it replicates at high rate. The molecular factors leading either to the maturation or to the rupture of the SCV remains unknown. Salmonella entry is concomitant with the formation of newly formed macropinosomes, termed “Infection Associated Macropinosomes” (IAMs) that originate from the closure of the membrane ruffles. As these IAMs are found in close proximity to the SCV at the entry site, we investigated the potential interaction between both compartments. We found that in most cases IAMs fuse with the SCV within minutes after bacterial internalization, and the small percentage of SCVs that do not fuse with IAMs ruptured, which resulted in Salmonella vacuolar escape. Taken together, these data support a model where SCV-IAM fusion is needed to maintain the SCV membrane integrity, and determines the intracellular fate of Salmonella. In order to identify the host factors involved in SCV-IAMs fusion, we isolated IAMs to high purity and we analyzed their proteomic composition. Comparing the IAM proteome with the previously obtained SCV proteome we identified proteins that coordinate their interaction. Currently, we use cutting-edge dynamic imaging techniques including super-resolution to characterize the interaction between SCV and IAMs. Our approach elucidates new mechanisms of host-pathogen interactions, and more broadly in cell biology defines how distinct endomembrane compartments interact.
Investigating the role of FAM21 in innate immune dendritic cells

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Vaccinia virus (VACV) belonging to Poxviridae family, is a large DNA virus with a wide host range to infect mammalian cells. Our previous studies with HeLa cells showed that Vaccinia mature virus is endocytosed into host cells and a cellular protein Fam21 is required for virus penetration. Fam21 is a component of WASP and Scar homolog (WASH) protein complex that mediates actin polymerization at the endosomal membranes to facilitate cargo-containing vesicles to be sorted out of endosomes. To study the in vivo function of Fam21 we generated a conditional Fam21 knockout mice in C57BL/6 background in which Fam21 was specifically knocked out in CD11c-positive dendritic cells (DC) population. Preliminary results indicated the role of Fam21 in antiviral functions of DC as Fam21 conditional knockout mice (CKO) were more susceptible to VACV infection through an intranasal infection route. We generated bone marrow derived dendritic cells (BMDC) from WT and Fam21 CKO mice and the data revealed a decreased phagocytic activity and antigen presentation function of DC isolated from Fam21 CKO mice, consistent with the endocytic role of Fam21 in HeLa cells. The CKO BMDC displayed cell spreading defects consistent with decreased surface levels of integrin CD11c on CKO BMDC. The detailed mechanism of Fam21 in DC development and function will be investigated in future.
F-BAR domain containing protein PACSIN2 regulates clathrin-independent endocytosis via membrane tubules.

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In addition to clathrin-dependent mechanisms, endocytosis also occurs by clathrin independent pathways. It is now clear that clathrin-independent endocytosis (CIE) transports a variety of endogenous cargoes and is also hijacked by viruses and bacteria to gain entry into the host cell. However, how CIE carriers form is not well understood, and little is known about the cellular machinery involved in this process. Recently, we showed that microtubules, dynein, and dynactin help bend the plasma membrane to form tubular CIE carriers. In the current study, we investigated whether BAR domain containing proteins, which are known to recognize and stabilize membrane curvature, also contribute to the biogenesis of these CIE carriers. Using an assay that stabilizes CIE carriers as surface-attached tubules, we identified the F-BAR domain-containing protein PACSIN2 (protein kinase C and casein kinase II interacting protein 2, also known as syndapin II) as a major tubule-associated protein. PACSIN2-positive tubules are also present under steady state conditions, suggesting they demarcate a constitutive CIE pathway. We show that the generation of PACSIN2-enriched tubules is regulated by the combination of microtubule, dynein, and dynactin and identify CD147 as an endogenous cargo molecule of this pathway. Together, these data suggest that PACSIN2 facilitates clathrin-independent endocytosis via membrane tubules and that the formation and maintenance of these structures is coordinately regulated by microtubules and the motor protein dynein.
Defining a novel role of distinct membrane trafficking regulators in blood glucose homeostasis

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The increase in the prevalence of type 2 diabetes has reached a qualified epidemic stage. The liver plays a central role in the development of type 2 diabetes due to its function in maintaining whole body glucose homeostasis. This is achieved by two cellular modules: glucose metabolism and endocytosis. Endocytosis is required for the uptake and signal transduction of growth factors, signaling receptors and hormones. The storage or release of glucose through a tight control of glucose metabolism allows maintaining whole body glucose homeostasis. These two modules need to be synchronized to collectively control glucose metabolism, where dysfunctions in this process promote the development of type 2 diabetes.

Recent evidences suggest that endocytosis participates in regulating glucose metabolism. First, overexpression of Rab25 in mammalian cells increased cellular glycogen stores to enhance cellular survival during energetic stress. Second, depletion of the endosomal PI-3-Kinase, Vps34 in mice in vivo led to hepatomegaly, hepatic steatosis and to a defect in glycogen storage. Third, knockdown of Rab5 in mouse liver caused hypoglycemia due to an inhibition of liver glucose production. Strikingly, loss of Rab5 in diabetic db/db mice rescued their elevated blood glucose levels highlighting a potential therapeutic application.

Here, we report a novel trafficking player in the control of liver metabolism, Rab24, which functions at the intersection between endoplasmic reticulum, endosomes and mitochondria and is required for autophagosome maturation. We find Rab24 to be upregulated in livers of high-fat diet mice and in diabetic patients with fatty liver disease. Depletion of Rab24 increases mitochondrial mass and function in primary mouse hepatocytes in vitro and improves glucose clearance and serum cholesterol levels in vivo, suggesting a regulatory role on glucose and lipid metabolism. Altogether, our data provide novel insights into the intracellular regulation of liver metabolism and pave the way for their exploitation in the control of hepatic glucose homeostasis and thus the treatment of type 2 diabetes.
The function of CD2AP in Aβ uptake and degradation

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CD2AP is a scaffold protein that has been shown to be involved in various cell functions including intracellular trafficking. In addition to the known function of CD2AP in T cell receptor regulation, recent GWAS showed that CD2AP is associated with Alzheimer's disease (AD). A hallmark of AD pathogenesis is the extracellular accumulation of Aβ, which is a product of Amyloid Precursor Protein (APP) cleavage. Dysregulation of Aβ production and/or clearance are thought to be critical in the pathogenesis of AD. To investigate the effect of CD2AP in Aβ accumulation, we generated CD2AP knockout (CD2AP KO) HeLa cells using CRISPR-Cas9 technology. Knockout of CD2AP in cells was confirmed by western blot and immunofluorescence staining. Compared to wild-type (WT) cells, CD2AP KO cells showed increased Aβ accumulation, which was due to delayed degradation of intracellular Aβ after uptake. To understand how CD2AP regulates Aβ uptake and degradation, we examined the localization of CD2AP in intracellular compartments. At steady state, CD2AP showed significant co-localization with MICALL1, a tubular endosome marker, suggesting the potential function of CD2AP in endocytic trafficking through this compartment. We hypothesize that CD2AP is required for delivery of cargos into lysosomes, and hence, CD2AP deficiency delays degradation of cargo, such as Aβ.
Melanin resides in mildly acidic and mildly degradative late endocytic compartments and resists degradation within keratinocytes

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Melanosome transfer from donor melanocytes to recipient keratinocytes and subsequent redistribution to the supra-nuclear area is a critical process in skin pigmentation and protection against ultra-violet radiation. However, the molecular mechanisms underlying intercellular transfer of melanin remain poorly characterized. We developed an in vitro system to analyze the mechanisms of melanin uptake and trafficking within keratinocytes and employed it to identify molecules involved in this process. For this, we used melanosomes secreted by melanocytes lacking the limiting membrane, which we termed melanocores and analyzed the role of endocytic Rab GTPases in melanocore uptake by XB2 keratinocytes. We found that the silencing of Rab5b impairs melanocore uptake by XB2 keratinocytes. Surprisingly, we observed that the silencing of the late endocytic regulators Rab7a or Rab9a does not affect melanocore uptake. To explore this finding, the acidic and degradative capacity of XB2 keratinocytes was assessed. For this, we evaluated the expression of late endosome/lysosome markers and observed that XB2 keratinocytes express them in amounts comparable to other cell types. Moreover, we used DQ-BSA and LysoTracker to examine the presence of degradative and acidic compartments, respectively and found that XB2 keratinocytes contain these types of compartments. Interestingly, we observed that melanocores reside in compartments that possess late endosome/lysosome markers but are neither highly degradative nor highly acidic. Hence, our results suggest that melanocores are stored in specialized late endocytic compartments within keratinocytes that are not highly acidic or degradative, allowing them to resist degradation for long periods.
Unravelling the cellular mechanisms controlling lipid nanoparticle mediated delivery of mRNA

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Modified mRNAs hold potential to offer a pioneering way of treating disease by triggering the body’s natural processes for protein production. However, one major challenge is productive delivery of the modified mRNA to the cell cytoplasm for protein translation. The use of lipid nanoparticle (LNP) based vehicles to aid delivery of nucleic acids is well documented, however key challenges remain with lack of efficacy observed in some target tissue types associated with poor uptake and endosomal entrapment.

This work aims to unravel the key mechanisms controlling cellular uptake and intracellular trafficking of LNPs and mRNA cargo across different cell lines. We first screened uptake of LNPs and productive delivery of mRNA cargo across a range of tumour cell types. Cellular uptake and cytoplasmic delivery was monitored using fluorescently labelled LNPs delivering mRNAs encoding a reporter protein (eGFP). We found delivery to be highly variable, with some tumour types completely refractile to LNP based delivery. We then applied a phenotypic screening approach using selected libraries of small molecule compounds alongside RNA interference strategies with the objective to identify pathways that if perturbed would enhance LNP mediated delivery.

This work has resulted in a greater understanding of the cellular mechanisms that control LNP based delivery and fate of mRNA cargo which will facilitate the design of next generation intracellular delivery technologies.
STAT3 and novel tumor suppressor SH2D4A are associated with endocytic trafficking in hepatocellular carcinoma

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Background and aim:
It was described previously that signal transducer and activator of transcription 3 (STAT3) localizes to lipid rafts and endosomal structures. However, the mechanism of STAT3 nuclear translocation via endocytic trafficking is poorly understood. In our previous study, we identified novel tumor suppressor genes SORBS3 and SH2D4A that are located on chromosome 8p. Hepatocellular carcinoma (HCC) represents the second most lethal cancer worldwide and chromosome 8p deletion which occurs in 45% of HCC patients is associated with poor outcome. Both tumor suppressors inhibit STAT3-mediated interleukin-6 (IL-6) signaling in HCC. Furthermore, we found that SH2D4A directly binds STAT3 in the cytoplasm, thereby reducing STAT3 transcriptional activity upon IL-6 stimulation. Thus, the aim of the current study is to analyze the role of SH2D4A in endocytic trafficking of STAT3.

Methods:
Live cell imaging was performed for tracking SH2D4A-STAT3 complexes that were visualized by using fluorescent labeling or bimolecular fluorescence complementation (BiFC). HCC cells were transfected with BiFC constructs and stimulated with IL-6. Combination of BiFC assay and staining of early endosomes was used to track localization of SH2D4A-STAT3 complexes upon cytokine stimulation. To study the impact of endocytosis on STAT3 trafficking, pharmacological inhibition was applied. Co-localization was additionally analyzed by subcellular fractionation of endosomes followed by western blot analysis.

Results:
A direct protein-protein interaction between SH2D4A and STAT3 was confirmed by using BiFC in HCC cell lines. In untreated cells, BiFC signal visualizing SH2D4A-STAT3 complexes was homogenously distributed in the cytoplasm. Upon IL-6 treatment the complexes accumulated close to the plasma membrane and translocated in a punctate pattern to the perinuclear region where the signal weakened suggesting a release of STAT3 from the protein-protein interaction enabling nuclear translocation. Chlorpromazine inhibited endocytosis leading to the disassembly of SH2D4A-STAT3 complexes.

Conclusion:
Tumor suppressor SH2D4A directly interacts with STAT3 and seems to be involved in endocytic trafficking of STAT3.
Endocytosis is involved in the initiation of pancreatic ductal adenocarcinoma.

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Activating Kras mutations induce neoplastic transformation of acinar cells through acinar-to-ductal metaplasia (ADM) that eventually develops to pancreatic ductal adenocarcinoma. The conditional activation of oncogenic KrasG12D in the pancreas of genetically engineered mice (GEM) leads to ADM and to pancreatic ductal adenocarcinoma development. However, signaling and cellular events taking place downstream of KrasG12D are poorly understood. Endocytosis controls both membrane dynamics and the association of signaling molecules within the cell; thus, endocytosis might influence neoplastic transformation of acinar cells.

We used GEM models and three-dimensional acinar tissue cultures to investigate the role of endocytosis in the development of preneoplastic lesions. We provide evidence that (i) acinar cells expressing oncogenic KrasG12D have an increased rate of fluid-phase endocytosis, (ii) that perturbation of the endolysosomal system, as well as inhibition of dynamin, impairs ADM, and (iii) that RAC1 regulates ADM formation partially through actin-dependent regulation of fluid-phase endocytosis. Acinar explants from mice with a pancreas-specific deletion of the Neural-Wiskott–Aldrich syndrome protein (N-WASP), a regulator of F-actin, in a KrasG12D background had decreased levels of fluid-phase endocytosis. In addition, N-WASP-deficient mice in the KrasG12D background did not develop ADM nor developed tumors emphasizing the in vivo relevance of our findings. Preliminary data suggested a link between early endosomes and enzymes of the methionine cycle in the pancreas. Furthermore, pharmacological inhibition of methionine adenosyltransferase, a key enzyme of the methionine pathway, inhibited ADM development.

Based on these results we hypothesize a link between endocytosis and the methionine cycle to support acinar-to-ductal metaplasia. Thus this work defines a new role of endocytosis as a tumor initiating mechanism.
Introduction: Mesenchymal stem cells (MSCs) have shown both anti-inflammatory and pro-regenerative activity in a variety of disorders. Recent studies support the notion that the signals responsible for these therapeutic effects are at least partially conveyed by extracellular vesicles (EVs). Despite growing interest in EVs as therapeutic tools, little information is available on the fate of these nanoparticles following in vivo administration because of methodological hurdles. The aim of the study was to optimize the method of EV visualization for biodistribution studies. Methods: The experiments were performed using human bone marrow mesenchymal stem cells (hBM-MSCs) (Lonza). hBM-MSCs were labelled with two different dyes: lipophilic stain PKH26 and iron nanoparticles conjugated with rhodamine (Molday) and co-stained with anti-CD9, -CD63 and -CD81 (tetraspanins) antibodies. EVs were isolated from the culture media of previously labelled hBM-MSCs. The size, morphology and biomarker expression of hBM-MSC-EVs were identified by NanoSight particle tracking analysis (NTA), high-resolution flow cytometric analysis (Apogee), transmission electron microscopy (TEM), superresolution structured illumination microscopy (SR-SIM) and MRI. Results: Our results revealed the presence of intracellular vesicles positively stained with PKH26 or iron nanoparticles visible inside hBM-MSCs. These structures co-expressed typical MSC markers i.e. CD44, CD73, CD90 and also CD9, CD63 and CD81 proteins specific for EVs. The isolated EVs represent heterogeneous population of a various size as confirmed by electron microscopy and NanoSight analysis. Co-culture of EVs with hBM-MSCs revealed their uptake by cells in vitro. The
positively stained exogenous EVs with PKH26 or Molday ION co-expressing tetraspanins were visualized inside hBM-MSCs. Conclusions: Both dyes PKH26 and Molday ION seem to be biocompatible with EVs and the labelling did not interfere with the capability of EVs to re-enter hBM-MSCs during in vitro co-culture studies. Molday ION may allow additional in vivo imaging of EVs delivery using MRI.

Supported by the Polish National Centre for Research and Development STRATEGMED1/235773/19/ NCBR/2016 “EXPLORE ME”
Escaping the bar: modulating dynamin function and T-tubule maintenance through SH3-PRD domain interaction

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Eukaryotic cells harness multiple endocytosis pathways to regulate cell signaling and nutrient uptake to cope with different physiological demands. Among different endocytic types of machinery, a membrane fission GTPase, dynamin, has been demonstrated to be a key regulator for several endocytic routes, and its function is believed to be positively regulated by interacting proteins, phosphatidylinositol-4,5-bisphosphate (PI4,5P2) and membrane curvature. In muscle cells, a huge part of the plasma membrane forms highly curved invaginations called T-tubules, by a muscle-specific membrane sculpting protein, Bin1. Bin1 is a Bin/Amphiphysin/Rvs (BAR) domain containing protein and also a dynamin-interacting partner. Bin1 binds to PI4,5P2 at plasma membrane and squeeze the membrane into highly curved T-tubules. These criteria make T-tubule an ideal substrate for dynamin-2 to sever, yet T-tubule integrity is preserved in physiologically normal cells, suggesting that there must be a mechanism to negatively regulate dynamin function. Despite its membrane-curvature-generation ability, we find that Bin1 would actually inhibit dynamin function in mediating membrane fission in vitro. This inhibition is due to SH3-PRD domain interaction, as truncated Bin1 lacking the SH3 domain would cause opposite result; it promotes GTP-dependent membrane scission of dynamin. In turns, membrane tubulation by Bin1 is inhibited in the presence of dynamin. This reciprocal inhibition happens through regulation of protein assembly on the membrane. We propose that phosphorylation in PRD domain of dynamin-2 would serve as a mechanism to modulate SH3-PRD domain inhibition. We generate dynamin-2 phosphomimetic mutant with lower binding affinity to SH3 domain of Bin1 and show that this mutant could relieve inhibition of both dynamin-mediated membrane fission and Bin1 membrane tubulation caused by SH3-PRD domain interaction. Together, our results provide a new insight into the regulation mechanism between dynamin and its binding partners via alteration of binding affinity. Our next step is to examine whether dynamin function in other endocytic pathways is controlled through similar mechanism.
Complex formation of Rab35 with myotubularin-related phosphatidylinositol phosphatases implicated in myelination

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Myelination, the membranous ensheathment of axons, is essential for neuronal health and survival in vertebrates. An imbalance in homeostasis or complete loss of myelin generating oligodendrocytes or Schwann cells result in neurodegeneration and cause neuropathies such as multiple sclerosis and Charcot-Marie-Tooth disease (CMT) in the central and peripheral nervous system, respectively. Strong genetic and cell biological data implicate myotubularin family phosphatases encoded by the CMT genes MTMR2, MTMR5, and MTMR13 in the control of myelination in the peripheral nervous systems in humans and in mice. Biochemical data argue that the formation of myelin outfoldings in knockout mice lacking MTMR2, MTMR5, or MTMR13 are due to their phosphatase activity towards phosphatidylinositol 3,5-bisphosphate [PI(3,5)P2], a lipid involved in late endosomal membrane homeostasis. However, the mechanism by which these enzymes regulate myelin formation is largely unknown.

Here we provide preliminary evidence that the small GTPase Rab35, which is involved in endomembrane trafficking, associate with CMT-related MTMRs and that complex formation between these proteins may regulates myelin biogenesis. We show that Rab35-GTP specifically binds to MTMR13/SBF2 and MTMR5/SBF1 and, via these, also to the active phosphatase MTMR2. To probe a potential role of Rab35 in regulating MTMR2/MTMR5/MTMR13 function we generated conditional Rab35 knockout (KO) mice that allow disruption of Rab35 expression upon tamoxifen addition. Acute KO in cultured oligodendrocyte precursor cells (OPCs), isolated from floxed mice, negatively affected cell survival and proliferation but had no impact on their ability to be differentiated into oligodendrocytes following hormone induction. Importantly, differentiated Rab35 KO oligodendrocytes displayed signs of hypermyelination, most notably increased sheath formation and elevated expression of key myelin proteins.

Current work is aimed at the generation of conditional KO mice specifically lacking
Rab35 in Schwann cells in the PNS and to probe the molecular mechanisms by which Rab35 complex formation with MTMRs regulates myelin biogenesis.
Alternative splicing of trafficking genes contributes myofiber structure and skeletal muscle physiology

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More than 90% of the human genes undergo alternative splicing, a posttranscriptional mechanism that explains how one gene can give rise to multiple protein isoforms. It has become apparent that coordinated splicing networks regulate organ development and that alternative splicing has physiological functions in developmental processes (Baralle & Giudice, 2017 Nature Reviews Molecular Cell Biology). Most conserved and tissue-specific splicing occurs on striated muscle and brain, raising the question of how developmental and tissue-specific splicing influences protein function. We have previously found that membrane trafficking genes undergo splicing transitions during heart development (Giudice et al., 2014 Nature Communications). Trafficking genes are mis-spliced in heart failure in mice (unpublished). Furthermore, alternative exons are gradually included in development in heart (atria, ventricle) and skeletal muscles (gastrocnemius, tibialis anterior, soleus, extensor digitorum brevis, flexor digitorum brevis (FDB), diaphragm) but not in other tissues (liver, pancreas, spleen, brain, intestine, lung, kidney) (unpublished), suggesting roles specific to striated muscle. In selected cases, we confirmed that mRNA transitions are translated into different protein variants (unpublished). We hypothesize that specific trafficking isoforms contribute to striated muscle biogenesis and cell architecture maintenance being important for cell type-specific functions. To start testing this hypothesis, we delivered morpholino antisense oligonucleotides into FDB muscles of adult mice to redirect splicing of four selected trafficking genes (Trip10/Cip4, Snap23, Cltc, Tmed2) to fetal isoforms. Splicing switch results in structural and functional defects including T-tubule disruption and DHPR and RYR1 mislocalization impairing excitation-contraction coupling and proper calcium handling and force generation (Giudice et al., 2016 Cell Reports).

As splicing transitions observed in development are robustly reproduced during C2C12 cell differentiation (myoblasts-myotubes), we are combining this system with the above-mentioned in vivo approach to investigate individual events. Depletion of specific trafficking proteins (by siRNAs) drastically reduces cell viability and differentiation, confirming their importance. Blocking Trip10/Cip4 (Cdc42-interacting
protein-2) or Snap23 (SNARE-complex member) endogenous splicing transitions (by morpholinos) increased myoblast fusion and produced myotube hypertrophy (unpublished), suggesting that precise splicing contributes to normal myotube formation.

In conclusion, our results demonstrate previously unrecognized roles for trafficking proteins in muscle homeostasis and specific requirements for adult splice variants.
Novel clathrin-independent endocytic routes: role of curvature sensing/inducing BAR domain proteins

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Endocytosis is an essential cellular process required for uptake of nutrients from cell environment and turnover of plasma membrane components. Clathrin-mediated endocytosis is by far the best characterized endocytic process. Since the mid-90's, the existence of endocytic routes independent of clathrin emerged. Therefore, the most challenging question in membrane biology rose: how could the plasma membrane be deformed in the absence of an organized clathrin coat? Until today, this process is not fully understood. First attempts to shed light into this topic demonstrated the requirement of glycosphingolipids for membrane deformation in lectin-driven endocytosis. Recently, BAR domain proteins (BAR stands for Bin/Amphiphysin/Rvs) have been described to be crucial for clathrin-independent endocytic routes. BAR domain proteins interact with membranes and act as curvature sensors/inducers. The function of BAR domain proteins remains unclear in the landscape of clathrin-independent endocytosis, and especially the endocytic pit formation. We hypothesize that this protein family constitute a module, which defines cargo specificity and is able to deform plasma membrane in clathrin-independent endocytic processes. To verify this hypothesis, we will perform a knock-down screen of various BAR domain proteins in mammalian cells and analyse the abundance of plasma membrane proteins via quantitative proteomics. Identified BAR domain proteins/potential plasma membrane cargoes couples will be selected, and deeply characterized using advanced cell biology techniques and model membranes: Do they constitute new clathrin-independent endocytic routes? What is the function of the BAR domain proteins in the new endocytic processeses?
Molecular control of integrin transport by Rab21 and EFHD2 in breast cancer cells

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The small GTPase Rab21 is a major regulator of endocytic transport of active integrins. However, it is not known how the Rab21-dependent integrin transport is regulated, due to the fact that only very few Rab21 interactors have been identified so far. Using a mass spectrometry-based screen to find interactors of Rab21-mediated integrin transport, we have identified a novel Rab21 interacting protein: EFHD2 (Swiprosin1).

The Ca²⁺-binding EF-hand domain containing protein 2 (EFHD2 or Swiprosin1) is an actin bundling protein necessary for cell spreading and migration by modulating lamellipodial dynamics. Using the invasive breast cancer cell line MDA-MB-231 as a model, we have observed that EFHD2 colocalises with β1-integrin at the cell leading front. Using Proximity ligation assays and live cell imaging approaches, we have observed that EFHD2 interacts with endocytosed β1-integrins and Rab21-containing vesicles. Moreover, knocking down EFHD2 affects β1-integrin endocytosis negatively and reduces the average speed of Rab21-containing vesicles. Both processes, integrin transport and the movement of Rab21-containing vesicles, are dependent on the actin binding function of EFHD2.

Cells with depleted levels of EFHD2 show decreased migratory potential measured with a scratch wound assay, as well as increased adhesion to extracellular matrix components laminin-1 and fibronectin. This suggests that the lack of EFHD2 increases the stability of integrin adhesions resulting in defective migration. Hence, we have hypothesised that expression of EFHD2 in breast cancer cells might enhance the turnover of integrin adhesion allowing the cells to efficiently migrate, increasing their invasive potential. Microarray analysis of 200 tumour samples showed that mRNA expression of EFHD2 is higher in less differentiated tumours (high grade tumours),
tumours with nodular metastasis and subtypes HER2+ and basal tumours. Future work will be focused on studying whether depletion of EFHD2 can reduce invasion of breast cancer cells in a mouse model. The results could lead to identify EFHD2 as a novel therapeutic target for metastatic breast cancer.
Clathrin-coated pits pinch collagen fibers to support 3D cell migration

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Migrating cells often use integrin-rich focal adhesions (FAs) in order to move. The structure, composition and dynamics of FAs have been extensively studied in cells migrating on two-dimensional (2D) or in 3D environments. While some cells migrate without FAs, alternative adhesion structures supporting cell migration have not been described so far. Here, we report a new mode of cell adhesion to the extracellular matrix (ECM) that depends on clathrin-coated pits (CCPs). CCPs are widely known to support endocytosis but we observed that they can also pinch collagen fibers in the 3D environment thereby providing anchoring points that are necessary for migration. We observed that both topological cues and local engagement of integrins are required for CCPs accumulation on collagen fibers. CCPs in contact with collagen fibers seemingly pinch those fibers by adopting a unique tube-like morphology. We also demonstrated that CCPs control cell adhesion to collagen fibers, in an endocytosis-independent manner. During migration in 3D, CCPs stabilize cellular protrusions by providing anchoring points to collagen fibers. We conclude that CCPs’ role during migration is much wider than previously anticipated and that, in coordination with FAs, CCPs-mediated adhesion is necessary for cell migration in 3D.
The alpha-arrestin Art2 mediates the endocytosis of yeast thiamin transporters

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In Saccharomyces cerevisiae, the endocytosis of PM proteins is essentially triggered by their ubiquitination. Ubiquitination of plasma membrane (PM) proteins in yeast is catalyzed by a single E3 ubiquitin ligase, Rsp5. In order to perform specific and regulated ubiquitination of about 240 PM proteins, Rsp5 is helped in his task by a set of thirteen cytosolic proteins called Arrestin-Related Trafficking (ART) adaptors (or alpha-arrestins): Art1 to Art10 and Bul1 to Bul3. The ART adaptors interact with Rsp5 and target it to their corresponding PM proteins following an endocytosis-triggering stimulus. In order to decipher the underlying relationship between PM proteins and the ART adaptors, we carried out a mass spectrometry-based quantitative proteomic analysis on yeast PM-enriched fractions. We compared the abundance of many PM proteins in absence of a single ART adaptor and in a WT strain after endocytosis induction using cycloheximide. This proteomic screening allowed us to find several new potential targets of the ART adaptors. From that screening, we focused on a specific target, the thiamin (vitamin B1) transporter Thi7, since the regulation of the endocytosis of vitamin transporters was poorly studied. Our screening revealed that the abundance of Thi7 at PM is affected by two ART adaptors, Art2 and Art9. We confirmed the involvement of Art2 in the stress and substrate-induced endocytosis of Thi7 by microscopy and immunoblotting, while the effect of Art9 is still under consideration. We extended our study to the endocytosis of two other PM thiamin transporters Nrt1 and Thi72 and showed that Art2 was also involved. Therefore, we are now investigating the mechanism underlying the thiamin-dependent regulation of Art2.
The endocytosis of the S. cerevisiae vitamin B6 transporter Tpn1 is regulated by several Arrestin-Related Trafficking (ART) adaptor proteins

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In the yeast S. cerevisiae, the signal inducing endocytosis of plasma membrane (PM) cargoes is ubiquitination, mostly catalyzed by the ubiquitin ligase Rsp5. Adaptor proteins assist Rsp5 in its function in order to specifically interact with hundreds of proteins. A family of twelve Rsp5-adaptors has recently been identified: the Arrestin-Related Trafficking (ART) adaptors. Today, their involvement in endocytosis has only been demonstrated for a few model cargoes and their regulation remains poorly understood.

In our lab, we study the endocytosis of the yeast vitamin transporters whose post-translational regulations and trafficking are unknown. Using a plasmid expressing a GFP-tagged transporter, we first showed that the PM vitamin B1 transporter Thi7 was endocytosed upon substrate addition. This down-regulation was dependent on Rsp5 and regulated by Art2. We then asked whether Art2 was a common regulator of vitamin transporters suggesting that endocytosis of all vitamin transporters might be regulated by the same downstream processes. Consequently, we have extended the study of the endocytosis to the PM vitamin B6 transporter Tpn1. Using the same methodology, we showed that the substrate-induced endocytosis of Tpn1 depends on Rsp5. However, we observed that in the twelve single artΔ strains, Tpn1 was still endocytosed and delivered to the vacuole upon substrate addition. Nevertheless, Tpn1 was stabilized at the PM in a strain lacking all ART adaptors (except one), indicating that several redundant ART adaptors regulate the endocytosis of Tpn1. In the future, we plan to characterize in detail the adaptor-mediated endocytosis of vitamin transporters to better understand how a yeast cell regulates these transporters at the cell surface.
The role of endocytosis in NF-κB signaling mediated by lymphotoxin beta receptor (LTβR)

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Cytokine receptors from the tumor necrosis factor receptor (TNFR) superfamily activate NF-κB signaling and undergo internalization upon ligand binding. We previously demonstrated that depletion of ESCRT-I components leads to endosomal accumulation of cytokine receptors (TNFRI, lymphotoxin beta receptor; LTβR) and their ligand-independent activation of NF-κB signaling. To further investigate a link between endocytosis and the NF-κB pathway we disturbed internalization and endosomal trafficking of LTβR.

Clathrin-mediated internalization was perturbed by depletion of clathrin, dynamin 2 and dynamin 1, whereas endosome-lysosome fusion - by depletion of HOPS complex components or Rab7A. We found the internalization step to be important for regulating canonical NF-κB signaling. Depletion of dynamins specifically augmented the canonical NF-κB route activated by ligand-bound LTβR, as judged by increased levels of active RelA transcription factor and expression of NF-κB target genes. However, the non-canonical NF-κB branch was not affected. In turn, knockdown of HOPS components or Rab7A caused local accumulation of ligand-free LTβR on endosomes, as observed upon ESCRT-I depletion. However, NF-κB signaling was not induced.

We propose that in unstimulated cells LTβR undergoes constitutive internalization that controls the plasma membrane (PM) level of the receptor. Impairment of internalization may lead to accumulation of the receptor on the PM resulting in over-activation of the NF-κB canonical pathway upon ligand stimulation. Moreover, our data from HOPS- or Rab7A-depleted cells indicate that endosomal accumulation of the receptor is not sufficient to activate NF-κB signaling in a ligand-independent manner. We hypothesize that the ligand-free receptor accumulated on endosomes signals intracellularly as long as it is exposed to the cytoplasm, which takes places e.g. upon knockdown of ESCRT-I. The receptor enclosed within multivesicular bodies (MVBs), as it may occur upon depletion of Rab7A, does not signal anymore.
A decrease in plasma membrane tension inhibits TOR Complex 2 activity via sequestration in PtdIns(4,5)P2-enriched Timmy domains

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The Target of Rapamycin Complex 2 (TORC2) plays a key role in maintaining the homeostasis of plasma membrane tension, but our understanding of the molecular interplay between the mechanical stimulus of altered membrane tension and the biochemical response, i.e. TORC2 kinase activity, was so far hindered by the lack of specific and potent tools to acutely impinge upon these parameters. Using a newly-identified indirect TORC2 inhibitor, Palmitoylcarnitine (PalmC), as a tool to manipulate membrane tension, we have discovered that decreased plasma membrane tension triggers a rapid and dramatic redistribution of phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P2) into discrete clusters, whose maintenance relies on the relocalization of the PtdIns(4)P 5-kinase Mss4. The consecutive recruitment of TORC2 to these structures induces the transient inhibition of the complex activity, until the Slm proteins also relocalize from eisosomes to the PtdIns(4,5)P2-Enriched domains to reactivate TORC2. These results reveal that TORC2 perception of an increase and a decrease of plasma membrane tension occur through fundamentally different mechanisms.
Signaling and endocytic trafficking of the receptor tyrosine kinase MuSK during neuromuscular junction development

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The neuromuscular junction is the central relay station that transfers neuronal information to skeletal muscle. It is formed as a result of reciprocal exchange of signals between muscle fibers and motor neurons. The receptor tyrosine kinase MuSK is the key regulatory molecule during the formation of the neuromuscular junction. MuSK is activated by the motor neuron-derived heparansulfate proteoglycan agrin. Agrin does not bind MuSK directly but interacts with Lrp4, a member of the LDL receptor family. Agrin binding results in the formation of a tetrameric agrin-Lrp4 complex that is capable of inducing MuSK dimerization and subsequent autophosphorylation of MuSK. Activation of MuSK kinase induces a signaling cascade leading to pre- as well as postsynaptic differentiation, which, most prominently, includes the clustering of acetylcholine receptors (AChRs) at synaptic sites. Besides its signaling activity, regulated MuSK endocytosis and degradation have also been implicated as crucial events for MuSK signaling activity pointing to a cross talk between signaling and endocytosis. We have used a live imaging approach to study MuSK endocytosis in heterologous cells. MuSK is internalized via a clathrin-, dynamin-dependent pathway and transported to Rab7-positive endosomes for degradation and recycled via Rab4- and Rab11-positive vesicles. Interestingly, MuSK colocalizes with actin and the small GTPase Arf6 at the cell surface and during endocytic trafficking. Disruption of the actin cytoskeleton or proper Arf6 function concentrates MuSK in cell protrusions. Moreover, inhibition of Arf6 or cytoskeletal rearrangements impairs AChR clustering and phosphorylation. These results suggest that MuSK uses classical as well as non-classical endocytic pathways that involve a variety of different components of the endocytic machinery. More recently, we have begun to study MuSK endocytosis in muscle cells. Here we use a combination of live imaging and biochemical analysis to determine the mechanisms that underlie MuSK endocytosis and their role in MuSK signaling.
The dynamics of ESCRT-mediated intraluminal vesicle formation

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The formation of multivesicular endosomes (MVE) is mediated by the ESCRT (endosomal sorting complex required for transport) machinery, which consists of four multiprotein subunits, ESCRT-0, -I, -II and –III and the enzymatically active VPS4 complex. This machinery mediates cargo sorting, membrane deformation and membrane scission on the surface of endosomes to generate intraluminal vesicles (ILVs). While the timing of other ESCRT-mediated scission processes in the cell, such as cytokinetic abscission and retroviral particle release have been elucidated, the dynamics of ILV formation remained unknown. In the current study, we utilized colocalization analysis and live-cell imaging of fluorescently tagged ESCRT proteins to elucidate their dynamics during ILV formation. We observed a coordinated and repetitive recruitment of ESCRT subunits to individual endosomes which lasts about one to three minutes per oscillation. We further show by electron microscopy that ILVs are consecutively formed starting immediately after endocytic uptake of EGF stimulated growth factor receptors and that one fluorescent oscillation corresponds to the formation of one ILV. These findings provide the temporal framework of ILV formation and increase our mechanistic understanding of this fundamental cellular process.
In HeLa cells treated with L-leucyl-L-leucine methyl ester cysteine cathepsins are not released to the cytosol but are inactivated in transiently permeabilized lysosomes

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L-leucyl-L-leucine methyl ester (LLOMe) induces lysosomal membrane permeabilization (LMP), which is enhanced by dipeptidyltransferase activity of cathepsin C. According to the current model of lysosomal apoptotic pathway, LLOMe-induced LMP enables release of lysosomal proteases cysteine cathepsins from permeabilized lysosomes into the cytosol, where their endopeptidase activities would induce apoptosis. When Hela cells are incubated with LLOMe overnight, we found that apoptosis is initiated only above 4 mM LLOMe. Both apoptotic (5 mM) as well as sub-apoptotic (1 mM) concentrations of LLOMe caused rapid and complete LMP, evidenced by loss of the proton gradient and release of internalized lysosomal fluorescent markers below 10K molecular weight into the cytosol. However, there was no evidence for release of cysteine cathepsins B and L to the cytosol. Instead, cysteine cathepsins remained within lysosomes, where they were rapidly inactivated and degraded. Like LMP, LLOMe-induced loss of cysteine cathepsin activities was dependent on cathepsin C activity. This suggests that when polymerized LLOMe can structurally destabilize the lysosomal limiting membrane as well as luminal hydrolases. When incubated with sub-apoptotic 1mM LLOMe concentration, lysosomes transiently lost protons but annealed and re-acidified within hours without the need for protein synthesis. However, long-lived protein degradation was impaired and full lysosomal function was regained only after new protein synthesis of cysteine cathepsins and other hydrolases.
Contributing to the complexity of Sonic Hedgehog secretory pathway: regulation within Shh amino and carboxy peptides in polarized epithelial cells

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Several polarized epithelial cells express and secrete Sonic Hedgehog (Shh) morphogen to activate Shh signaling with roles in patterning, stem cell maintenance and neoplastic disease. Shh is synthesized as a precursor protein in the endoplasmic reticulum (ER), where it becomes cleaved in amino- (ShhN) and carboxy- (ShhC) peptides. ShhN is covalently conjugated with fatty acid and cholesterol moieties generating the widely studied secreted signaling morphogen (ShhNp). ShhC is glycosylated and its function is thought to be restricted to catalyse autocleavage of precursor protein. However it was recently shown that polarized targeting decision of ortholog Hedgehog (HhNp) is controlled by HhC-mediated proteolysis in developing Drosophila visual system. Here we present evidences of the influence of the ShhN fatty acid modification in ShhC trafficking in polarized MDCK cells. Culture media analysis of cells showed apical secretion of ShhNp and ShhC. However, while apical secretion of ShhNp is the result of basolateral to apical transcytosis (assayed with cell surface biotinylation and antibody-tagging experiments), ShhC is sorted apically from the Golgi apparatus. Biosynthetic trafficking analysis evaluated by microinjection showed separate transport carriers of ShhN and ShhC from ER. However a mutant ShhN that lacks fatty acid modification (ShhNpC24S) showed (1) higher co-localization with ShhC along its biosynthetic trafficking and (2) apical secretion of both peptides. Co-immunoprecipitation assays from apical and basolateral culture media indicate that ShhN and ShhC interact only when Shh lacks its fatty acid (ShhNpC24S). On the other hand, disruption of ShhC secretion by tunicamycin treatment does not modify ShhN polarized secretion. Our results indicate that Shh processing and secretion are highly complex, regulated events. Specific mechanisms for the various tissue and/or developmental stages where Shh is expressed warrant further investigation.

(Basal Project PFB 12/2007 from CONICYT, FONDECYT grant# 1141127 and Postdoctoral Project # 3150603).
A new actin cytoskeleton structure around endocytic Shigella-containing vacuoles regulates bacterial release into the host cytosol

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The Gram-negative bacterium Shigella flexneri is the causative agent of bacillary dysentery and a model for intracellular pathogenesis. Like many enteroinvasive pathogens, Shigella forces its endocytic uptake into non-phagocytic epithelial cells through the translocation of type 3 effector proteins. These effectors actively hijack (i) the host actin cytoskeleton and (ii) endomembrane trafficking to establish an intracellular niche for bacterial survival and replication. So far, studies of Shigella-induced actin subversion focused on the rearrangement of cortical actin during early invasion steps and the formation of actin tails for cytosolic movement. Our laboratory recently developed new imaging tools that allow with an unprecedented resolution level the investigation of key steps between Shigella’s epithelial entry and its escape from the bacteria-containing vacuole (BCV). One of the most exciting discoveries is that Shigella is coated by an actin “cage” formed in situ around its endocytic vacuole. This previously unknown dynamic F-actin structure is rapidly assembled and disassembled in the short timespan after the bacterial entry and before vacuolar rupture, respectively.

Here, we first reveal that disassembly of this newly identified actin structure is a necessary prerequisite for bacterial release into the host cytosol. We previously found direct contacts between the BCV and Shigella-induced endocytic vesicles to be required for vacuolar rupture. The actin cage prevents these membrane contacts and may exhibit a gatekeeper-like function to regulate pathogen entry into host cells. Its assembly as well as regulation has so far not been studied, and the overall role in Shigella pathogenesis needs to be determined. Second, we identified host factors that are originally involved in actin cytoskeleton rearrangements during endocytosis and that are hijacked for the re-organization of the actin cage. We further determined these actin regulators and actin binding proteins at the Shigella-containing vacuole. Finally, we identified Shigella effectors that delay vacuolar rupture specifically affecting either the dynamics of the actin cage assembly, or the probability of its formation. Together, we aim to unravel the molecular mechanisms underlying the dynamics of the actin cage, and to gain novel insights into how Shigella manipulates this structure to control vacuolar rupture and its subsequent cytoplasmic propagation.
Actin filament networks are involved in cellular processes that move or shape membranes. To understand the force-generating mechanisms applied by actin networks to bend membranes, we studied the role of different actin network components in shaping the plasma membrane during clathrin-mediated endocytosis in yeast.

We used a hybrid imaging approach that combines fluorescence microscopy and correlative electron microscopy. Fluorescence microscopy was used to visualize endocytic events as diffraction limited patches that are located on the yeast plasma membrane. The dynamics of those patches can be tracked over time very precisely. Correlative electron microscopy allowed us to resolve the morphology of the plasma membrane invagination at the different stages of its growth. Together, these techniques were used to resolve defects in membrane invagination and vesicle formation that were induced by the deletion of different components of the actin system. These defects allowed us to understand what are the properties of the force-generating mechanism that the actin network uses to reshape the membrane during endocytosis.

Our data shows that endocytic adaptor proteins are not sufficient to initiate membrane bending and that the actin network needs to be physically linked to the membrane for it to be bent. Interestingly, Sac6, which is an actin filament cross-linker, is required both for efficient initiation of membrane bending and for the transition from the latest stage of invagination growth to the vesicle scission. Finally, cells lacking Bbc1, which regulates the yeast N-WASP homolog Las17, fail in controlling actin filament nucleation leading to an increase in the size of the actin network that surrounds the growing invagination. However, this large network affects only the scission of the
vesicle and its movement into the cytoplasm but not the growth of the plasma membrane invagination. These results allow us to identify two phases in the actin network lifetime underlain by two different mechanisms driving the growth of the plasma membrane invagination: First, the actin network initiates membrane bending by surmounting a force threshold; the nucleation and polymerization of the actin filaments pull the plasma membrane invagination. Then, when nucleation and polymerization of filaments drop before scission, the actin network expands and the plasma membrane invagination undergoes scission into a vesicle.
The PI3P-binding protein Phafin2 escorts macropinosomes through the cortical actin cytoskeleton

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Macropinocytosis is an endocytosis mechanism that leads to the formation of large vesicles filled with extracellular fluids and soluble macromolecules. Formation of macropinosomes is a stochastic process that is caused by actin and membrane remodeling. The resulting membrane ruffles can collapse, fuse and pinch off by yet unknown mechanisms to form large vesicles. During the transition from membrane ruffles to vesicles, the phosphoinositide composition of the membrane changes - a phosphatase cascade metabolizes PtdIns(3,4,5)P3 by way of PtdIns(3,4)P2 and PtdIns(3)P to PtdIns. We have identified an effector of the transient PtdIns(3) pool generated during these early steps of macropinocytosis, the PI3P-binding protein Phafin2. Cells lacking Phafin2 show reduced macropinocytosis and defects in actin cytoskeleton organization. We could show that Phafin2 localizes transiently to newly formed macropinosomes directly after scission of the vesicle from the plasma membrane. This localization occurs prior to recruitment of early endocytic markers such as Rab5 and APPL1, indicating that Phafin2 labels macropinosomes during their transition from plasma membrane identity to endosomal identity. We identified an interactor of Phafin2, the actin crosslinking protein Filamin-A. Life cell imaging shows that Phafin2 colocalizes with both Filamin-A and actin on newly formed macropinosomes. Nascent macropinosomes are coated with a dense meshwork of cortical actin which is crosslinked by Filamin-A. Arrival of Phafin2 coincides with a weakening of this coat and the liberation of the macropinosome from the surrounding actin network. Deletion of Phafin2 results in persistent Filamin-A recruitment and ultimately causes the collapse of the nascent macropinosome. We found that Phafin2 interacts with the actin-binding domain of Filamin-A, suggesting that it might disrupt the actin-binding ability of Filamin-A and thereby liberates the newly formed macropinosome from the cortical actin cytoskeleton. This constitutes a critical step during the maturation of macropinosomes, as it allows the macropinosome to enter the endolysosomal pathway.
Regulation of the retromer complex by mTOR kinase

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mTOR kinase is a major regulator of cell growth and function, and it requires proper subcellular localization to become activated. Recent findings suggest that mTOR is not only regulated by, but also influences various membranous compartments in the cell. Mass spectrometry analyses of the mTOR-dependent phosphoproteome, performed by several labs, have suggested components of the retromer complex as potential mTOR pathway targets. The retromer orchestrates the transport of proteins from the endosomes to the Golgi, termed retrograde transport. Proteins sorted via the retrograde transport pathway include membrane receptors, ion channels or protein transporters. Additionally, some toxins, e.g. Shiga toxin, exploit the retromer to reach the Golgi and ER, and disturbed retromer function was linked to Alzheimer’s and Parkinson’s disease.

The aim of this work was to investigate whether mTOR activity affects the retromer complex. In the first step, a known retromer cargo, Shiga toxin B-subunit (STxB) and immunofluorescence analysis were utilized to check whether blocking mTOR activity with rapamycin could influence retromer function. Upon treatment with rapamycin, fluorescently labeled STxB reached the Golgi at a decreased rate, suggesting an interplay between mTOR and the retromer. Our subsequent search of possible mTOR targets has found TBC1D5, a negative regulator of the retromer, to be phosphorylated by mTOR. This phosphorylation was revealed by an in vitro kinase assay and predicted phosphorylation sites were confirmed by mass spectrometry analysis. Currently functional analysis of TBC1D5 phospho-mutants is ongoing in order to confirm the importance of these phosphorylation sites in the regulation of retromer by mTOR.

The results of this study have revealed the mTOR kinase as a potential regulator of the retromer complex, providing novel insight into protein sorting mechanisms within the cell.

This work has been supported by Polish National Science Centre OPUS grant 2012/07/E/NZ3/00503. JJ and KS are recipients of the Foundation for Polish Science "Mistrz" Professorial Subsidy and Fellowship, respectively.
Molecular Mechanisms of Membrane Tethering and Fusion – Reconstituting Rab5 Domains

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The small GTPase Rab5 is a master regulator of Early Endosome (EE) biogenesis that acts by recruiting a diverse protein machinery to regulate membrane tethering and fusion. We have identified and reconstituted a minimal machinery necessary for membrane tethering and fusion. Further, by correlative super-resolution and Electron Microscopy (SuperCLEM) we could establish that the Rab5 machinery is spatially segregated into distinct domains on the EE membrane.

Many critical questions remain as to the formation and maintenance of these Rab5 domains as well as how they are coupled to other endosomal functions. Using a combination of single particle tracking, high-throughput membrane recruitment assays, computational modelling, fluorescence and cryo-electron microscopy, we investigated the molecular mechanisms governing domain organization of the Rab5 machinery. We aimed to 1) define a minimal machinery required for domain formation, 2) investigate the roles of each component in driving/maintaining domain formation and 3) investigate how this specialized domain relates to tethering/fusion and other functions at the endosome. We have found that 1) in vitro GDP Dissociation inhibitor (GDI) is sufficient to extract Rab5 from a supported lipid bilayer 2) the presence of the GEF:Effector complex, Rabex:Rabaptin, and GTP, but not GDP, is sufficient for the removal of Rab5 from the Rab5:GDI complex and insertion into the membrane. This system constitutes the basis of the minimal machinery of domain formation, as the specific membrane recruitment of active Rab5 and the nature of the GEF:Effector complex should lead to spatial patterning of the Rab5 machinery. It is likely that the mechanisms governing membrane compartmentalization and specialization at the EE are generally applicable to different membrane systems.
GPCRs shape their own destiny in constitutive endocytosis through surface activity of the amphipathic helix8

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The existence of helix8 has been shown in a large number of class A GPCRs through functional and structural studies, and the motif has been accepted as a generic motif in this family. Helix8 has been implicated in localization, constitutive trafficking and agonist-induced signaling and trafficking, however, while a consistent protein machinery has been extensively mapped in regard to agonist induced signaling and internalization, no unifying pattern has emerged for the role of helix8 in the localization and constitutive internalization.

We here identify a pivotal role of helix8 in the constitutive trafficking properties of GPCRs. We show that helix8 confers the autonomous internalization properties to the full-length receptors, and demonstrate that chimera where only the helix8 has been switched also transform their constitutive internalization propensities to the full-length receptors. Amphipathic helices not associated with the endocytic machinery as well as an artificial amphipathic can likewise drive autonomous internalization. We further extract a bioinformatic parameter from an empirical combination of hydrophobicity and amphipathic moment. This parameter strongly predicts the extent of autonomous constitutive internalization and is used as a tool predictive tool and for evolutionary analyses. This novel pattern can be used to rationalize previous experimental observations and understand biological behaviors of GPCRs across biology.

Here we demonstrate that membrane-anchored helix8 stimulate constitutive internalization of the Transferrin Receptor in a manner that correlate with their calculated surface activity. This trans effect is regulated through membrane tension and is reproducible with an artificial helix that lack natural protein interaction partners in the cells. We apply the pulsed pH TIRF setup and discern a promiscuous mechanism that act through multiple known endocytic markers. We concomitantly use state-of-the-art three-dimensional STORM microscopy to identify and characterize the morphologies of the helix-induced internalization events both in conjunction with common endocytic markers and in what appears to be independent endocytic event of distinctive tubular morphologies. Altogether, we elucidate a mechanism in which the helix8-containing cargo regulates their own internalization rates through a combination of curvature sensitive recruitments and membrane deformation in several stages of endocytic trafficking.
Identification of genes required for hepatocyte polarity

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Hepatocytes are the major parenchymal cells of the liver. They are polarized and their apical membranes collectively build a highly ramified 3D tubular network, the bile canaliculi, where the bile is secreted and flows towards the bile ducts. In contrast to the classical epithelial cells, hepatocytes have a peculiar cell polarity as they have multiple basal and apical domains. Very little is known on how hepatocyte polarity is established and how is the apical surface organized to form the bile canaliculi. To address these mechanisms, we followed hepatoblasts differentiation into hepatocytes during embryonic liver development. In parallel, we established cultures of isolated primary hepatoblasts, where the cells differentiate into hepatocytes and recapitulate hepatocyte specific polarity by forming bile canaliculi in vitro. To identify candidate genes required for hepatocyte polarity, we compared gene expression profiles of unpolarized hepatoblasts, polarized hepatocytes and classical epithelial cells (bile duct cells) by RNA seq. We tested the requirement for such genes for hepatoblast differentiation and hepatocyte polarization by siRNA-mediated gene knock-down. Among the candidate genes, we found that the small GTPase Rab35, known to be involved in endosomal recycling, plays an important role. Upon silencing of Rab35, hepatoblasts still differentiated and polarized, however their apical membrane was strikingly enlarged and failed to form properly structured bile canaliculi. These results suggest that modulation of the endocytic/recycling pathway is crucial to control the size and the shape of the apical membrane domain of hepatocytes and to form a correctly organized bile canalicular network.
Regulation of TLR4 trafficking by an ER-localized membrane protein

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Toll-like receptor 4 (TLR4) is an important player in innate immune defense, and signals upon recognition of LPS in the membrane of Gram-negative bacteria. Cellular trafficking can regulate proper response from this receptor. Whereas inflammatory responses are activated by ligand binding to surface-localized TLR4, through activation of NF-κB, recruitment of TLR4 to the bacterial phagosome from Rab11a positive recycling endosomes (Husebye et al., 2010) leads to activation of type I interferon expression. Here, we show that knock-down of protrudin, an ER-localized protein involved in trafficking of late endosomes to the plasma membrane, inhibits signaling from TLR4. Protrudin knock-down inhibits recruitment of TLR4 to E.coli-containing phagosomes, and secretion of both TNF-alpha and IFN-beta upon TLR4 stimulation. The mechanisms of TLR4 regulation by protrudin are currently being investigated.
Ubiquitylation by the E3 ligase RNF115 regulates phagosome maturation and innate immune responses.

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Phagocytosis is an evolutionarily conserved process performed by a numbers of immune cells such as macrophages for host defence and homeostasis. During phagocytosis, particles are recognised by cell surface receptors that trigger rearrangement of the actin cytoskeleton and internalization of the bound particle into a de novo, membranous organelle known as the phagosome. In order to degrade the internalised particle the phagosome fuses with a number of intracellular organelles such as endosomes and the lysosome in a process called phagosome maturation. In the past, we have shown that macrophage activation by the pro-inflammatory cytokine Interferon-γ (IFN-γ) induces a remarkable delay in phagosome maturation. Now, using proteomics tools, we can show that IFN-γ increases substantially ubiquitylation of phagosomal proteins, including a large number of proteins regulating vesicle trafficking. Using a specific E2-ligase inhibitor, we demonstrate that ubiquitylation plays an important role in the maturation process of the organelle. Furthermore, we show that the E3 ligase RNF115 is induced and recruited to the phagosome upon IFN-γ stimulation thereby regulating K63 ubiquitylation of phagosomal proteins. Knockdown of RNF115 promotes phagosome maturation indicating that RNF115 is a negative regulator of phagosome maturation. We can furthermore show that this dysregulation of phagosome maturation induces pro-inflammatory responses demonstrating that tight regulation of vesicle trafficking in the endocytic system is key to innate immune responses to pathogens.
EEA1 and APPL1 interactions with endosomes after stimulation of EGR receptor endocytosis depend on cell type.

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To date involvement of endocytosis in signal transduction is widely appreciated. In particular, it was shown that just formed endosomes bearing active EGF-EGFR complexes interact with APPL1 the protein participating in chromatin remodeling and early response genes activation, and EEA1 tether mediating early endosomes fusions. However, experimental data on EEA1 and APPL1 mode of interaction with endosomes allow several interpretations, from APPL1 replacement by EEA1 onto membrane of the same endosome, to different endosomal subpopulations specifically bound to APPL1 or EEA1 or the both. We suggest that this pattern may be not universal but specific for certain cell type. Indeed, we have analyzed dynamics of co-localization of EGFR -loaded endosomes with the two proteins in proliferating cells of quite different origin – HeLa Kyoto transformed cell line and human endometrial mesenchimal stem cells (hEMSC). It was found that 5 min after EGF endocytosis stimulation in HeLa cells two subpopulation of EGFR containing endosomes can be registered, the first decorated with EEA1 and the second bearing APPL1. In 15 min only first subpopulation was present. In case of hEMSC after 5 min EGF-EGFR complexes endosomes were co-locolazed with EEA1, but minor fraction of vesicles contained cargo and APPL1. By 15-30 min enlarged endosomal structures mostly around nucleus contain all three proteins in separated domains. Interestingly, such structures were reported for cells overexpressing activated Rab5. We also found differences in dynamics of ERK1/2 activation correlating with dynamics of EGFR phosphorylation. Our findings suggests that proliferative programs of different cell types can be supported by specific patterns of endosomal signaling.

The research was supported by research resource center «Molecular and cell technologies» of St. Petersburg State University, by the grant from the Russian Science Foundation (project №14-50-00068) and with financial support from the Federal Agency of Scientific Organizations (Russia).
Identification of the MYO6 interactome using functional proteomics

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MYO6 is a motor protein unique among the myosin family because it moves towards the minus end of actin filaments and, as the sole myosin with this directionality, is critically important in a number of biological processes. Indeed, loss or overexpression of MYO6 in humans is linked to a variety of pathologies including deafness, cardiomyopathy, neurodegenerative diseases as well as cancer. The diverse cellular roles of MYO6 are mediated by a number of cargo adaptor proteins that interact with the C-terminal cargo-binding domain and target the motor to different cellular compartments. Among the known MYO6 binding partners are DAB2, GIPC, TOM1 and LMTK2, which mediate distinct functions of different MYO6 isoforms along the endocytic pathway as well as the selective autophagy receptors optineurin, TAX1BP1 and NDP52.

In order to understand the spatial and temporal regulation of motor/cargo attachment and to identify the complete MYO6 interactome, including the constituents of the larger multi-protein complexes associated with this motor, we have utilised advances in functional proteomics methods. Due to the dynamic nature of motor proteins their adaptor-cargo interactions are often weak, transient, and highly regulated, thus the larger complexes they associate with are often inaccessible. To overcome this issue we have focused on the use of in-situ proximity labelling strategies such as BioID and have successfully identified a number of novel MYO6 adaptors and multi-protein complexes. Protein interaction profiling in combination with detailed functional analysis resolved a number of distinct MYO6-associated protein complexes, which highlight the pivotal role of this myosin in coordinating receptor trafficking with endosome dynamics and cytoskeletal architecture. Most notably we identified two complexes containing RhoGEFs which appear to have roles in the regulation of the actin cytoskeleton linked to receptor trafficking at early endosomes or modulation of the septin cytoskeleton.
The ErbB3 growth factor receptor is required for endocytic recycling of β1-integrin

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The outcome of growth factor signalling is in part determined by vesicular trafficking of their receptors in and out of the cell. We now show that the growth factor receptor ErbB3 does not merely constitute vesicular cargo but is an integral component of the endocytic recycling machinery per se. We found that ErbB3 is required for endocytic recycling of β1-integrin in breast epithelial cells in a manner that does not require ligand-induced tyrosine phosphorylation of ErbB3. Consequently loss of ErbB3 leads to impaired cell migration. ErbB3 co-localizes with integrin β1 in Rab4-positive but not Rab11-positive vesicles, suggesting a role of ErbB3 in the fast recycling route. The ErbB3 function in endocytic recycling does not appear to be cargo specific since RNAi-silencing of ErbB3 also impaired recycling of transferrin. The molecular mechanism by which ErbB3 regulates vesicular trafficking will be discussed. These unexpected findings demonstrate that ErbB3 functions independently of canonical phospho-tyrosine-dependent signalling to drive recycling of integrin β1.
The role of human MTMR9 in intracellular trafficking

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Myotubularin and myotubularin-related proteins (MTMRs) are lipid phosphatases that dephosphorylate PtdIns3P and PtdIns(3,5)P2 at position 3. By modulating the lipid composition of various intracellular membranes, MTMRs play an important role in establishing vesicular identity and regulating vesicular transport.

Previous studies in C. elegans established a link between MTMRs and the Wnt signalling pathway. Wnt proteins are secreted from Wnt-producing cells. There Wnt binds to the transmembrane receptor Wntless (GPR177) in the endoplasmic reticulum (ER) and is transported through the Golgi apparatus towards the cell surface for release. Vacated Wntless is recycled through the endosomal system in a retromer-dependent manner back into the ER for another round of Wnt secretion. Experiments in C. elegans uncovered that specific MTMRs play a role in Wntless recycling, probably at the level of retromer-dependent Wntless retrieval.

To test whether human MTMR family members play a role in Wnt signalling we screened for MTMR proteins which influence Wls trafficking. While knock-down of individual MTMR members by siRNA did not consistently lead to changes in overall Wls protein level, overexpression of MTMR9 led to partial redistribution of Wls into MTMR9-positive vesicles. MTMR9 is an inactive phosphatase which forms a heterodimer with an active MTMR to form a functional complex. We also observed partial co-localization of MTMR9 with markers of the Golgi apparatus, with Rab8, Rab11 and PtdIns(3,5)P2. We found no co-localization with PtdIns3P or markers of early or late endosomes or lysosomes. These findings suggest that, unlike in C. elegans, human MTMR9 does not affect trafficking at the endosomal, retromer-dependent step. We favour a model where MTMR9 regulates secretion rather than the retrograde transport. This notion is supported by our finding that MTMR9 co-localizes with VSVG along the secretory route. Assays to monitor the activity of the Wnt pathway in cells with compromised MTMR9 function are currently in progress.

Supported by grant 16-17966Y from the Czech Grant Agency
Endolysosomes are the principal sites of acid hydrolase activity

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Lysosomes are dynamic, terminal organelles of both the endocytic and autophagic pathways which play a role in macromolecule degradation, nutrient sensation, signalling to the cell nucleus, and membrane repair. Lysosomes are classically described as having an acidic lumen which enables efficient function of their resident acid hydrolases; however many studies indicate heterogeneity of lysosomal pH within individual cells including lysosomes with a neutral luminal pH. Material from the cell surface destined for lysosomal degradation has to travel through the endocytic pathway, culminating in fusion between late endosomes and lysosomes to form the hybrid endolysosomal compartment from which lysosomes can be reformed. Through use of fluorescent reporters of cathepsin activity and acid phosphatase cytochemistry combined with immunofluorescence of endosomal and lysosomal markers, we have found that acid hydrolase activity occurs predominantly in the endolysosomal compartment. After loading terminal endocytic compartments with fluorescently-tagged dextran, endolysosomes can be distinguished from re-usable terminal lysosomes by their accumulation of cathepsin activity reporters and fluorescent acidotropic probes, neither of which accumulate in more neutral terminal lysosomes. We demonstrated the dynamic equilibrium between these compartments by allowing cells to endocytose sucrose, resulting in the accumulation of sucrose-laden osmotically swollen endolysosomes (sucrosomes) and depletion of the terminal lysosome pool within the cells. These cells were subsequently allowed to endocytose invertase, enabling the hydrolysis of sucrose, which resulted in tubulation from the sucrosomes and eventual reformation of the normal proportion of re-usable terminal lysosomes. The functional differences between endolysosomes, maturation stages of reforming lysosomes, and terminal lysosomes are not entirely clear, but the respective capacities of each compartment for luminal acidification, and homo- or hetero-typic fusions are likely to be controlled by machinery on the lysosomal membrane. To explore these aspects of lysosomal function, we are investigating the distribution of V-ATPase subunits and phosphoinositides between organelles and the SNARE requirements for fusions between late endosomes and endolysosomes or terminal lysosomes. Our studies on the molecular mechanisms underlying the heterogeneity of acidity and function within the endolysosome/lysosomal compartment will provide insights into the pathogenesis of lysosomal diseases and the effects of the lysosomal accumulation of many weakly basic, amphiphilic drugs, which can affect drug pharmacokinetics, drug-drug interactions and off-target toxicity including drug-induced phospholipidosis.
TORC1 Organised in Inhibited Domains (TOROIDs) regulate TORC1 activity

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The Target of Rapamycin complex 1 (TORC1) protein kinase is a master regulator of eukaryotic growth and metabolism and its dysregulation in humans has been implicated in cancer and metabolic syndrome. GTPases, responding to signals generated by abiotic stressors, nutrients, and, in metazoans, growth factors, play an important, but poorly understood role in TORC1 regulation. We found out that, in budding yeast, glucose withdrawal, which leads to an acute loss of TORC1 kinase activity, triggers a similarly rapid Rag GTPase-dependent reorganisation of TORC1 in the cell. We could identify, both by super-resolution optical microscopy and 3D reconstructions of cryo-electron micrograph (cryo-EM) images, that inhibited TORC1 molecules organise in a novel structure which we name a TOROID (TORC1 Organised in Inhibited Domain). Guided by this new structure, we could describe the first molecular mechanism of TORC1 inhibition in yeast.
Palmitoylation – Emerging roles in TNF-R1 endocytic trafficking and signaling

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TNF-R1 as well as other members of the TNF-Receptor superfamily and their ligands have been described to be involved in various physiological processes ranging from regulating immune response to organ formation. On the other hand they have pathological function i.e. in cancer formation, chronic inflammatory and neural diseases. It is known that TNF-R1 can induce diametrically opposed biological effects upon ligand binding: inflammation/proliferation versus cell death. The differences in signal quality depend on the localization of the receptors. Plasma membrane resident receptors activate pro-inflammatory/survival signals, while endocytosed receptors can induce cell death.

We recently showed that TNF-R1 ubiquitination is mandatory for signaling bifurcation and that signaling cascades are regulated by protein complexes, which are assembled in distinct membrane enclosed compartments upon ligand binding. One mechanism to induce or modulate membrane localization of proteins is their post translational modification by palmitoylation. We here present unpublished data showing that TNF-R1 itself is palmitoylated, it induces biological effects which can be modulated by the application of (de-)palmitoylation inhibitors and that it interacts with palmitoyl transferases (PAT) and thioesterases (PTE). Thereby, activation of TNF-R1 induces dynamic changes in the palmitoylation status of various trafficking associated proteins, highlighting their role in endocytic TNF-R1 signaling. Functional characterization of the involved proteins may allow targeted manipulation of the TNF-R1 signaling for disease treatment in future.
Studying the Endocytosis Mechanisms Involved in Nanoparticle Uptake for Drug Delivery

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Nanoparticles have drawn a lot of interests in recent years both in drug delivery and in nanosafety studies due to their unique physicochemical characteristics and biological properties. Their high cellular uptake efficiency and their ability to interact with the cellular machinery in new ways compared to conventional drugs has made them promising candidates to be used as drug delivery systems. However a clear understanding of the mechanisms of cellular recognition, internalization and processing of these objects is often still missing. Only with this knowledge it will be possible to design truly targeted nanomedicines and control their localization, uptake and fate inside cells.

Within this context, the aim of our study is to characterize the endocytic mechanisms involved in the internalization of nanosized materials. We use model nanomaterials of different sizes, such as amorphous silica, because of their stability and their well-defined properties. We investigate nanoparticle uptake mechanisms in cell lines typically used to study cellular pathways, such as HeLa cells, but also in primary Endothelial Cells (HUVEC) organized in cell monolayers that can mimic endothelial barriers encountered by nanomedicines after administration into the blood stream. In our study the mechanisms of internalization of nanomaterials are investigated in the presence of relevant biological media, such as human serum, at concentrations resembling in vivo conditions. In fact it has emerged that the environment in which nanosized materials are dispersed confers them new properties that influence remarkably their cellular interactions. RNA interference directed toward key proteins involved in different endocytosis mechanisms is used to determine the role of specific pathways or molecules in the uptake, together with commonly known chemical inhibitors for endocytosis.

The results obtained so far indicate that proteins adsorbed on the nanoparticle surface have a key role in triggering cellular recognition and to direct nanoparticles towards specific endocytic routes. It has emerged that many components of different endocytic pathways are involved in the uptake of nanomaterials and that those pathways depend not only on the cell type but also on cell polarization.
Epsin-dependent ligand endocytosis activates Notch by force

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DSL ligands activate Notch by inducing proteolytic cleavage and shedding of the receptor ectodomain — an event that requires ligand to be endocytosed in signal sending cells by the adaptor protein Epsin. Two classes of explanation for this unusual requirement are: (i) recycling models, in which ligand must be endocytosed to be modified or repositioned before it can bind Notch, and (ii) pulling models, in which ligand must be endocytosed after it binds Notch to exert force that exposes an otherwise buried site for cleavage. We demonstrate in vivo that ligands that cannot enter the Epsin pathway nevertheless bind Notch but fail to activate the receptor because they cannot exert sufficient force. This argues against recycling models and in favor of pulling models. Our results also suggest that activation depends on competition between ligand internalized via Epsin, which signals, and uptake of ligand by receptor, which aborts the incipient signal.
Phospho-Regulation of Las17/WASP function during Endocytosis in S.cerevisiae

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Regulation of actin polymerization is critical to ensure actin filaments only form at specific regions of the cell. In the budding yeast Saccharomyces cerevisiae, the WASP homologue Las17 plays an important role in activating Arp2/3 to drive the rapid actin filament polymerization required to drive membrane invagination during endocytosis. In previous studies we have also identified an Arp2/3-independent actin nucleating activity of Las17 that requires G-actin binding motifs in its polyproline region but not its C-terminal WCA region. Mutation of motifs in this polyproline region causes an early defect in membrane invagination, while mutations in the WCA domain appear less critical, reducing the rate of invagination but having less impact on the proportion of successful invagination events per se. In order to investigate the interplay of the Arp2/3-dependent and –independent functions of Las17 we undertook an analysis of phosphorylation sites in Las17 by mass spectrometry. We identified several sites including sites within the WCA domain and more specifically in the G-actin binding WH2 region. The WH2 motif normally bind G-actin and this binding activity is required for Arp2/3-dependent actin polymerization. In this study we have mutagenized phosphorylation sites of Las17 and demonstrate that mutation of a single phospho-site in the WH2 domain can compromise G-actin binding. In vivo analysis of endocytosis reveals that the phosphorylation mimetic change causes defects in the rate of membrane invagination supporting the idea that functions of Las17 during single endocytic events can be regulated by phospho-control of monomeric actin binding.
During phagocytic uptake by macrophages, role of Golgi apparatus or vesicles derived from it has never been established. Using fluorescently tagged Mannosidase-II, a marker for Golgi-derived vesicles, we show these vesicles are recruited during uptake of diverse targets including latex beads, E. coli, Salmonella Typhimurium and Mycobacterium tuberculosis in both human and mouse macrophages. The recruitment of Mannosidase-II vesicles occurred very early during phagocytosis, which was mediated by focal exocytosis. The focal movement of Mannosidase-II vesicles required Ca2+ from both extra- and intra-cellular sources apart from PI3Kinase, microtubules and dynamin-2. At the site of uptake voltage-gated Ca2+ channels help establish a Ca2+-dependent local PIP3 gradient, which guide the focal movement. Mannosidase-II vesicles also contained Neuronal Calcium Sensor-1 (NCS1) and resembled secretory vesicles. Depleting NCS1 blocked the recruitment of Mannosidase-II vesicles and inhibited phagocytic uptake of diverse targets. We propose Golgi-derived vesicles provide membrane for phagosome biogenesis and are universally required for phagocytosis, the key innate defense function.
Function of Rab 10 interacting isoform of Myosin Vb is important for the maintenance of peridermal cell morphology in the developing zebrafish epidermis

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Determination as well as maintenance of the tissue size relies on dynamic balance between cell number and cell size. Under various altered physiological conditions and pathological states this balance is disturbed resulting in compensatory changes in cell size and proliferation. Whether and how the regulators of intracellular transport contribute toward maintenance of this balance between cell size and number is poorly understood. In the recent past, using zebrafish as a model, we have shown that Myosin Vb is an important regulator of intracellular transport in the epidermis. The function of this motor is essential to maintain plasma membrane homeostasis, which directly controls the cell size and proliferation. Here, we show that the reduction in cell proliferation results in increased cell spreading, which is characterized by increased cross sectional area, in the periderm which is the outer layer of the developing zebrafish epidermis. Further cytometric characterization revealed an increase in the surface area of the apical domain but relatively no effect on the basolateral domain of the cells in the periderm, resulting in decreased height of the periderm. These observations suggest that epidermal cells becomes more squamous in absence of cell proliferation to cover surface of the entire organism. In an attempt to identify the regulation of this change in cell morphology, we investigated the function of a specific Rab10 interacting isoform of Myosin Vb, which is shown to function in membrane biogenesis in neurons. Our analyses indicate that knockdown of this particular isoform of Myosin Vb results in change in the Golgi morphology and accumulation of ceramide rich vesicles indicating altered membrane biogenesis. We also observe increase in late endosomes as well as recycling endosomes. Interestingly, the knockdown of Myosin Vb isoform, during normal development leads to reduced apical surface area and increased height or basolateral surface area and under cell proliferation inhibition paradigm, cells can't spread in apical directions but show increase in basolateral domain hence acquiring a columnar morphology in both the cases. Taken together, our results suggest that Rab10 interacting isoform of Myosin Vb functions in regulating squamous cell morphology in the zebrafish epidermis, during normal development as well as when cell-proliferation is inhibited.
Internalization of antibody fragments targeting FGFR1

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Fibroblast growth factors (FGFs) and their plasma membrane-localized receptors (FGFRs) regulate signaling pathways that control metabolism and developmental processes. Numerous tumors are characterized by the elevated level of FGFR and this is considered a bad prognostic factor for patient survival. Antibody drug conjugates (ADCs) targeting cancer cells with the overproduced FGFR represent one of the most promising therapeutic strategies. ADCs are composed of the antibodies developed against tumor-specific biomarkers linked to the highly cytotoxic drugs. After selective binding to the cancer cells ADCs are internalized and delivered to the lysosomes by intracellular vesicular transport system. The lysosomal proteolysis of ADCs causes release of the cytotoxic drugs, which subsequently leads to the cell death. A prerequisite for an ADC approach is efficient internalization of the antibody-target complex. Although the biology of FGFRs and their ligands has been broadly studied, the requirements for the effective internalization of antibodies that target FGFR remain elusive.

We analyzed the internalization of antibody fragments in various formats that target FGFR1. The antibody fragments in the monovalent scFv format bind to FGFR1, but are not internalized into the model cells that overproduce FGFR1. In contrast, the same scFv proteins in the bivalent scFv-Fc format are efficiently internalized via FGFR1-mediated, clathrin and dynamin dependent endocytosis. Interestingly, antibody fragments in the scFv-Fc format induce FGFR1 endosytosis in the absence of growth factor. The receptor kinase function of FGFR1 is dispensable for endocytosis of scFv-Fc-FGFR1 and FGF1-FGFR1 complexes. Since binding of the bivalent scFv-Fc induces FGFR1 dimerization without simultaneous receptor activation, our data imply that oligomerization of FGFR1 constitutes the trigger for receptor endocytosis. Summarizing, our results may facilitate design of highly-internalizing antibody fragments for ADC therapy of FGFR1 overproducing cancers.

This work was supported by grant FUGA 2015/16/S/NZ3/00363 from the National Science Centre awarded to Lukasz Opalinski.
Membrane shaping in yeast endocytosis is facilitated by a host of proteins that are recruited to endocytic sites in stereotypic sequence. Among these proteins is Rvs167/161 (Rvs), a heterodimeric Bin-Amphiphysin-Rvs (BAR) domain protein complex. While BAR domains are known to sense or induce membrane curvature in vitro, and Rvs is implicated in endocytic membrane scission, how the properties of Rvs mechanistically effect the scission process remains unknown. Here we study the contribution of Rvs to scission by combining mutagenesis with live-cell tracking of fluorescent proteins. Centroids of fluorescent proteins are tracked as the proteins accumulate and depart from endocytic sites; multiple centroid tracks are then averaged to yield information about the dynamics of these proteins at endocytic sites at high spatial and temporal resolution.

We first studied the recruitment of Rvs as a function of its different domains. We found that the localization is based, as expected, on a curvature-sensitive interaction between the BAR domain and curved membranes, but also unexpectedly, on an interaction mediated by the SH3 domain. Live-imaging showed that the SH3 domain contributes to recruitment and dynamics of Rvs at endocytic sites, and also affects growth of the membrane invagination and the actin network during endocytosis.

Interestingly, the membrane in rvs167Δ cells can retract without producing a vesicle, but the majority of endocytic sites undergo scission earlier than in wild type cells, implying that Rvs delays scission. Thus, an Rvs structure around the invaginated tubule, assembled by both BAR and SH3 domains, may keep the underlying membrane stable and prevents premature scission. Disassembly of this structure is concomitant with scission, suggesting that triggering disassembly could allow regulation of the scission time point. We are investigating regulation of the Rvs structure, and therefore scission, by studying the effects of several proteins known to interact with the Rvs complex and the underlying membrane, on this process.
Tumor Susceptibility Gene 101 (TSG101), an ESCRT-I protein, can mitigate Endoplasmic Reticulum (ER) Stress mediated apoptosis via ESCRT machinery accessory proteins.

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ESCRT (endosomal sorting complex required for transport) proteins are implicated in myriad cellular processes, including endosome formation, fusion of autophagosomes/amphisomes with lysosomes. Further, components of the ESCRT machinery are suggested to be a bridge between the endo-lysosomal system and cell-death. The role played by these proteins in either facilitating or protecting against apoptosis is still unclear. In this study, while trying to understand how deficiency of Mahogunin Ring Finger 1 (MGRN1) affects cell viability, we uncover a novel role for its interactor, the ESCRT-I protein, TSG101 – it directly participates in mitigating ER stress mediated apoptosis. The association of TSG101 with ALIX prevents predisposition towards apoptosis while ALIX-ALG-2 interaction pushes towards a death phenotype. Altered Ca2+ homeostasis in cells and a simultaneous increase in the protein levels of ALIX and ALG-2 are required to elicit apoptosis by activating ER stress associated caspase 4/12. We further demonstrate that in the presence of membrane-associated disease-causing prion protein CtmPrP, increased ALIX and ALG-2 levels are detected along with ER stress markers and associated caspases in transgenic brain lysates and cells. These effects were rescued by overexpression of TSG101 – thus also making it pathologically relevant. Our analysis identifies a yet unidentified role of TSG101, where it directly participates in alleviating ER stress mediated apoptosis. Depletion of TSG101 in cell culture systems causes upregulation of ER stress. Levels of marker proteins for smooth ER (Rtn3 and Nogo A and B) go down and marker proteins for rough ER (TRAP-α and Sec-61β) go up during TSG101 depletion; mixing of smooth and rough ER occurs. Total protein load at the ER also increases during absence of TSG101. We believe that in absence of TSG101, the translocation of protein from ER to golgi bodies gets delayed and that causes accumulation of proteins in the ER. This causes ER stress and also leads to its aberrant morphology and expansion. This study unravels a unique function of TSG101, where it is required to maintain proper morphology of ER and protect against ER stress.
Multi-modal Endophilin interactions with closed, uncoupled membranes monitored with novel assay: FRAIM

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PROBLEM
N-BAR proteins such as Endophilin sense and induce membrane curvature through a recursive and multi-modal process of hydrophobic insertions of their N-terminal amphipathic helix (AH) as well as scaffolding mediated by the crescent shaped BAR domain. The role of these two agents in determining the synergic membrane modulating effect of N-BAR proteins has been a matter of dispute – partly due to studies of membranes with different intrinsic properties. Most studies have been conducted on tubules pulled from open and coupled giant unilamellar vesicles (GUVs). Only few have addressed the function of N-BARs on spherical, uncoupled and closed membranes, although such membrane compartments are involved in most cellular trafficking. Consequently, the lack of robust assays for dissecting protein interactions with closed, uncoupled membranes has halted progress and consensus in the field.

OUR SOLUTION
Here we address the recursive interactions between endophilin A1 and uncoupled and closed lipid compartments using a novel flow cytometry based assay, which we term FRAIM (Flow cytometry of Recursive Amphiphile-Interactions with Membranes). FRAIM allows for simultaneous assessment of multiple cardinal features of protein interactions with spherical membranes, which let us discern the individual contribution of the AH from the synergetic N-BAR domain of endophilin.

FINDINGS
We find that Endophilin binds to vesicles and induce vesiculation in a concentration and time dependent sequential process. Helix0 is sufficient to induce binding, sensing and deformation on spherical membranes. Interestingly, Helix0 recapitulates the full curvature sensing paradigm of the full length Endophilin construct, though it binds to the membranes with less affinity. Highlighting the flexibility of the assay, we further show that the membrane interactions of Endophilin may be severely halted at low incubation temperatures, presumably due to increased membrane rigidity.

We expect FRAIM to be an easily implemented assay that may help establish consensus on the mechanics of N-BAR protein interactions with closed uncoupled membranes as opposed to the their more well defined role in open coupled membrane systems.
Endosomal compartmentalisation and Notch signal regulation in Drosophila melanogaster

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In eukaryotic cells, the endocytic pathways play important roles in modulating the activity of membrane signalling receptors. It down regulates signalling by removing receptors from access to ligands at the cell surface, and delivering them to the lysosome for degradation. Moreover, the endocytic pathway can also have a positive affect on signalling by bringing necessary components together on the endosomal perimeter membrane into an appropriate signal-competent environment. It is clear that such an endocytic regulation of signalling must rely on an ordered endosomal architecture including specialised compartments and regulated flux between them. However we only have a limited understanding of how the sub-compartmentalisation of the endosomes, and the trafficking of cargo to and between these different sub-domains, is linked to their various biological roles in signalling regulation, or how misregulation of endosomal architecture is linked to aberrant signalling activity and thus to human diseases.

Our recent research on the cell biology of Notch using the Drosophila model system has provided a new overview of the way Notch signal levels are set by its sorting through alternate endocytic routes, to distinct Glycosylphosphatidylinositol (GPI)-anchored protein negative or positive endosomal domains respectively, in which Notch is activated by distinct mechanisms. We have now found that disruption of different Endosomal sorting complexes (ESCRTs) is correspondingly linked to Notch misactivation by the different mechanisms, which argues strongly for selective consequences of individual complexes on Notch endosomal compartmentalisation. In fact, the two Notch endocytic routes form distinct endocytic subdomains recruiting different ESCRTs and other proteins. Our findings are highly significant because disruptions to different endosomal sorting components have been linked to a number of different trafficking, developmental and pathological consequences including tumour formation and neurodegenerative disease, in ways that are heterogeneous and not well understood.
Characterization of the compartments disrupted by endosomal escape enhancing small molecule drugs

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Nucleic acid based drugs, e.g., siRNA drugs, are a potential novel class of drugs that offer the prospect to revolutionize medical therapy in general and cancer therapy in particular. However, efficient intracellular delivery of small double-stranded RNAs to the cytosol of target cells remains a substantial obstacle to their widespread and effective use. Non-viral delivery vehicles, with their RNA cargo, are initially endocytosed. Most of the endocytosed material is retained within endosomal vesicles and only a small fraction subsequently exits the endosome and enters the cytosol – a process known as “endosomal escape”. One commonly used strategy to try to enhance the endosomal escape of various drug delivery vehicles is co-administration of lysosomotropic small molecule drugs. Such drugs are believed to selectively disrupt lysosomes, however experimental data clearly demonstrating this is lacking. We have developed a sensitive live cell imaging approach to characterize the identity of the compartments disrupted by a collection of lysosomotropic small molecule drugs, including the classical agent chloroquine. Using fluorescent galectin-9 accumulation as a sensor, we could time the moment of membrane disruption with a precision of a few seconds. The disrupted compartment was only in a minority of events positive for LAMP1. Instead, the autophagy marker LC3 was more commonly associated with the disrupted structures. Taken together, our data suggest that autophagic structures are more sensitive to pharmacological disruption than the commonly assumed target – lysosomes – with implications for efforts to enhance delivery of non-viral nucleic acid based drugs.
Leishmania donovani modulates endo-lysosomal pathway by upregulating Rab5a for its successful survival in macrophages

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Successful intracellular pathogens avoid their lysosomal transport by modulating the intracellular trafficking pathways for their successful survival in the host cells. However Leishmania is suggested to successfully carve its niche and reside in a phagolysosomal compartment called Leishmania Parasitophorous vacuole (PV) by virtue of acquiring Lamp1, vacuolar ATPase, and CathepsinD. However, in the present study we have found that Leishmania donovani infection specifically upregulates the expression of Rab5a in THP-1 differentiated human macrophages and human PBMCs. Subsequently, L. donovani recruits and retains Rab5a and its effector EEA1 on its PV to reside in modified early endosomes in THP-1 differentiated human macrophages and human PBMCs. Consequently, we also have observed that recruitment of higher amounts of Rab5a by fusing with Lamp1 and Pro-CathepsinD containing vesicles. Thus, Leishmania PV contain Lamp1 and Pro-CathepsinD which results into reduce proteolytic capacity of the PV in infected cells. Furthermore, siRNA mediated knockdown of Rab5a in human macrophages significantly inhibits the survival of the parasite. These results provide the first mechanistic insights how Leishmania remodeling of endo-lysosomal trafficking to reside in a specialized early endocytic compartment. Furthermore this study also raises the possibility of modulating endo-lysosomal pathway in parasite infected cells by small molecules to divert trafficking of Leishmania probably to lysosome which might be useful for developing future therapeutic intervention.
A direct link between membrane physical properties modulations and ESCRT polymerisation on endosomes

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The endocytic pathway is a regulator of the sensitivity of the cell to external stimuli. Once internalised at the plasma membrane, material merges with endosomal compartments where a second mechanism of internalisation occurs. This process is called intraluminal vesicle (ILV) formation and can serve to limit sustained receptor signalling by sending them for degradation in the lysosome. Biogenesis of ILVs is achieved by a machinery called Endosomal Sorting Complex Required for Transport (ESCRT). This process is very well described in yeast while it is more obscure in mammals. The goal of this study is to better understand the role of mammalian ESCRTs in the formation of ILVs by determining the ESCRT subunits involved in this process and analysing the link between physical membrane properties and ESCRT polymerisation on endosome. We found that by playing with membrane properties, we can trigger a fast recruitment of a specific set of ESCRT subunits on early endosomal membrane. We can also recapitulate this process in vitro using GUVs and purified proteins, confirming the direct link between membrane physical properties modulations and ESCRT polymerisation.
Follicle stem cell maintenance in the fly ovary is regulated by alternative and competing endocytic routes.

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We are interested in how different endocytic routes of Notch trafficking, mediated by two different ubiquitin ligases, a Nedd4 family member Su(dx) and a ring finger containing Deltex, alter the activity of the Notch signalling pathway in vivo in Drosophila melanogaster. Deltex endocytosed Notch can be activated by a non-canonical pathway that is independent of its standard Delta and Jagged ligands. This involves retention of Notch on the limiting membrane of the late endosome and the release of Notch intracellular domain transcription factor upon fusion to the lysosome. Su(dx) opposes this function by transfers Notch in to the intraluminal vesicular compartments by a ubiquitination-dependent step. This overall signaling levels of Notch depends on the relative balance of fluxes through different pathways. Using a genetic approach we are investigating the physiological outcomes of altering this flux balance in the malleable in vivo system of the fly ovary. Developing eggs become covered in epithelial like follicle cells that act to isolate egg chambers from one another while keeping them linked together. The production of the germ line must be in tune with the manufacture of these follicle cells in order to maintain egg production throughout the fly’s life and synchronize their response to environmental effects such as diet. We found that flies mutant for Su(dx) and Deltex, were unable to maintain egg production as the fly aged, or shifted between poor and rich food, with many of the developing egg chambers containing multiple follicles. We reasoned that the Su(dx) mutant defect could be due to a deficiency in follicle stem cell maintenance and/or replacement. Follicle cells are the progeny of a pair of stem cells. Their daughter cells can repopulate any empty niche. Using mitotic marked clones we show that Su(dx) has a strong loss of follicle stem cell retention over time compared to wild-type which can be rescued by reducing the dosage of Deltex by half.
Substrate stiffness determines cell rear membrane tension to activate RhoA via Caveolae and promote rear retraction of migrating cells

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Protrusion and traction force generation in cell migration has been studied in great detail, and several modes of forward movement of the leading edge have been defined in 2D and physiological 3D environments. However, how the rear of the cell actively translocates is not well understood. The cytoskeletal regulator RhoA is highly active at the rear of actively moving cells, but how it is activated there and how RhoA signals to control rear retraction is not clear.

We have identified a mechanism of recoil retraction of the migrating rear in cells moving in 3D-matrix. Caveolae, membrane domains associated with buffering membrane tension and with endocytosis, accumulate at the rear of the cell and co-localise with active RhoA. Caveolae are required for the activation and localisation of active-RhoA in migrating cells, and for retraction of the cell rear. Furthermore, increasing membrane tension, by reducing osmolarity of the media, prevents formation of Caveolae to the cell rear, activation of RhoA and translocation of the trailing edge. Downstream of RhoA, both ROCK1 (but not ROCK2) and PKN2 are required for the formation and alignment of F-actin cables to form a contractile axis and allow the rear of the cell to be actively moved forward.

Interestingly, on 2D surfaces of uniform stiffness, rear polarity is not well defined, Caveolae are excluded from the cell front and localise to the rear and sides of the cell (together with cortical F-actin). However, in cells within a gradient of stiffness, protrusions orient toward the stiff matrix, and Caveolae toward the soft, suggesting that cells sense differences in substrate stiffness and polarise accordingly. Our data suggest that in migrating cells, differences in substrate stiffness are transduced via the cytoskeleton to decrease membrane tension at the cell rear, promote Caveolae formation and activate RhoA to propel the rear for the cell forward.
Subversion of the Rab35-Rab11 cascade by Shigella promotes bacterial vacuole disassembly

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The intracellular bacterial human pathogen Shigella triggers its entry in epithelial intestinal cells within tight phagosome-like bacterial containing vacuoles (BCV). Simultaneously to its internalisation, the bacterium also induces massive formation of macropinosomes in the vicinity of the BCV. Shortly after invasion, the bacteria escape from the BCV to reach the host cytosol. This key event is necessary for subsequent bacterial growth and spread to neighbouring cells. We have recently demonstrated that macropinosomes are not required for bacterial uptake, as previously hypothesized, but for bacterial vacuolar escape. In particular, the establishment of contact sites between the BCV and macropinosomes are needed for efficient membrane rupture during this process.

Here, we identify the involvement of a Rab GTPases cascade associated with recycling pathways, namely through Rab35 and Rab11, which is involved in the disassembly of the Shigella containing vacuole. We employ a variety of cell biology tools and imaging techniques to address: (i) how these Rabs are specifically relocated either to the BCV or surrounding macropinosomes, (ii) how these Rabs mediate the tethering between BCV and macropinosomes, (iii) the recruitment of host effectors involved in vesicular transport along cytoskeletal tracks, such as class I FIPs and different motor proteins, and (iv) the generation of directional movements of the tethered macropinosomes away from the BCVs after having made contact that eventually provokes a physical disruption of the BCV. This shows that the pathogen is capable to divert the host recycling trafficking to the newly induced macropinosomes and use them to exert mechanical forces that strip off the BCV membranes from the bacterium. With this intricate strategy, Shigella reaches its cytosolic niche that is required for bacterial growth and mobility.
Dynamical features of spatially distributed EGFR-PTP network determine growth factor response

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The intrinsic epidermal growth factor receptor (EGFR) kinase activity opposed by protein tyrosine phosphatases (PTPs) determines its phosphorylation state and thereby its signaling capacity after epidermal growth factor stimuli. The continuously maintained balance between kinase and phosphatase activity has to be spatially regulated during the course of receptor trafficking, when EGFR meets PTPs at distinct cellular locations. Combining functional imaging and dynamical systems theory, we identified a single EGFR-PTP network with specific topology that controls spatial-temporal EGFR phosphorylation patterns. Moreover, the dynamical features of this network determine a sensory system that regulates how and when cells respond to growth factors.
Autonomous activation and trafficking of c-Met promotes anchorage independent survival of pancreatic cancer cells

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The Receptor Tyrosine Kinase c-Met and its ligand, the hepatocyte growth factor (HGF), promote cell proliferation, migration and survival. c-Met pathway is activated during embryogenesis and wound healing, and its deregulation contributes to cancer development and progression. c-Met is over-expressed in 60-80% of Pancreatic Ductal Adenocarcinoma (PDAC), the most common type of pancreatic cancer. As PDAC has very poor survival rates and limited therapy options, c-Met represents a major target in PDAC. However, in PDAC, c-Met signalling is poorly characterized and its endocytic trafficking has not been reported.

It is assumed that c-Met signals through a paracrine mode in pancreatic cancer. Indeed, we have detected HGF secretion from stromal pancreatic stellate cell cultures. In several PDAC cell lines, c-Met gets activated through binding to exogenous HGF, leading to its internalisation and progressive degradation. Our results suggest that, HGF-bound-c-Met signals to ERK1/2 and STAT5 on endosomes, leading to cell migration. Surprisingly, in a subset of PDAC cell lines (5 over 8 tested), c-Met is constitutively activated, constitutively internalized – recycled, in association to a slow degradation rate. These PDAC cells possess a unique mechanism to activate c-Met, independently of HGF but dependent on endocytosis. The constitutively activated c-Met form cooperates with HER3 to promote cell survival in anoikis conditions. Strikingly, we have detected c-Met constitutive activation and localisation on endomembranes in 5 over 6 tested PDAC cells isolated from patients, indicating the relevance of our results in cell lines.

Our results suggest that targeting the trafficking machinery may constitute a new way of targeting c-Met in PDAC. Futhermore we report that, unexpectedly, a subset of PDAC cells express a constitutively activated and constitutively endocytosed-recycled c-Met. Our results suggest that this autonomous c-Met activation is endocytosis dependent and may confer a survival advantage to detached cancer cells during the metastatic process.
Impaired axonal lysosome transport contributes to Alzheimer’s pathology

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Through a comprehensive analysis of organellar markers in mouse models of Alzheimer’s disease (AD), we documented a massive and robust accumulation of lysosome-like organelles at amyloid plaques and establish that the majority of these organelles reside within swollen axons that contact the amyloid deposits. Notably, we discovered that lysosomes that accumulate in such axons are lacking in multiple soluble luminal proteases and thus predicted to be unable to efficiently degrade proteinaceous cargos. Of relevance to AD, β-secretase (BACE1), the protein that initiates amyloidogenic processing of the amyloid precursor protein (APP), is a substrate for these proteases and builds up at these sites. Furthermore, through a comparison between the axonal lysosome accumulations at amyloid plaques and neuronal lysosomes of the wildtype brain, we identified a similar, naturally occurring, population of lysosome-like organelles in neuronal processes that is also defined by its low luminal protease content. In conjunction with emerging evidence that the lysosomal maturation of endosomes and autophagosomes is coupled to their retrograde transport, our results suggested that extracellular β-amyloid deposits cause a local impairment in retrograde axonal transport, leading to the accumulation of lysosome precursors and a blockade in their further maturation.

In testing this hypothesis, we have identified JNK interacting protein 3 (JIP3) as an important regulator of both axonal lysosome abundance and maturation state. JIP3 KO neurons accumulate lysosomes within focal axonal swellings that closely resemble the dystrophic axons at amyloid plaques- including high levels of APP processing enzymes (BACE1 and presenilin 2) and are accompanied by elevated Aβ peptide levels. We tested our hypothesis that these traffic jams of axonal lysosomes could thus potentially serve as disease-relevant sites of APP processing by depleting JIP3 in a mouse model of AD. We observed that JIP3 haploinsufficiency strongly increased both the abundance and size of amyloid plaques. These results establish a critical role for efficient axonal lysosome transport and maturation in protecting the brain from amyloid plaque pathology.
Molecular mechanisms of ESCRT recruitment to damaged endosomal membranes

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Damage in the plasma membrane or endomembranes is associated with development of human diseases. Therefore, repair of the damaged cellular membranes is an important mechanism that contributes to our health. Recent findings implicate components of the endosomal sorting complex required for transport (ESCRT) machinery in sealing holes in the damaged nuclear envelope (Raab M et al., 2016; Denais CM et al., 2016) and plasma membrane (Jimenez et al., 2014). The ESCRT machinery, originally identified for its involvement in vacuolar protein sorting in budding yeast, is crucial for endosomal sorting of growth factor and chemokine receptors in mammalian cells. In addition, ESCRT components have been found to mediate budding of enveloped viruses, abscission of the intercellular bridge that connects two daughter cells during cytokinesis, and sealing of the reformed nuclear envelope during mitotic exit. Common to all these processes is that they involve membrane involution and scission of a cytosol-containing membrane stalk. Thus, the ESCRT machinery has evolved to perform a number of related functions in membrane dynamics, suggesting that the ESCRT machinery could, in addition, be specialized for sealing small holes in cellular membranes. However, the exact molecular details of the process remain elusive. Herein, we aim to investigate whether the ESCRT machinery mediates sealing of damage-induced holes in endosomal membranes, and, if so, to identify the underlying molecular mechanisms. Our results will be used to explore the concept of using ESCRT components as potential diagnostic markers and therapeutic targets in cancer cells.
Characterization of GAS6-induced AXL endocytosis

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Upon ligand binding, receptor tyrosine kinases (RTKs) undergo endocytosis, via different, often parallel, internalization pathways. Importantly, endocytosis is considered as an important organizer of cellular signaling activated by RTKs. AXL, a member of the TAM (TYRO3, AXL, MER) receptor family, and its ligand growth arrest specific gene 6 (GAS6) are overexpressed in a variety of different cancers, which correlates with poor prognosis, metastasis and drug resistance. Moreover, a growing body of evidence suggests that AXL is also implicated in viral infections and serves as a cofactor for cellular entry of Zika virus.

In the present study we generated tools for visualization of GAS6-induced AXL internalization and investigated endocytic traffic of this receptor. We found that following uptake, ligand-activated AXL accumulates rapidly on endosomes. Moreover, within 15 min the number of AXL-positive vesicles decreases, in contrast to internalized platelet-derived growth factor receptor β (PDGFRβ). Western blot analysis showed that this decrease in intracellular accumulation of AXL did not result from its degradation. Interestingly, during continuous cell treatment with GAS6 we observed cyclic changes in AXL accumulation on endosomes over time which may reflect recycling and multiple rounds of AXL internalization.

Finally, our data suggest that AXL is internalized via clathrin-independent mechanisms, as depletion of clathrin and the AP-2 adaptor protein, key components of clathrin-mediated endocytosis machinery, did not affect AXL internalization. Altogether our results provide a preliminary characterization of endocytosis of AXL, a process which has not been previously studied for any of receptors that belong to the TAM family.
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