Associazione di **B**iologia **C**ellulare e del **D**ifferenziamento

Membrane Trafficking and Organelle Biogenesis

Programme & Abstracts

Bertinoro (FC), 20-21 April 2012

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Organisers

Bice Chini (Chair) - CNR Institute of Neuroscience

Paolo Remondelli (co-Chair) - University of Salerno

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PROGRAMME

Friday, 20 April

12:30 **Registration**

13:00-14:00 **Lunch**

14:30-16:30 **Session 1**

Chair: Simona Paladino and Chiara Zurzolo (Naples)

14:30-14:50 *Arianna Crespi (Milan)*
Role of POF1B in epithelial polarity

14:50-15:10 *Carolina Crespo (Milan)*
The PAR polarity complex controls the directional migration of medaka macrophages to sites of tissue injury *in vivo*

15:10-15:30 *Maria Svelto (Bari)*
Knockdown of BBS10 in renal cells affects apical targeting of AQP2: a possible explanation for the polyuria associated with Bardet-Biedl Syndrome

15:30-15:50 *Grazia Tamma (Bari)*
A novel regulatory mechanism of AQP2 trafficking to explain the syndrome of inappropriate antidiuresis

15:50-16:10 *Alessia Castagnino (Genoa)*
ErbB2 membrane redistribution induced by Trastuzumab in SKBR3 cells

16:10-16:30 *Elisa Duregotti (Padua)*
Calpains participate in nerve terminal degeneration induced by spider and snake presynaptic neurotoxins

16:30-17:00 **Coffee break**

17:00-18:20 **Session 2**

Chair: Nica Borgese (Milan)

17:00-17:20 *Annalea Conte (Lecce)*
Biogenesis of the yeast cytochrome bc1 complex

17:20-17:40 *Laura Pancrazi (Pisa)*
Rett Syndrome and FOXG1 subcellular localization

17:40-18:00 *Francesco Consolato (Milan)*
Mitochondrial network fragmentation caused by enhanced OPA1 processing leads to impaired mitochondrial calcium uptake in *Afg3l2* null cells

18:00-18:20 *Grazia Della Sala (Pisa)*
Cellular mechanisms of Niemann Pick type C disease

18:30-19:30 **Plenary Lecture**

Pietro De Camilli (New Haven, CT, USA)

Membrane dynamics and phosphoinositide signaling in the endocytic pathway

19:30-20:00 **General discussion and election of next chair**

20:00-21:30 **Dinner**

21:30 **Poster Session, Wine and Dessert**

Saturday, 21 April

8:30-10:30 **Session 3**

Chair: Stefano Bonatti (Naples) and Paolo Remondelli (Salerno)

8:30-8:50 *Andrea Orsi (Milan)*

Monitoring differential UPR activation by over-expression of ER client proteins

8:50-9:10 *Sara Colombo (Milan)*

Assembly, trafficking and up-regulation of the α3β4 neuronal nicotinic receptor

9:10-9:30 *Giulia Papiani (Milan)*

ER-derived cytoplasmic inclusions generated by mutant, Amyotrophic Lateral Sclerosis-associated VAPB are cleared by the proteasome

9:30-9:50 *Alexandre Mironov (Milan)*

COPII vesicles are not transport carriers

9:50-10:10 *Rossella Venditti (Naples)*

Sedlin binds Sar1p and controls type II procollagen trafficking by modulating the cycle of COPII

10:10-10:30 *Carmen Faso (Zurich, Switzerland)*

ER exit sites exist independently of the Golgi in the highly reduced parasitic protozoan *Giardia lamblia*

10:30-11:00 **Coffee break**

11:00-13:00 **SESSION 4**

Chairs: Carlo Tacchetti (Genoa) and Francesco Filippini (Padua)

11:00-11:20 *Paola Bagnato (Genoa)*
Role of ErbB1 in Trastuzumab-induced ErbB2 signaling and internalization

11:20-11:40 *Giusi Caldieri (Milan)*
Identification of novel candidate proteins involved in the non-clathrin endocytic pathway of the epidermal growth factor receptor

11:40-12:00 *Rosa Molfetta (Rome)*
Syk kinase activity regulates the coupling of ubiquitinated FcεRI to the endocytic pathway

12:00-12:20 *Maria De Luca (Lecce)*
RILP and V1G1: functional interaction in mammalian cells

12:20-12:40 *Adriana Di Benedetto (Bari)*
Nuclear translocation of oxytocin receptor mediates increased expression of osteoblast specific genes

12:40-13:00 *Giuseppe Di Noto (Brescia)*
Immunoglobulin free light chains trafficking pathway

13:00 **LUNCH AND DEPARTURE**

ABSTRACTS

Oral Presentations

in alphabetical order
(presenting authors are shown underlined)

Role of ErbB1 in Trastuzumab-induced ErbB2 signaling and internalization

P. Bagnato[*1,2,3], A. Rabellino[*1,2,3] , A. Castagnino[1,2,3], M. Di Benedetto[1,3], P. Bianchini[1] , M. Bono[1,2,3], K. Cortese[1,2,3], C. Tacchetti[1,2,3,4]
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*these authors contributed equally to this work

The ErbB family of receptors comprises four closely related members: EGFR (ErbB1 or HER1), ErbB2 (HER2), ErbB3 (HER3), and ErbB4 (HER4). These receptors play important roles in cell proliferation, survival, migration and differentiation, and also in development and progression of cancer. In particular, ErbB2 is over-expressed in approximately 30% of invasive breast cancers, which often display a highly invasive and metastatic phenotype, resistance to conventional chemotherapy and hormone-therapy. The anti-proliferative effect of the ErbB2 specific antibody Trastuzumab in cells over-expressing ErbB2 has lead to its effective use in current therapeutic protocols. However, notwithstanding the extensive use, the mechanism of action of this antibody has not been defined yet and some controversial data have been reported. We concentrated our study on Trastuzumab effect in SK-BR-3 cells, following ErbB2 molecules directly bound to the drug on short term treatments. We found that Trastuzumab: i) induces the phosphorylation of ErbB2; ii) promotes ErbB1/ErbB2 heterodimerization; iii) triggers a signaling pathway leading to ERK 1/2 phosphorylation followed by Akt down-regulation and G1 arrest, iv) promotes the internalization and the recycling of ErbB2, v) heterodimerization with ErbB1 is responsible for the endosomal sorting/recycling of ErbB2 by subtracting it from a degradative compartment. Within this scenario, in cells treated with Trastuzumab, low levels of ErbB1 lead to a more efficient down-regulation of ErbB2 and a higher sensitivity to the drug, compared to high levels. Our results represent an important finding to further support the possibility to introduce ErbB1 as a marker of treatment feasibility, evaluating its levels in screenings of patients for Trastuzumab therapy.

Identification of novel candidate proteins involved in the non-clathrin endocytic pathway of the epidermal growth factor receptor

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Epidermal Growth Factor Receptor (EGFR) can be endocytosed through different entry routes depending on ligand concentration. At low doses of EGF, the receptor is not ubiquitinated and internalized exclusively through clathrin-mediated endocytosis (CME). At higher concentrations of ligand a substantial fraction of the receptor becomes ubiquitinated and is endocytosed through non-clathrin endocytosis (NCE). Importantly, the two pathways have distinct receptor functions: CME is mainly involved in receptor recycling and signalling, while NCE targets the majority of the receptors to degradation.
The molecular mechanisms involved in NCE of the EGFR are mainly unknown. Essentially, NCE is defined by its insensitivity to functional ablation (KD) of clathrin and for its sensitivity to cholesterol-interfering drugs, hence its definition as a "raft-dependent pathway".
A procedure based on the preparation of EGFR-containing vesicles in condition of clathrin-KD, coupled to a quantitative proteomic approach, provided us with a list of potential molecular components of NCE. Their role in EGFR NCE was tested through a large scale RNA interference approach, with the aim to identify specific functional regulators. We found 15 genes able to selectively impair the pathway upon KD. Among non-functional regulators, however, we scored a number of candidates that co-localize with the EGFR in NCE vesicles. The identification of these co-trafficking proteins further corroborate our procedure and could provide new potential NCE markers.

ErbB2 membrane redistribution induced by Trastuzumab in SKBR3 cells

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ErbB2 is a receptor tyrosine kinase (RTK) member of the EGFR family (ErbB1-4). ErbB2 over expression has been identified in approximately 25-30% of primary breast cancers and correlates with poor prognosis and cancer relapse. For these reasons, ErbB2 represents an attractive target for immunotherapy. Trastuzumab (Tz) is a humanized monoclonal IgG1 against ErbB2 currently used in advanced breast cancers therapy, and it exerts anti-proliferative effects on ErbB2-positive breast tumor cell lines. Despite its therapeutic success, resistance to Trastuzumab has been frequently observed. Therefore, the study of the molecular mechanisms involved in Trastuzumab action represents an important aim of our research work. In particular we have found that Trastuzumab short treatment induces ErbB2 phosphorylation, signaling and ErbB1/ErbB2 heterodimerization, promoting the ErbB1/ErbB2/Tz complex endocytosis.
We observed, as an ealy response to treatment with Trastuzumab, a dramatic ERK 1/2 dependent redistribution of ErbB2 in the plasma membrane, and the formation of circular dorsal ruffles (CDRs). CDRs are transient actin-based structures that assemble and disappear on the dorsal plasma membrane very quickly. CDRs (waves) are involved in the reorganization of actin cytoskeleton necessary to prepare a static cell for motility and in the internalization of RTKs, such as EGFR and PDGFR, after stimulation by a variety of growth factors.
We focused our studies on the characterization of this redistribution event, by co-IP e immunofluorescence studies of Tz/ErbB2 complex with markers of CDR such as N-WASP and Cortactin. The use of specific inhibitors of CDRs formation support the hypothesis of CDRs formation after Tz treatment. We are interested in the study of the possible involvement of this event in Trastuzumab induced ErbB2 internalization, and the role of these “waves” in the complex mechanism of action of this drug.

Assembly, trafficking and up-regulation of the $\alpha_3\beta_4$ neuronal nicotinic receptor

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The $\alpha_3\beta_4$ nicotinic receptor is the major subtype in the autonomic ganglia and adrenal medulla but is also present in some areas of the central nervous system (CNS) such as the medial habenula, pineal gland and retina. Most of our knowledge of nicotinic receptors and nicotine addiction is based on studies on the $\alpha_4\beta_2$ subtype that accounts for 90% of nicotinic receptors in mammalian brain. Recently a lot of interest in the study of $\alpha_3\beta_4$ subtype has emerged due to several genetic studies that suggest a strong link with nicotine dependence, smoking, and lung cancer. To investigate the effects of nicotine on $\alpha_3\beta_4$ receptors we transfected the nicotinic subunits both in a heterologous system (Hela cells) and in a more physiological one (SHSY-5Y cells). We studied with biochemical, binding and imaging experiments the assembly of the pentamers, the trafficking of the receptor from the ER membrane and the effects of nicotine treatment.
We demonstrate that In Hela cells most of the receptor, although assembled as a pentamer is retained at the level the ER and is rapidly degraded via the proteasome.
Chronic treatment with nicotine has a double effect. On the one hand, nicotine doubles the amount of the receptor, an effect already seen for other subtypes, but this effect is not due to a direct effect on the proteasome. The same up-regulation is obtained with the chronic exposure to other agonists or antagonists provided they are permeable and competitive for nicotine binding site. On the other hand, nicotine facilitates the exit of the newly-synthetized receptors from the ER and their arrival at the plasma membrane. To obtain these two effects, however, nicotine must be present during, or immediately after, synthesis of the receptor subunits. Since nicotine has no effect on pre-synthetized receptors we speculate that during the assembly of pentamers the nicotine binding favors a conformational state that is preferably released from the ER for transport to the plasma membrane.

Mitochondrial network fragmentation caused by enhanced OPA1 processing leads to impaired mitochondrial calcium uptake in *Afg3l2* null cells

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The mitochondrial protease AFG3L2 forms homo-oligomeric and hetero-oligomeric complexes with paraplegin in the inner membrane, named m-AAA proteases. These complexes exert protein quality control and chaperone like activity on respiratory chain complexes. Moreover, they control mitochondrial morphology by regulating the cleavage of OPA1, which mediates mitochondrial fusion. Missense mutations in *AFG3L2* cause spinocerebellar ataxia type 28 (SCA28), while homozygous mutations in *AFG3L2* have been associated to a severe neurological syndrome of childhood. We provided evidence that *Afg3l2* knockout mice recapitulate features of patients carrying homozygous mutations. Accordingly, *Afg3l2* haploinsufficient mouse resembles SCA28 patients, showing progressive ataxia due to dark degeneration of Purkinje cells (DCD-PC). Alterations of mitochondrial metabolism, increased mitochondrial fragmentation and disruption of cristae morphology witness the organellar origin of the disease. In Afg3l2 haploinsufficient mice mitochondrial dysfunction mediates DCD-PC a phenomenon originating by increased intracellular calcium concentration. We hypothesize that fragmented SCA28 mitochondria have defective calcium-buffering capacity and this causes DCD-PC. We demonstrated that fragmentation of the mitochondrial network in *Afg3l2* knockout murine fibroblasts (MEFs) reduces the mitochondrial calcium uptake by lowering the organellar capacity and calcium wave propagation. This defect is not a consequence of global alteration in cellular calcium homeostasis or the reduced driving force for calcium internalization. The recovery of mitochondrial fragmentation by overexpression of wild type OPA1 or constitutively active form of OPA1 (OPAQ297V) in *Afg3l2* knockout MEFs rescues the defective mitochondrial calcium buffering. These results propose a functional link between mitochondrial morphology and PC degeneration in the SCA28 mouse model.

Biogenesis of the yeast cytochrome bc_1 complex

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The cytochrome bc_1 complex, or complex III, is a component of the mitochondrial respiratory chain. This complex catalyzes the transfer of reducing equivalents from quinol to cytochrome *c* and this process brings about proton translocation from the mitochondrial matrix to the intermembrane space. Each monomer of the yeast dimeric cytochrome bc_1 complex is composed of ten different protein subunits inserted into or bound to the inner mitochondrial membrane. Three of these subunits, cytochrome *b*, cytochrome c_1 and the *Rieske* iron–sulfur protein (ISP) contain redox centers and participate in electron transfer. The remaining seven subunits are non-redox subunits and their function is largely unknown.
In our previous studies, we demostrated that biogenesis of the bc_1 complex occurs via a multiple-step pathway involving both catalytic and non-catalytic subunits in distinct subcomplexes. Here, we used epitope tagged proteins to purify the native late core assembly intermediate and to define its composition. In fact, this subcomplex could represent either a monomeric bc_1 complex in association with various unidentified assembly factors or a dimeric bc_1 complex perhaps associated with the monomeric chaperone protein Bcs1. We found that the late core assembly intermediate is a dimeric complex and so concluded that the subsequent insertion of ISP subunit is not essential to induce bc_1 complex dimerization.

Role of POF1B in epithelial polarity

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POF1B is a candidate gene for the Premature Ovarian Failure (POF), a disorder that affects about 1% of the general female population before the age of 40 years. POF1B is mainly expressed in polarised epithelial tissues, but its function and the relationship with the disorder are still unknown. It was observed that the tight junction localization was maintained by the human POF1B stably expressed in the MDCK polarised epithelial cell line, whereas it was lost by the POF1B R329Q variant associated with POF. Localization of apico-basal polarity markers and ultrastructure of the tight junctions were maintained in cells expressing the mutant. However, tight junction assembly was altered, cells were dysmorphic and the monolayer organization was found altered in the three-dimensional culture system, demonstrating a role of POF1B in planar cell polarity. Moreover, cells expressing the POF1B R329Q variant showed defects in ciliogenesis and cystogenesis as a result of misorientation of primary cilia and mitotic division. All of these defects were explained by interference of the mutant with the content and organization of F-actin at the junctions. Indeed, while the wild-type almost completely colocalized with F-actin, the mutant showed only a partial colocalization and a lower content of actin (evaluated by FACS analysis). A role for POF1B in the regulation of the actin cytoskeleton was further verified by shRNA silencing of the endogenous protein in human intestinal Caco-2 cells. The downregulation of the protein led to an alteration of the monolayer: the silenced cells completely lost their apico-basal polarity and detached from the monolayer, showing also a reduction of the actin content. Taken together, these data indicate that localization of POF1B to tight junctions has a key role in the organization of epithelial monolayers by regulating the actin cytoskeleton.

The PAR polarity complex controls the directional migration of medaka macrophages to sites of tissue injury *in vivo*

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The establishment and maintenance of cell polarity is fundamental for leukocyte responses to inflammatory cues. The evolutionary conserved partitioning defective (PAR) polarity complex consisting of PAR3, PAR6 and atypical Protein kinase C (aPKC) temporally and spatially control polarization in a wide variety of contexts. To investigate the physiological requirement of the PAR complex in leukocyte polarized migration *in vivo*, we used as model system membrane-tethered YFP-labelled medaka (*Oryzias latipes*) macrophages exposed to a mechanical wound in the larva tailfin. We combined a cell-specific transient transgenesis strategy to target the complex in a mosaic fashion with *in vivo* live imaging to access overall parameters of stimulus-induced macrophage dynamics. Compared to PKCζWT macrophages, the expression of either the dominant-negative or the constitutively active PKCζ mutants decreased macrophage directional motility to the injured tailfin. The genetic displacement of PAR6 or PAR3/aPKC interactions also impaired wound-triggered macrophage directional motion, although the effects were comparatively less pronounced. To visualize in detail the impact of the PAR complex in specific patterns of the cell cytoskeleton, we developed a double transgenic line amenable to the *in vivo* assessment of the dynamics of F-actin and the microtubule networks selectively in medaka macrophages. Preliminary analysis on high-resolution time-lapse image sequences provides evidence of an altered periodicity of F-actin front-rear waves and on MTOC positioning in the perinuclear region of wound-activated macrophages interfered for the PAR complex activity or assembly. In addition, correlation studies revealed that each of the above perturbations negatively impacted wound-directed speed.
Collectively, our findings reveal the existence of a functional role for core components of the PAR complex in regulating the directional migration of myeloid cells responding to inflammation *in vivo*.

Membrane dynamics and phosphoinositide signaling in the endocytic pathway

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Endocytosis plays a fundamental role in all cells and a highly specialized role at neuronal synapses. In axon terminals, the efficient endocytic recycling of synaptic vesicle membranes after exocytosis makes possible the reliable function of synapses even during high frequency stimulation, in spite of their distance from the cell body, where new proteins are synthesized. Postsynaptically, endocytosis plays a key role in the regulation of the number of surface exposed neurotransmitter receptors. While much has been learned about endocytosis, our understanding of this process lags behind the field of exocytosis due in part to the multiplicity of endocytic mechanisms. We study such mechanisms using a variety of complementary approaches, which include reconstitution experiments with purified endocytic proteins and lipid membranes, broken cell preparations, intact cells, model synapses and genetically modified mice. In my talk I will focus on studies of mechanisms underlying membrane deformation and membrane fission at early stages of the endocytic pathway, with emphasis on the role of the GTPase dynamin and its functional partnership with BAR domain containing proteins, endophilin in particular. BAR domains are protein modules that bind the lipid bilayer and have curvature sensing and curvature generating properties. I will also discuss the regulatory role of phosphoinositide metabolism in the progression of membranes along early stations of the endocytic pathway.

RILP and V1G1: functional interaction in mammalian cells

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Endocytosis and trafficking of receptors are dependent on an acidic intra-endosomal pH that is maintained by vacuolar H+-ATPase (V-ATPase) proton pump. The V-ATPases are large multisubunit complexes organized into two domains, operating by a rotary mechanism. The V1 domain is peripheral, it is located on the cytoplasmic side of the membrane and carries out ATP hydrolysis. The membrane V0 domain, instead, is responsible for translocation of protons from the cytoplasm to the lumen or extracellular space. V-ATPases have central roles in normal physiology, modulating pH homeostasis in a number of intracellular sites. Deficient functions of V-ATPases and defects of vesicular acidification are recognized as important mechanisms in a variety of human diseases. The interaction with specific partners plays a crucial role in controlling V-ATPase activity. We have identified the G1 subunit of the V-ATPase as a RILP (Rab-Interacting Lysosomal Protein) interactor. Here we show that V1G1 directly interacts with RILP. RILP is a specific regulator of V1G1, it regulates V1G1 stability, via a proteasome-dependent mechanism, and its endosomes/lysosomes localization. RILP is required for biogenesis of MVBs (multivesicular bodies) and, together with Rab7, acts in the regulation of late endocytic traffic. Acidification, triggered by V-ATPase in early endosomes, is required for the formation of MVBs. To clarify the molecular pathway through which RILP regulates V1G1, we have analyzed the role of Rab7 in this process. Our data show that Rab7 over-expression doesn't modulate V1G1 stability in contrast with Rab7 silencing where V1G1 stability decrease. Interestingly, the concomitant over-expression of RILP and Rab7 restores normal V1G1 levels suggesting that the interaction between Rab7/RILP could positively modulate V1G1.

Cellular mechanisms of Niemann Pick type C disease

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Over 200 disease-causing mutations have been identified in the NPC1 gene. NPC1 is a 1278 amino acid protein with 13 transmembrane domains that is crucial for normal cholesterol homeostasis. The most prevalent mutation, $NPC1^{I1061T}$, is predicted to lie within the cysteine-rich luminal domain and is associated with the classic juvenile-onset phenotype of Niemann-Pick type C disease.
To gain some insights on how loss of NPC1 function leads to neurodegeneration we have examined the global pattern of expression the protein in HN9.10e a neuronal cell line. In this research we studied first the distribution of the protein in the cell and motility and then we focused on the cell suborganelles seeking evidence for mislocalization of the protein as well as structural damage.
Our research shows that the $NPC1^{I1061T}$ protein fails to advance in the secretory pathway and remains trapped into the endoplasmic reticulum with consequent alteration in the reticulum structure and function. Further analyses reveal that the presence of missense mutation of NPC1 protein affects also mitochondria functions.
Understanding the behaviour and properties of organelles containing NPC1 will help to address the substantial mysteries of NPC1 function. This work could be useful to understand the basis of Niemann-Pick disease and devise prevention or treatment strategies.

Nuclear translocation of oxytocin receptor mediates increased expression of osteoblast specific genes

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We reported that the neuro-hypophyseal hormone oxytocin (OT) is a novel anabolic regulator of bone mass and up-regulates expression of osteoblast markers and transcription factors, as Osterix (Osx), Schnurri, Atf-4, Osteocalcin and BSP (Tamma et al., 2009). These effects are mediated by the seven-transmembrane G-protein coupled receptor OTR (OT receptor) expressed by osteoblasts. Recently an increasing number of GPCRs have been demonstrated to be targeted to the nuclear membrane; (Boivin et al., 2008). Accordingly we found OTR in osteoblast nuclear protein extracts after OT stimulation (15-30 min), confirmed by nuclear localization observed by confocal microscopy and immunogold staining at TEM (transmission electron microscopy). Exogenous OTR-EGFP, transfected in primary osteoblasts, colocalized with β-arrestin1/2 within 2-3 min after OT treatment. Thereafter the receptor, dissociated from β-arrestins, was found in endosomal vesicles and eventually sorted to the nucleus.
MALDI-TOF analysis confirmed this finding revealing the presence of four peptides corresponding to OTR intracellular loops. We hypothesized as possible role for nuclear OTR the regulation of transcription and/or transcription factors. By immunoprecipitation, we found physical interaction of OTR with the osteoblast transcription factor Runx-2, transcription co-activators Schnurri-2 and Smad4 in the nucleus. The blockage of OTR endocytosis by β-arrestins silencing, or by using a not-internalizing OTR mutant, prevented OT induced up-regulation of Osx, ATF-4, BSP and Osteocalcin mRNA. Similarly OTR nuclear localization as well as the up-regulation of Osx, ATF-4, Osteocalcin and BSP mRNA in response to OT were impaired by Transportin-1 silencing. Taken together these data suggest that OT anabolic effect on bone could be mediated, at least in part, by a novel mechanism initiated by β-arrestin mediated OTR internalization and Transportin-1 dependent nuclear translocation.

Immunoglobulin free light chains trafficking pathways

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INTRODUCTION: Monoclonal serum Free Light Chains (FLCs) are present in the serum and urine of many patients with plasma cell proliferation diseases. The physical-chemical characteristics of FLCs vary among patients and to date the different tissue damage potential is not fully understood. FLCs are bioactive molecules that induce antigen specific hypersensitivity and interact with several cell lines by activating different intracellular trafficking patterns.
GOAL: The mechanism of internalization and trafficking of FLCs is not well understood, therefore, we aimed to characterize this pathway. We have developed an experimental protocol to identify the FLCs membrane tropism and their pathogenicity by analysing FLCs interaction with different cell lines (endothelial, epithelial and myocardial).
METHODS: In order to identify a correlation between the FLCs phenotype and pathogenicity, we have investigated the internalization rate of different lambda and kappa FLCs (from hospitalized patients) in different cell lines. The mechanisms of internalization in different cellular subtypes might be an important key in the study of different pathological conditions strictly correlated to FLCs (systemic and not only kidney related) metabolism. Moreover we performed a sucrose density gradient protocol to isolate, quantify, and characterize vesicles containing paraproteins.
RESULT: Our results highlight that the monoclonal FLCs of every patient behave differently in different cellular lines and demonstrate that cells other than epithelial cells are able to process paraproteins. We could show that FLCs distributes in differently sized intracellular vesicles: the FLCs colocalized with Lamp1 (Lisosomal associated membrane protein) and c-Src , a proto-oncogene that belongs to a family of non-receptor tyrosine kinases involved in the Nf-KB activation pathway.

Calpains participate in nerve terminal degeneration induced by spider and snake presynaptic neurotoxins

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Alpha-latrotoxin and snake presynaptic phospholipases A2 neurotoxins affect the neuromuscular junction causing paralysis. These neurotoxins have different biochemical activities, but similarly alter the presynaptic membrane permeability causing Ca2+ overload within the nerve terminals, which in turn induces nerve degeneration. In this study we show that the calcium-activated proteases calpains are involved in the cytoskeletal rearrangements which we have previously observed in cultured neurons exposed to α-latrotoxin and to snake presynaptic phospholipases A2 neurotoxins. Our results indicate that calpains, activated by the massive calcium influx from the extracellular medium, target fundamental components of neuronal cytoskeleton such as spectrin and neurofilaments with ensuing nerve terminal degeneration.

ER exit sites exist independently of the Golgi in the highly reduced parasitic protozoan *Giardia lamblia*

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ER exit sites (ERESs) and Golgi are tightly-associated hallmark structures of the eukaryotic secretory pathway. In organisms with multiple dispersed ministacks, ERESs are in close proximity to *cis*-Golgi cisternae, whereas in mammalian cells the vesiculo-tubular compartment serves as an interface between ERESs and the single perinuclear Golgi complex. In the highly-reduced parasitic protozoan *Giardia lamblia*, a classical Golgi apparatus was lost due to secondary reduction. However, Golgi analogues termed encystation specific vesicles (ESVs) are generated de novo in a stage-specific manner when trophozoites differentiate to cysts.
In this work we ask the question whether ERESs are maintained as distinct ER subdomains in *G. lamblia*, despite the absence of a steady state Golgi. Due to its peculiar subcellular compartmentalization and inducible Golgi genesis, this organism provides us with a unique opportunity to investigate the fundamental principle of ERES to Golgi correspondence.
To approach this question we measured the localization and dynamics of conserved ERES and COPII components in *G. lamblia* cells. We identified distinct ERES domains and characterized them in terms of quantity, dynamics and activity. Furthermore, we could show that, similarly to Golgi biogenesis, ESV neogenesis is closely associated to ERESs. Furthermore, ectopic expression of non-functional Sar1GTPase variants caused ERES depletion and ESV ablation, leading to impaired parasite differentiation.
Thus, our data indicate that ERESs remain conserved as subdomains even after secondary reduction of the Golgi apparatus. Furthermore, we demonstrate how minimized eukaryotes such as *G. lamblia* can be successfully employed to tackle questions pertaining to fundamental cellular mechanisms such as protein export from the ER.

COPII vesicles are not transport carriers

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Lipids, secretory and most of the membrane proteins are synthesized in the endoplasmic reticulum (ER) and then are transported from the ER to the Golgi apparatus. Mechanisms of the ER-Golgi transport remains under a hot discussion. Until now, ER- Golgi transport is considered as executed by COPII-dependent vesicles.
Here, we found that at the steady state, within ER exit sites, COPII-coated buds and vesicles present were depleted of procollagen-I (PCI), albumin, and G protein of the vesicular stomatitis virus (VSVG), whereas COPI-coated buds and vesicles visible there were depleted of ER resident proteins. PCI aggregates and chylomicrons, both of which are larger than typical COPII vesicles, did not co-localize with COPII proteins. The ER protrusions containing chylomicrons in hepatocytes and similar protrusions filled with PCI in fibroblasts were not coated and never appeared as being isolated. Inhibition of membrane fusion did not induce accumulation of COPII vesicles near ERES suggesting against their role as carriers. Under these conditions, VSVG and PCI but not albumin exited from ER. Transient connections between consecutive compartments and the bolus-like mechanism of transport exist within ER-Golgi interface. After depletion of Sar1A and B with the help of siRNA interference, the cells remain viable. In these cells, the Golgi apparatus is fragmented, and Golgi stacks did not contain the cis- and trans-most cisternae. The ER exit sites were hypertrophied. COPII-coated buds were not visible on the ER. Transport of VSVG and albumin, the well-established cargo proteins is almost normal whereas exit of procollagen I from the ER was blocked. The sensitivity of cells to brefeldin A was not changed significantly.
These data argue against the role of COPII vesicles as anterograde carriers and the existence of COPII-dependent mega-vesicles and evidence in favor of the kiss-and-run model of ER-Golgi transport.

Syk kinase activity regulates the coupling of ubiquitinated FcεRI to the endocytic pathway

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Syk plays a central role in signal propagation upon aggregation of the high affinity IgE receptor (FcεRI) on mast cells and basophils, and it is also required for ubiquitination and down-regulation of engaged FcεRI complexes. However, the Syk substrates that regulate FcεRI endocytic traffiking are not understood.
By siRNA-based approaches combined with FACS and microscopic analysis, we initially demonstrated, on the rat mast cell line RBL-2H3, that Syk depletion dramatically affects the initial step of FcεRI internalization and prevents the delivery of engaged receptors to lysosomes. We then concentrated our attention on the ubiquitin-binding protein Hrs as potential Syk substrate, since its serves as critical regulator for FcεRI entry into lysosomes. Using biochemical approaches, we showed that Hrs undergoes antigen-dependent tyrosine phosphorylation and ubiquitination, and we identified Syk as the kinase responsible for Hrs phosphorylation. Syk was also required for Hrs ubiquitination catalyzed by c-Cbl E3 ligase.
Finally, we demonstrated that Syk-dependent regulation of Hrs modifications, without affecting protein stability, controlled Hrs localization: the majority of phosphorylated Hrs forms are observed only in membrane compartments, whereas ubiquitinated Hrs are predominantly cytosolic, suggesting that both modifications might impact on Hrs function.
In summary, we provided evidence that Syk, commonly known as a positive regulator of FcεRI-mediated signal transduction, is also capable of limiting FcεRI-triggered signals by regulating the endocytosis of ubiquitinated receptors. Syk-dependent Hrs phosphorylation and ubiquitination is required to drive FcεRI endocytosis, thus targeting activated receptors to lysosomes for degradation.

Monitoring differential UPR activation by over-expression of ER client proteins

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The unfolded protein response (UPR) not only allows cells to cope with acute stresses that provoke protein misfolding in the ER lumen, but also governs ER expansion when precursor cells differentiate into professional secretory cells, such as during B to plasma cell differentiation. Remarkably, these differentiation programmes can activate the IRE1, PERK and ATF6 branches of the UPR independently from one another. So far, the complexities of UPR signal integration have been little appreciated, because most studies employed drugs that induce generic ER stress, resulting in a monolithic UPR.
We recently established that activation of the IRE1α branch entails its oligomerization into distinct foci at the ER membrane. To study UPR activation in a more physiological context we have developed a lentivirus-based protocol to visualize foci formation of fluorescently-tagged UPR reporters in live cells. By this method, we targeted a wide variety of cells, including those most refractory to conventional transfection protocols. Key to the usefulness of the fluorescent IRE1 reporter was that we fine-tuned its expression to near-endogenous levels via the doxicyclin inducible promoter.
Immunoglobulin μ heavy chain (Ig μ) cannot assemble into oligomeric antibody in the absence of light chain and, consequently, is retained in the ER. We report that over-expression of Ig μ drives IRE1 activation. Surprisingly, a mutant version of Ig μ lacking the first constant domain (Ig μ?CH1) failed to do so. Ig μ?CH1 aggregates into Russell bodies that are often found in aberrant plasma cells in multiple myeloma and autoimmune disease. Our data highlight the crucial role for the CH1 domain in Ig quality control and give the first description of UPR activation driven by an ER client protein alone. Moreover, we posit that escape from UPR recognition of misfolded ER client proteins, which eventually accumulate either in aggregates or expanded ER cisternae, may be a common theme in ER storage diseases.

Rett Syndrome and FOXG1 subcellular localization

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The Forkead Box G1 (FOXG1) gene encodes for a DNA–binding transcription factor, essential for the development of the telencephalon in embryonic mammalian forebrain, its main role being the maintainance of the appropriate cell cycle kinetics in progenitor cells. FOXG1 sequence contains three know domains: a Forkhead Domain (FHD), a GROUCHO-TLE Binding domain (GTB) and a JARID1B Binding Domain (JBD). Mutations in the autosomic gene FOXG1 were recently implicated in the onset of Rett Syndrome (RS), a neurodevelopmental disorder that is one of the most common causes of mental retardation in girls, for which sequence alterations of the X–linked genes MECP2 and CDKL5 have long been known.
The FoxG1-GFP fusion showed an unexpected and intriguing subcellular localization.

ER-derived cytoplasmic inclusions generated by mutant, Amyotrophic Lateral Sclerosis-associated VAPB are cleared by the proteasome

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VAPB (Vesicle-Associated Membrane Protein-Associated Protein B) is a ubiquitously expressed, ER-resident tail-anchored protein that functions as adaptor for lipid-exchange proteins. Its mutant form, P56S-VAPB, is linked to a dominantly inherited form of Amyotrophic Lateral Sclerosis (ALS8). P56S-VAPB forms intracellular inclusions, whose role in ALS pathogenesis has not yet been elucidated. We recently clarified the ultrastructure of those inclusions and their precise relationship with the ER, demonstrating that they are formed by a profound remodeling of ER membranes with pairs of ER cisternae separated by a thin layer of electron-dense cytosol. Here, we used stable HeLa-TetOff cell lines inducibly expressing wild type (wt) and P56S-VAPB to investigate the dynamics of inclusion generation and degradation. Shortly after synthesis, the mutant protein forms small, polyubiquitinated clusters, which then concentrate in large juxtanuclear structures independently from the integrity of the microtubule cytoskeleton. The rate of degradation of the aggregated mutant is higher than that of the wt protein and the inclusions are cleared only a few hours after cessation of P56S-VAPB synthesis. At variance with other inclusion bodies linked to neurodegenerative diseases, clearance of P56S-VAPB inclusions involves the proteasome, with no apparent participation of macro-autophagy. Moreover, we investigated a possible involvement of the p97/VCP AAA-ATPase in the extraction of poly-ubiquitinated P56S-VAPB from the ER membrane. Transfection of a dominant-negative p97 mutant stabilizes P56S-VAPB, indicating that it is degraded by the ER-Associated Degradation (ERAD) pathway. Our results suggest that the slow onset of P56S-linked familial ALS is not a consequence of the progressive accumulation of the mutant protein over time and may be related to a reduction of the intracellular levels of wt VAPB.

Knockdown of BBS10 in renal cells affects apical targeting of AQP2: a possible explanation for the polyuria associated with Bardet-Biedl Syndrome

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Bardet-Biedl syndrome (BBS) is a autosomal-recessive ciliopathy characterized by defects in multiple organ systems. The clinical phenotype consists of major features that are considered the hallmarks of the disorder: retinal degeneration, obesity, hypogonadism, polydactyly, mental retardation, and renal dysfunction. In particular, polyuria and polydipsia, with impairment of renal concentration capacity, are the earliest signs of renal dysfunction.
Multiple lines of evidence have indicated that the BBS phenotype is largely a consequence of ciliary dysfunction since most BBS proteins localize to the basal body and the ciliary axoneme. However, recent evidences revealed that BBS proteins might also be involved in non-ciliary-related microtubule-based transport.
Among the 14 identified genes (*BBS1-14*), mutated in BBS patients, *BBS10* alone contribute approximately 20% of all known mutations.
In order to investigate whether the polyuria associated with BBS might be related to a defect in the shuttling of the water channel AQP2 in the kidney collecting duct, we studied the effect of selective *BBS10* knockdown in AQP2-expressing renal cells.
Interestingly, apical surface biotinylation indicated that *BBS10* siRNA dramatically and specifically prevented the forskolin-induced exocytosis of AQP2 at the apical membrane.
In the same experimental condition, immunofluorescence followed by confocal analysis showed that *BBS10* silencing strongly affected the organization of the microtubules cytoskeleton within the cell. As a consequence, we observed that, upon FK, treatment AQP2 mostly redistributed to the basolateral membrane with negligible increase at the apical membrane.
Taken together, these results suggest that lost of proper microtubule-based polarized transport in the collecting duct cells cause basolateral misrouting of AQP2 and might explain the polyuria associated with mutations of *BBS10* causing BBS.

A novel regulatory mechanism of AQP2 trafficking to explain the syndrome of inappropriate antidiuresis

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Kidney water reabsorption is regulated by the hormone vasopressin (AVP) which binds to its V2 receptors causing activation of protein kinase A (PKA), phosphorylation of AQP2 at serine 256 (pS256) and AQP2 translocation to the plasma membrane. Besides S256, AVP action coincides with dephosphorylation of S261. Bioinformatical analysis suggests that cdk1/5 may phosphorylate S261. In ex-vivo kidney slices and MDCK-AQP2 cells, the cdk1/5 inhibitor roscovitine, increased pS256 and decreased pS261 resulting in AQP2 translocation to the apical membrane, independently of PKA activation.
Since the phospho-status of a protein is dynamically controlled by protein kinases and counteracting phosphatases, the activity of phosphatase 1 (PP1) was evaluated. Compared to control condition roscovitine treatment caused a reduction of PP1 activity which has been demonstrated to be regulated by DARPP32. DARPP32 is a bifunctional signal transduction molecule integrating the activities of PP1, cdks and PKA through the phosphorylation of Thr34 and Thr75, respectively. Interestingly, incubation with roscovitine increased pThr34, and reduced pThr75 independently of forskolin stimulation. These data indicate that PP1 inhibition, via the cdks-DARPP32 signaling, may represent a novel pathway for increasing AQP2 abundance to the apical plasma membrane independently of cAMP.
Of note, in a mouse model of inappropriate antidiuresis (Pkd1+/-) characterized by increased cell surface expression of pS256-AQP2 despite unchanged level of cAMP, AQP2 and V2R, we found a significant reduction of pS261 paralleled by an increase in pS256. Furthermore, in Pkd1+/- kidneys the phosphorylation level of DARPP32 at Thr34 increased.
To conclude we have identified a novel regulatory mechanism of AQP2 trafficking that might explain the molecular bases of several disorders of inappropriate antidiuresis characterized by water retention such as, congestive heart failure and liver cirrhosis.

Sedlin binds Sar1p and controls type II procollagen trafficking by modulating the cycle of COPII

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Genetic defects of sedlin, a conserved component of TRAPP complex, cause spondyloepiphyseal dysplasia tarda (SEDT), a condition characterised by impaired chondrogenesis that results in short stature, flattening of the vertebrae, and premature osteoarthritis. Prompted by the consideration that sedlin is ubiquitously expressed but that sedlin mutations cause cartilaginous-restricted dysfunctions, we hypothesized that sedlin should exert a role in the transport of chondrocyte-specific cargoes, such as type II procollagen (PCII). This hypothesis was reinforced by the fact that certain mutations in PC II give rise to autosomal dominant forms of spondyloepiphiseal dysplasia. We tested this hypothesis by analyzing the involvement of sedlin in the transport of different classes of secretory cargoes and found that sedlin is selectively required for PCII to exit the ER, while it is not essential for ER exit of small soluble and membrane-associated cargoes. We have also identified the molecular mechanism underlying this role of sedlin in its ability to bind the GTPase Sar1 and to control the membrane–cytosol cycle of Sar1 itself and of the COPII coat complex at the level of the ER exit sites. Sedlin depletion and/or mutation in SEDT patients slower the cycle of Sar1 and prolong the membrane association of Sar1-GTP at the ER exit sites, thud inducing constriction and premature fission of nascent carriers which fail to incorporate the large PC protofibrils but are still competent for smaller secretory cargoes.

ABSTRACTS

Poster Presentations

in alphabetical order
(presenting authors are shown underlined)

Characterization of RTN3, a Novel Candidate Involved in Non-Clathrin Endocytosis of the EGFR

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Epidermal Growth Factor Receptor (EGFR) can be internalized through different pathways depending on ligand concentration. At high EGF concentrations (20-100 ng/ml), a fraction of the receptor becomes ubiquitinated and is internalized through non-clathrin endocytosis (NCE). As opposite to clathrin-mediated endocytosis (CME), that targets EGFR to signaling and recycling (already active at low EGF), NCE commits the majority of the receptors to degradation. Integration between CME and NCE is therefore crucial to determine EGFR signal intensity.
The molecular mechanisms involved in NCE of the EGFR are mainly unknown. In order to identify the molecular components of NCE, we have previously employed a large-scale proteomic approach and RNA interference-based validation studies, that provided us with a list of potential molecular components of NCE. From these initial experiments, we have preliminary data indicating that reticulon-3 (RTN3) exerts a critical functional role in NCE, but is not involved in CME. Reticulons mainly localize at the ER membrane and are involved in ER tubulation pathway. However, they have also been shown to be localized at the PM and to share several properties with other endocytic proteins, such as caveolins and flotillins. We are currently studying the impact of RTN3 on EGFR fate and downstream signaling response. In parallel, we are also characterizing proteins with no functional role in NCE, which might represent EGFR co-trafficking cargoes and novel markers of the pathway.

Evidence of ligand selective recruitment of β-arrestin2 in a G-protein coupled receptor

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Oxytocin receptor (OTR) is a G-protein coupled receptor (GPCR) that undergoes desensitization and internalization upon agonist stimulation. Agonist-induced internalization is due to receptor phophorylation and recruitment of a member of the β-arrestin family. In particular, β-arrestin1 and β-arrestin2 are ubiquitously expressed and target most GPCRs for endocytosis. Our previous data indicated that the stable OTR/β-arrestin2 interaction plays an important role in determining the rate of recycling of human OTRs, but does not determine the fate of endocytosed receptors (Conti et al AJP-EM 2009). To further characterize the role played by β-arrestins in intracellular receptor trafficking, we investigated the OTR-induced recruitment of β-arrestin1 and β-arrestin2 by means of a BRET-based assay. Our data indicate that the OTR is able to recruit both proteins although with different kinetics (Busnelli 2012 JBC). However, a selective recruitment of β-arrestin2 was observed in presence of a particular peptidic ligand which differs from oxytocin by a single aminoacidic substitution. A fluorescent derivative of this analog was also demonstrated to recruit β-arrestin2 but not β-arrestin1 by *in vivo* confocal experiments. These data indicate that selective agonist-induced receptor conformations underly β-arrestin1 and β-arrestin-2 recruitment. To gain further insight into the molecular determinants of selective β-arrestin1 and β-arrestin2 recruitment, we are now investigating receptor variants in the serine clusters located in the C-terminal-tail of the OTR. Furthermore, as two independent OTR genes coding for two receptors with very diverse C-terminal ends have been recently identified in Zebrafish, we are currently characterizing their β-arrestin recruitment and internalization properties.

NKCC2 trafficking and activity: the role of interacting proteins

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The Na+-K+-2Cl- cotrasporter type 2 (NKCC2) is exclusively expressed in the apical membrane of the thick ascending limb (TAL) in the kidney where is involved in the vectorial transepithelial salt reabsorption. We focused our research on the identification of NKCC2-interacting proteins possibly involved in the trafficking of NKCC2. Using NKCC2 stably transfected LLC-PK1 cells we found that NKCC2 functionally interacts at the apical membrane with MAL/VIP17, a member of the tetraspan family of proteins. We demonstrated that this interaction stabilizes NKCC2 at the apical membrane by inhibiting its constitutive endocytosis and increasing its susceptibility to be activated (Carmosino et al., 2011). In addition we identified moesin, a protein belonging to ERM family, as an NKCC2-interacting regulatory protein and as a crucial player in the insertion of NKCC2 in the apical membrane.
It has been reported that perturbation of NKCC2 membrane expression and activity might have a crucial role in the onset and maintenance of Na+-sensitive hypertension. Indeed, we tried to identify the NKCC2 regulatory partners in spontaneously hypertensive rats (SHR) to shed light on the role of NKCC2 in the pathogenesis of hypertension. Using a proteomic approach we found that the NKCC2-containing macromolecular complexes in SHR rat TAL membrane preparations were different from that observed in age-matched control animals suggesting a different supramolecular arrangement of NKCC2 in the membranes of SHR rat kidneys.
Taken together, these findings highlight the importance of investigating the dynamics of NKCC2 membrane expression in order to gain insights into the regulation of Na+ and Cl- NKCC2 mediated absorption in the kidney in physiopathological conditons.

Regulation of intracellular targeting of tail-anchored proteins

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A group of membrane proteins called tail-anchored (TA) have a transmembrane domain near the C-terminus and an N-terminal cytosolic moiety; because of this topology they do not have access to the SRP-mediated co-translational insertion pathway, and must be inserted into their target membranes (ER and outer mitochondrial membrane (MOM)) posttranslationally. Recently, a novel chaperone, TRC40 (Get3 in yeast) has been implicated in ER targeting of TA proteins, however, not all ER-targeted TA proteins depend on this chaperone.
To investigate how discrimination between the MOM and ER occurs, we used mammalian cytochrome b5, a TA protein existing in two, MOM or ER localized, versions (b5 ER and b5 MOM). However, the in vivo targeting specificity is not reproduced in cell-free systems. We have therefore set up an in vivo system to investigate the mechanism underlying specific targeting within cells, consisting in microinjection into the cytoplasm of cultured cells (CV1) of the two b5 isoforms, as purified recombinant proteins. 15 minutes after microinjection each isoform is correctly localized, indicating that no ribosome-associated factors are involved in the targeting. We are using this system to investigate possible factors involved in TA targeting. With different treatments, we observed two types of effect: (i) inhibition of insertion, obtained with cholesterol overloading and with the GSH oxidant diamide; (ii) mistargeting of the ER form to mitochondria, observed with Calmodulin, Hsp70 inhibitors, and with TRC40 silencing. Interestingly, none of these treatments interfere with b5 RR localization.
In conclusion, a number of treatments that affected b5-ER targeting/insertion do not affect the correct localization of b5-MOM, indicating that the two isoforms interact with different chaperones and membrane components. In the future we will investigate the role of these factors with biochemical assays.

Features of autophagy in a Niemann-Pick disease type B cell line

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Lysosomal storage disorders (LSD) are a group of more than 60 genetic diseases in which a deficiency of specific lysosomal enzymes produce an accumulation of undegraded substrates in lysosomes. Recent studies have establish that many LSD are associated with a general dysfunction of autophagy, due to damaged lysosomes. In this study we examine the induction of autophagic pathway and its involvement in lymphocytes of Niemann-Pick disease type B (NPB), a LSD caused by a lack of acid sphingomyelinase activity. By flow cytometry, western blot, fluorescence and electron microscopy we analyzed pathway of autophagy (AP) in a NPB B-Lymphocyte cell line. EBV-transformed B Lymphocytes from patients with Niemann-Pick type B and from healthy donor were treated with nocodazole (NZ) and wortmannin (WM), two inhibitors of AP, and rapamicyn (RM), an autophagic inductor. We observed an increase in Acridine Orange, Lysotracker Green and Monodansylcadaverine staining in NPB cells in respect of their normal counterparts (fold of increase: 1.7). This reflects the major number of autophagic vacuoles (AVs), demonstrated also by TEM analysis, suggesting an imbalance between rates of AV formation and degradation ("autophagic stress"). Furthermore the lowest NAO intensity fluorescence reveals dysfunctional mitochondria in pathological cells, also enclosed into AV, as shown by TEM. However NPB lymphocytes treated by NZ and RM showed an opposite trend than that observed in normal cells for vesicular staining, confirming the impairment of autophagic process. In this work, we show evidences highlighting that AP basal level and flux are increased to the detriment of specific AP induction, furthermore lysosomal clearance is clearly impaired in NPB. We propose the existence of an imbalance among induction, flux and clearance through the autophagic pathway probably contributing to clinical picture of Niemann-Pick disease type B.

Rab7 interacts with peripherin: implications for the Charcot-Marie-Tooth type 2B neuropathy

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Charcot-Marie-Tooth type 2 (CMT2) is an axonal (non-demyelinating) hereditary peripheral neuropathy characterized by distal muscle weakness and atrophy, mild sensory loss, and normal or near-normal nerve conduction velocities.
Four missense mutations (Leu129Phe, Lys157Asn, Ans161Thr and Val162Met) that target highly conserved amino acids in Rab7, a ubiquitous small GTPase, which controls transport to endocytic degradative compartments, cause the CMT2B form of the disease.
We previously demonstrated that CMT2B-causative Rab7 mutant proteins have higher Koff for nucleotides compared to the wt protein (particularly high for GDP), and, as a consequence, lower GTPase activity. In addition, these mutant proteins are predominantly GTP-bound in cells, and are able to rescue Rab7 function when expressed in Rab7-silenced cells.
Interestingly, expression of CMT2B-causing Rab7 mutant proteins in PC12 and in Neuro2A cells lines impairs neurite outgrowth and neuronal differentiation (up-regulation of GAP43 in PC12 cells and of NeuN in Neuro2A cells).
Recently, we have identified peripherin as a new Rab7 interacting protein by the two-hybrid system, using a dorsal root ganglion (DRG) neurons cDNA library. Peripherin is a type III intermediate filament protein expressed predominantly in the peripheral nervous system. In injured nerves, a peripherin increase has been observed, suggesting that peripherin plays a role in neuronal regeneration and nerve repair. In particular, peripherin may be involved in the regrowth of axons.
The interaction was confirmed by co-immunoprecipitation and pull-down, and direct interaction was demonstrated using bacterially expressed recombinant proteins. Moreover, we observed that overexpression of Rab7 wt and mutant proteins modifies peripherin assembly suggesting that alterations of the interaction between Rab7 and peripherin might be responsible for the development of the disease.

Modulation of endosomal trafficking by ErbB2 and geldanamycin

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The ErbB2 receptor is a clinically validated cancer target whose internalization and trafficking mechanisms remain poorly understood. HSP90 inhibitors and monoclonal antibodies, such as Geldanamycin (GA) and Trastuzumab (Herceptin), have been developed to target the receptor for degradation or to modulate downstream signaling. Despite intense investigations, the entry route and post-endocytic sorting of ErbB2 upon GA stimulation have remained controversial. We report that ErbB2 levels directly impact on clathrin-mediated endocytosis (CME) capacity so that the low ErbB2 internalization rate observed in SK-BR-3 is due to the receptor overexpression. In addition, we have examined ErbB2 internalization and trafficking stimulated by GA by light and (3D) electron microscopy. We show that ErbB2 internalization occurs via clathrin-mediated endocytosis (CME), and that GA is profoundly affecting early/recycling endosome (EE/RE) morphology and function, perturbing protein trafficking irrespective of HSP90 interaction. Indeed, EE/RE assume aberrant tubular/elongated morphology, suggesting that GA exerts widespread effects on endosomal sorting and organization.

Defining the cellular and molecular basis of Lowe syndrome for the identification and validation of targets for pharmacological intervention

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Lowe syndrome is a X-linked disorder characterized by congenital cataracts, mental retardation, and renal ion and low-molecular-weight protein transport defects (Fanconi's syndrome). There is no specific therapy available.
Lowe syndrome is caused by mutations in ocrl1, a gene encoding for OCRL, a phosphatidylinositol 4,5-bisphosphate 5-phosphatase that associates with the endosomal compartment and the Golgi complex. Although we still have no complete understanding of the role of OCRL, it is likely to be a key component in sorting receptors that traffic through the early endosomes. To note, knock-out of OCRL induces the peripheral redistribution of the mannose-6-phosphate receptor (MPR) and impairs the internalisation of ligands like transferrin and RAP (a specific ligand for LDLR-related proteins, including megalin, the multiligand receptor responsible for protein reabsorption by kidney proximal tubule cells).
We exploited the endocytic defects induced by OCRL knock-down (KD) (i.e. MPR redistribution) to set up a "phenotypic" cell- and microscope-based assay suitable for high content screen (HCS) of small molecules to search for correctors of the MPR phenotype. These correctors represent potential drug targets for Lowe syndrome. We screened small molecules from the LOPAC collection of Sigma (an annotated library of 1280 Pharmacologically-Active Compounds) in a stable OCRL-KD-inducible HeLa cell line. The screen was performed in a 96-well format incubating OCRL KD cells with 10μM of each compound for 16 h. Automated image analysis (performed by the Olympus ScanR screening platform) have revealed 51 positive hits that will be evaluated for biological relevance and confirmed as bona fide hits by the secondary screen.

Biogenesis of mitochondrial carrier proteins: role of the chaperones Hsc70 and Hsc90

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Metabolite carrier proteins carry targeting signals to mitochondria in their transmembrane domains. In addition, some carrier proteins possess cleavable presequences which are dispensable for mitochodrial targeting, but have some other function before import. The cytosolic chaperones Hsc70 (heat-shock cognate 70) and Hsp90 (heat-shock protein 90) bind to carrier precursors and interact specifically with the Tom (translocase of the mitochondrial outer membrane) 70 import receptor to promote import.
In this study, we examined the interactions of the mature and precursor forms of PiC (phosphate carrier) and CIC (citrate carrier), and for comparison, of OGC (oxoglutarate carrier), which naturally lacks a presequence, with the cytosolic chaperones. The binding of each protein to Hsc70 and Hsp90 was tested in relation to solubility before import. The function of chaperones in import was analysed by inhibition of Hsc70 and by competition of Tom70 targeting using an Hsp90 fragment. We found that the presequences of PiC and CIC improve import competence by different mechanisms, as PiC provides a binding site for a particular chaperone, Hsc70, and CIC reduces the aggregation of the polypeptide independent of any external chaperone activity.

LIN7 regulates the filopodia and neurite promoting activity of IRSp53

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The insulin receptor substrate protein of 53 kDa (IRSp53) is critically involved in the formation of filopodia and neurites through mechanisms that have only in part been clarified. Here, we investigated the role of the small scaffold protein LIN7, an interactor of IRSp53. We found that the formation of actin-filled filopodia and neurites depends on motifs mediating the LIN7:IRSp53 association and filopodia tip localization. We further showed that coexpression of LIN7 with IRSp53 enhanced the formation of filopodia protrusions in neuronal NSC34 cells. On the contrary, LIN7 silencing prevented the extension of both filopodia and neurites, induced by ectopic expression of IRSp53 or by serum starvation, respectively in undifferentiated and differentiated neuronal N2A cells. The expression of full length IRSp53 ?or LIN7 mutants lacking its domains for association with IRSp53 (PDZ domain) and plasma membrane protein complexes (L27 domain) was unable to restore neuritogenesis in LIN7 silenced cells. Conversely, defective neuritogenesis could be rescued by the expression of RNAi-resistant full length LIN7 or chimeric L27-IRSp53 fusion mutant. Finally, LIN7 silencing prevented the recruitment of IRSp53 into Triton X-100 insoluble complexes, otherwise occurring in differentiated cells. Collectively these data indicate that LIN7 is a novel regulator of IRSp53, and that their association is required to promote the formation of actin-dependent filopodia and neurites.

Mechanisms of transmembrane domain-dependent transport of membrane proteins at the ER-Golgi interface

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Even though cargoes are usually recruited to ER Exit Sites (ERES) by a sequence-dependent mechanism, it is known that other factors contribute to protein export from the ER. By using two fluorescent tail-anchored proteins differing only for the length/hydrophobicity of the TMD and without any export signal, our group demonstrated that the longer TMD (FP22) is enriched at the ERES, travels through the secretory pathway and finally reaches the plasma membrane, while the short TMD (FP17) is retained within the ER because of its exclusion from ERES. In order to clarify the molecular mechanism underlying this TMD-dependent transport, we applied a cDNA microinjection protocol combined with live cell imaging, and compared the transport of an export signal-bearing (VSV-G) with FP22. FP22 and VSVG are recruited together to ERES, but VSVG reaches the plasma membrane more rapidly than FP22. . After an incubation at 20°C, VSV-G accumulates at the Golgi apparatus, while FP22 remains distributed between the ER and the Golgi, suggesting that it may be recycling between the two compartments. In agreement, we observed a fluorescence recovery of the Golgi after FP22 bleaching at 20°C (half time of 3 min), despite the fact that FP22's fluorescence at the Golgi is stable before bleaching. After repeated bleaching of the Golgi, a slow ER emptying and a partial recovery of the Golgi fluorescence after each bleaching has been observed. Taken together, these data suggest that the absence of an export signal determines an inefficient transport because of the inclusion into futile cycles between the Golgi to the ER so that only a small fraction of FP22 within the Golgi can escape towards the plasma membrane.
In order to generalize this mechanism, we plan to study a signal-deleted mutant form of VSV-G, which is still able to travel through the secretory pathway thanks to the physicochemical features of its TMD similar to those of FP22.

Novel signaling pathways linking chemokine stimulation to the modulation of integrin function in extravasating leukocytes

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The multi-step leukocyte extravasation process is governed by adhesion molecules and chemotactic factors dynamically interplaying in the presence of shear forces. Responsiveness to chemotactic ligands is mediated by G protein-coupled receptors (GPCRs) which are regulated by beta-arrestin. On this basis, we investigated the physiological role of ARRB2 in chemokine-driven dynamics associated with leukocyte extravasation, with special interest to the activation of the Rap1 small GTPase, recently emerged as master regulator of integrin function. The analysis of KC (Keratinocyte-derived Chemokine) induced Rap1 activation profile in RBL (Rat Basophilic Leukemia) cells expressing mCXCR2 shows a bimodal kinetic, with the first peak at 30" and the second at 5'. RNA interference-mediated depletion of ARRB2 specifically inhibits Rap1 activation. Rap1-GTP formation is catalyzed by guanine nucleotide exchange factors (GEFs). We showed that KC stimulates the rapid tyrosine phosphorilation of endogenous C3G and that knockdown of C3G by siRNA inhibits the KC-dependent Rap1-GTP formation. Finally, to examine the physiological contribution of the beta-arrestin 2 dependent axis to leukocyte extravasation, we used murine neutrophils differentiated ex vivo from immortalized myeloid progenitors. In agreement with previous data, neutrophils depleted of beta-arrestin 2 showed impaired adhesion strengthening to TNF-alpha-activated endothelial cells in in vitro adhesion assays under flow. In the same context, we are currently investigating the functional requirement of signaling intermediates involved in Rap-1 activation in leukocyte firm arrest and transendothelial migration. Together, these results provide a model whereby ARRB2, switching from G-protein dependent to independent signaling regulates Rap1 activity during leukocyte extravasation.

Intercellular communication: a new role for Fas signalling

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In a multicellular organism, biological functions are performed by complex groups of cells, whose actions must be coordinated by intercellular communication. To this regard, the exchange of signals is usually ascribed to specific molecules (soluble or immobilized) and their corresponding cognate receptors, and may require a direct cell-to-cell contact or gradients formed by soluble (paracrine) mediators. Recently, other different mechanisms for the exchange of molecular information between cells have been documented, such as the exchange of membrane fragments, formation of tunneling nanotubes (TNTs) and the release of microvesicles (MVs). In our investigation, CD4+T cells were stained with different dyes (PKH67, CFSE and DiI), and treated with FasL at the final concentration of 0.5 μg/ml for the following times:30 min, 60 min, 120 min. Our approach, combining flow cytometric (FC) with confocal microscopy (CM) analyses, highlights a social dimension for Fas/FasL interactions between CD4+T cells. Indeed, we show that FasL treatment promote intercellular communication in CD4+ lymphocytes in different ways. In particular, we found an exchange of cytosolic material (CFSE, DiI) at the early stage of treatment, mainly through TNT and MVs formation, while the exchange of membrane fragments (PKH) was rather poor. Furthermore Fas stimulation strongly increased the release of MVs and exosomes from stimulated T cells, successively uptaken by unstimulated ones. These data were achieved by FC procedures of absolute counting and CD63 labelling of small particules (≤1 μm). Pharmacological agents as latrunculin and nocodazole interfere with cytoskeleton elements, and strongly affected the CFSE and DiI transfer: this may be due to their action on both TNT formation and MVs release. In conclusion, our data suggest a new role for Fas signalling linking the apoptotic pathway with intercellular communication, also suggesting a phenomenon identified as transcellular apoptosis.

Tetanus and Botulinum Neurotoxins need two feet to jump into neurons

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Tetanus and botulinum neurotoxins are di?chain proteins that cause neuroparalysis by inhibiting neuroexocytosis. These neurotoxins enter into nerve terminals via endocytosis inside synaptic vesicles, whose acidic pH induces a structural change of the neurotoxin molecule that becomes capable of translocating its L chain (LC) into the cytosol, via a transmembrane protein?conducting channel made by the H chain (HC). This is the least understood step of the intoxication process primarily because it takes place inside vesicles within the cytosol. We developed an assay to render this passage accessible to investigation by making it to occur at the surface of neurons. The neurotoxin, bound to the plasma membrane in the cold, is exposed to a warm low pH extracellular medium and the entry of the L chain is monitored by measuring its specific metalloprotease activity with a ratiometric method. The pH dependence of the conformational change of tetanus neurotoxin and botulinum neurotoxins type B, C and D is similar and takes place in the same slightly acidic range, which comprises that present inside synaptic vesicles. Based on these experimental evidences and bioinformatic analysis deriving from the alignment of all available neurotoxins sequences, we have speculated that this conformational change could be driven by some aminoacids which are conserved in strategic portion of the tridimensional structure. Thus, we decided to construct some mutants to challenge with our cell based assay and to determine whether those aminoacids are crucial for toxin structural change and membrane insertion.

Intracellular trafficking of AQP2 in renal cells: from physiology to pathology

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The water channel Aquaporin 2 (AQP2) is responsible for the vasopressin (VP)-dependent water reabsorption occurring in the kidney during antidiuresis.
X-linked nephrogenic diabetes insipidus (XNDI), a severe rare disease characterized by impaired urine-concentrating ability of the kidney, is caused by inactivating mutations in the V2 type VP receptor (V2R) gene. Mutation prevents the VP-induced shuttling of AQP2 from intracellular storage vesicles to the apical plasma membrane of kidney collecting duct principal cells. This, in turn, dramatically reduces water reabsorption resulting in severe polyuria and constant risk of dehydration. Unfortunately, the current pharmacological approach for handling XNDI is unable to rescue AQP2 membrane expression.
We have previously reported that the cholesterol-lowering drug lovastatin increases AQP2 membrane expression in renal cells in vitro.
More recently we reported that, in mice, fluvastatin increases AQP2 membrane expression in the collecting duct in a VP-independent fashion and greatly increases the amount of water reabsorbed in the kidney.
Additional experiments in vitro, performed on a cell culture model recapitulating AQP2 trafficking, indicate that this effect of fluvastatin is most likely caused by the statin-dependent inhibition of protein prenylation of key regulators of AQP2 trafficking in collecting duct cells. We identified members of the Rho and Rab families of proteins as possible key players whose reduced prenylation might result in the accumulation of AQP2 at the plasma membrane, by modulating the basal rate of exocytosis and/or endocytosis.
Most importantly, preliminary results obtained using the conditional mouse model of human XNDI, characterized by severe polyuria and low urine osmolality, indicate that fluvastatin treatment significantly reduces diuresis and increases urine osmolality.
Taken together, these results strongly suggest that statins may prove useful in the therapy of XNDI.

Kinetic protein retention in the early secretory pathway

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ERp44 is a multifunctional chaperone of the PDI family, primarily localized in the ERGIC and cisGolgi. It binds client proteins (Ero1alpha, IgM, SUMF1, Adiponectin) non-covalently and covalently, via cysteine 29. It operates in a pH dependent manner, binding much better its clients at pH 6.5 (as in the cis-Golgi) than at 7.1 (as in the ER). The crystal structure of ERp44 revealed a clover structure composed of 3 thioredoxin-like domains and a C tail, whose opening is modulated by pH. In the cis-Golgi, C-tail opening likely exposes both the active site and RDEL motif, allowing KDELR to retrieve ERp44-client complexes into the ER. Increasing the cisGolgi pH by silencing of Golgi pH regulator (GPHR) specifically weakens the retention of ERp44 clients (submitted).
Analyses of many deletion or replacement mutants suggest that pH sensing depends on the coordinate activity of numerous key residues. Amongst these, a highly conserved, unstructured and histidine-rich region plays an important role in ERp44 function. Deleting the entire loop (ΔHis) or mutating histidines 332-333 increases retention efficiency and abolishes pH sensitivity. Surprisingly, these hyperactive mutants form fewer covalent bonds with client proteins at steady state. In contrast, mutants lacking the whole tail (ΔTail) or part of it (Δß16) bind Ero1alpha with much greater affinity, but are less efficient in retaining it. This reverse correlation implies that disulfide bonds with clients need be continuously formed and reduced to exert full activity. When overexpressed, the tail mutants (ΔTail and Δß16) accumulate in the ER; in contrast, the hyperactive ΔHis and H332-333A mutants are found primarily in ERGIC and cisGolgi. The His-rich region might interact with ER resident proteins. Taken together, our findings suggest a kinetic model for retention by ERp44, whose stringency, localization and possibly signaling properties can be modulated during cell stress or differentiation.

Two internal di-leucine motifs drive the intracellular trafficking of Ring Finger Protein 11 (RNF11)

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RNF11 is a small E3-ligase (154aa) that has been reported to be overexpressed in several tumor tissues. The mature protein is anchored, via a double acylation, to the membranes of early and recycling endosomes and is subjected to several post-translational modifications, among them ubiquitinations and phosphorylations. Even though the membrane anchoring mechanism has been clearly described, the intracellular trafficking of RNF11 and the subcellular targeting signals responsible for its localization remain to be clearly elucidated. In this study, we describe the presence of two acidic cluster/di-leucine (Ac-LL) motifs, respectively mapping at the N-terminal (aa 11-DDISLL-16) and at the C-terminal end (aa 495-DDISLL-500). Our data provide evidences that both motifs are involved in the interaction with the VHS domains of GGAs. Accordingly, mutating the LL to di-alanine (L15,16/AA) causes the mislocalization of RNF11 which mainly accumulates at the plasma membrane, while the C-terminal Ac-LL mutant is partially missorted and predominantly clustered in peripheral sorting vesicles. Our study also show that RNF11 and GGAs, when overexpressed in HeLa cells, colocalize mainly in peripheral vesicles which are close to the plasma membrane, suggesting that GGAs could act as regulators of the RNF11 membrane trafficking both at the trans-Golgi Network (TGN), where the newly synthesized protein is packaged in vesicles directed to the endosome compartment, and also along a more peripherical route involving the plasma membrane and the early and recycling compartments. By biochemical and immunofluorescence methods, we demonstrate that RNF11 is the first GGA binding partner with E3-ligase function identified so far, and we propose a model describing the intracellular route of RNF11 as the result of the recognition of linear motifs in the amino acid sequence of RNF11, in a way that is strictly dependent on both post-translation modifications and the integrity of the RING domain.

Design and layout

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