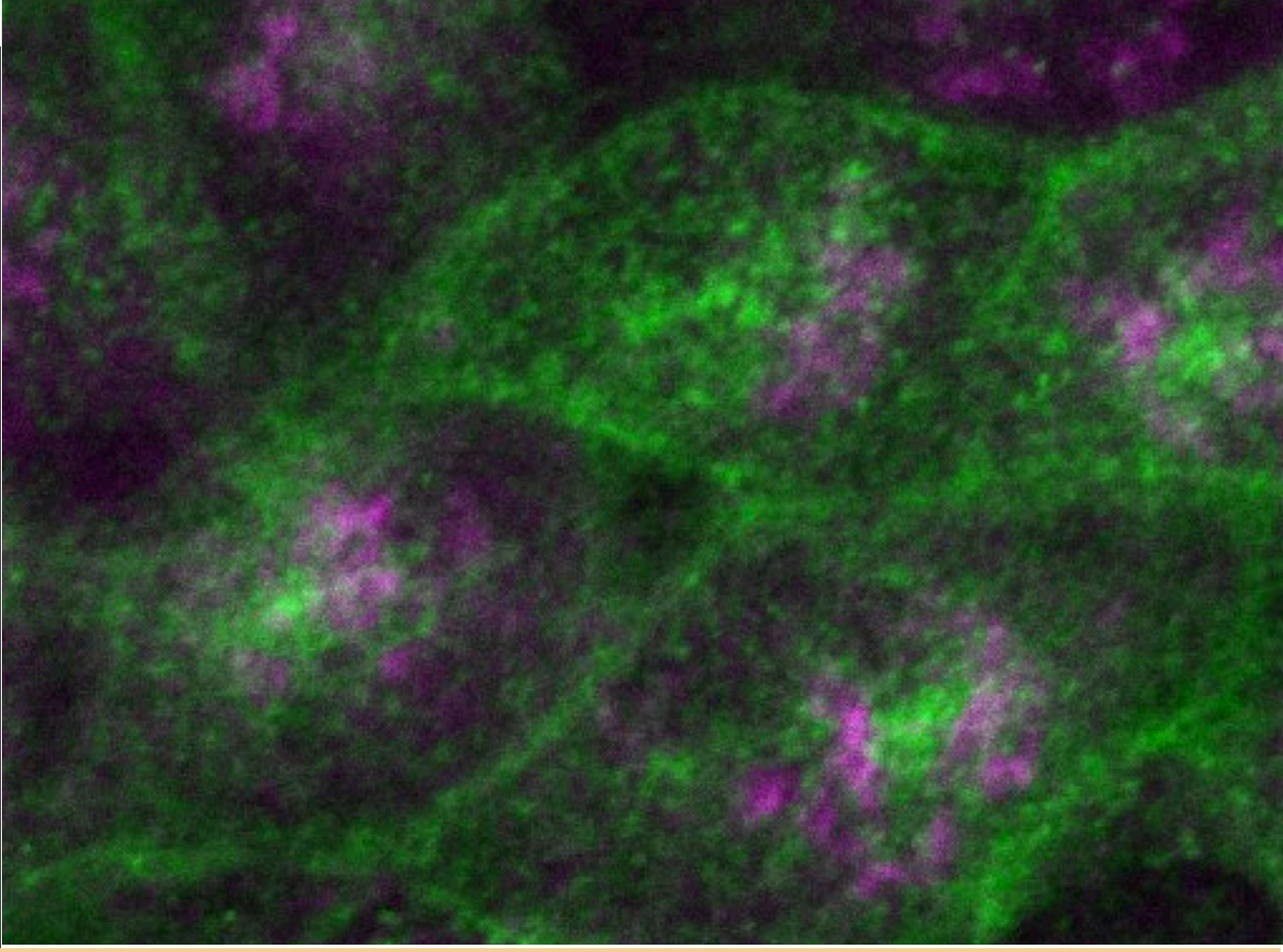

Sara Filipa Pereira Cruz

The Role of SPCA1 in the Traffic of Glycosylphosphatidylinositol-Anchored Proteins

Dissertação para a obtenção do grau de Mestre em Investigação Biomédica sob orientação científica do Doutor Henrique Manuel Paixão dos Santos Girão e co-orientação da Doutora Chiara Zurzolo e apresentada à Faculdade de Medicina da Universidade de Coimbra.

Maio 2017

UNIVERSIDADE DE COIMBRA

On the front page:

MDCK GFP-FR shSPCA1 stained with Furin/Giantin antibodies (purple).

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This work was performed in the Laboratory of Membrane Traffic and Pathogenesis, Institut Pasteur, Paris.

“Nothing in life is to be feared, it is only to be understood. Now is the time to understand more, so that we may fear less.”

- Marie Curie

Acknowledgements

I am thankful to Professor Henrique Girão, first for presenting me the protocol between the Faculty of Medicine and Institute Pasteur, and for all the availability and support.

I would also like to thank Chiara Zurzolo for accepting me in her laboratory, creating the necessary conditions for me to develop this project and for always stimulating me to question my results and be able to fully understand them.

A special thank you to Stéphanie Lebreton, who supported me throughout the whole development of this project and taught me everything I needed to see this journey through.

I would also like to acknowledge to all my colleges at the Laboratory of Membrane Traffic and Pathogenesis, for being so keen on sharing their experience and knowledge whenever I might need it. And also for being so kind to me.

It would not be fair not to highlight the friendship and much needed help in times of distress of Miguel Lemos, who will always be my favourite personal assistant.

To my friends in Paris who made this city home and turned this experience more joyful.

And to my parents, to whom I owe my perseverance and for, from so little, giving me so much.

Contents

Abbreviations

AEE	Apical Early Endosome
AP	Apical Carriers
ARE	Apical Recycling Endosome
ARE	Apical Recycling Endosome
ATP	Adenosine Triphosphate
ATP2C1	Ca^{2+} transporting type 2C, member 1
aPKC	Kinase Atypical Protein Kinase C
BEE	Basolateral Early Endosome
BL	Basolateral
BSA	Bovine serum albumin
CRE	Common Recycling Endosome
DAF	Decay-Accelerating Factor
DLG	Discs Large
DMEM	Dulbecco's modified Eagle's medium
ECM	Extracellular Matrix
EDTA	Ethylene Diamine Tetra Acetic Acid
EGTA	Ethylene Glycol Tetraacetic Acid
elF4A	Eukaryotic Initiation Factor-4A
ER	Endoplasmatic Reticulum
EPP	Epithelial Polarity Program
FBS	Fetal Bovine Serum
FRT	Fisher Rat Thyroid
GFP-FR	Green Fluorescent Protein-Folate Receptor

GPI	Glycosylphosphatidyl-Inositol
GPI-AP	Glycosylphosphatidyl-Inositol-Anchored Proteins
HHD	Hailey-Hailey disease
IF	Immunofluorescence
IGF1R	Growth Factor 1 Receptor
LGL	Lethal Giant Larvae
MDCK	Madin Darby Canine Kidney
PAR	Partitioning Defective
PAR-3	Partitioning Defective 3
PAR-6	Partitioning Defective 6
PBS	Phosphate Buffered Saline
PFA	Paraformaldehyde
pIgR	Polymeric Immunoglobulin Receptor
PLAP	Placenta Alkaline Phosphatase Apical
PVDF	Polyvinyl Difluoride
RE	Recycling Endosomes
SERCA	Sarco/Endoplasmic Reticulum Ca^{2+}-ATPase
S.D.	Standart Desviation
SPCA1	Secretory Pathway Calcium ATPase 1
TBS	Tris-buffered saline
TfR	Transferrin Receptor
TGN	trans-Golgi Network
Tub	Tubulin
WB	Western Blot

Abstract

Oligomerization of glycosylphosphatidyl-inositol-anchored proteins (GPI-APs) in the Golgi is crucial for the correct sorting of these proteins to the apical plasma membrane of polarized MDCK (Madin Darby Canine Kidney) cells. Cholesterol and calcium play an important role in the regulation of the clustering of these proteins in the Golgi.

In this work, we focus on the Secretory Pathway Calcium ATPase 1 (SPCA1), Golgi Ca^{2+}-ATPase responsible for the uptake of Ca^{2+} into this compartment. More precisely, SPCA1 is predominantly found and active in the trans-Golgi where clusters of GPI-APs form.

The aim of this project is to unravel the role of SPCA1 pump in the trafficking of GPI-APs in polarized cells.

We firstly characterized the endogenous SPCA1 localization and expression both in polarized and non-polarized MDCK cells. We found that in both conditions SPCA1 is localized to the Golgi apparatus and we further report that polarized MDCK cells exhibit a higher amount of endogenous SPCA1 compared to non-polarized MDCK cells.

Next in order to directly address the involvement of SPCA1 in the trafficking of apical GPI-AP, we used stable MDCK cell line overexpressing an apical GPI-AP, GFP-FR, with either shscramble (CTRLi) or shSPCA1 (SPCA1i). By performing immunofluorescence experiments and confocal acquisition, we revealed that GFP-FR seems to be more localized intracellularly in SPCA1i cells compared to CTRLi cells.

Finally, we performed an exocytosis experiment in order to monitor the sorting of GFP-FR from the Golgi to the plasma membrane and highlighted that the traffickinf of GFP-FR is delayed in SPCA1i cells compared to CTRLi cells.

Our data indicates that SPCA1 may have a role in controlling the traffic of GPI-APs from the Golgi to the cell surface through the regulation of the levels of Ca^{2+} in the Golgi.

Resumo

A oligomerização de GPI-APs (*Glycosylphosphatidyl-Inositol-Anchored Proteins*) é crucial para o correto tráfego destas proteínas para a membrana plasmática de células MDCK (*Madin Darby Canine Kidney*) polarizadas desempenhando o colesterol e o cálcio, um importante papel na regulação da formação destes clusters no Golgi.

Neste projeto, incidimos na SPCA1 (*Secretory Pathway Calcium ATPase 1*), uma Ca^{2+}-ATPase do Golgi, responsável pelo influxo de Ca^{2+} para este compartimento, sendo esta a principal bomba de Ca^{2+} encontrada no trans-Golgi, onde se formam os clusters de GPI-APs.

O objectivo deste projeto é revelar o papel da bomba SPCA1 no tráfego de GPI-APs, em células polarizadas.

Primeiramente, caracterizámos a localização e expressão endógena da bomba SPCA1, em células MDCK polarizadas e não polarizadas. Descobrimos que, em ambas as condições, esta se encontrava no Complexo de Golgi com maior expressão em células polarizadas comparando com as não-polarizadas.

De seguida, para estudar diretamente o envolvimento da SPCA1 no tráfego de GPI-APs, utilizámos linhas celulares estáveis de MDCK que sobrexpressavam uma GPI-AP apical - GFP-FR - e também shscramble (CTRLi) ou shSPCA1 (SPCA1i). Após a realização de imunofluorescências e a consequente aquisição de imagens, revelámos que as células SPCA1i contêm mais GFP-FR intracelularmente que as células CTRLi.

Por último, concretizamos uma experiência de exocitose, com intuito de monitorizar o tráfego do GFP-FR desde o Golgi até à membrana plasmática, com vista a evidenciar o atraso do tráfego de GFP-FR, em células SPCA1i, face às células CTRLi.

Em suma, os nossos dados demonstram que a SPCA1 poderá ter um papel no controlo do tráfego de GPI-APs - do Golgi para a membrana plasmática -através da regulação dos níveis de cálcio neste compartimento.

CHAPTER 1- Introduction

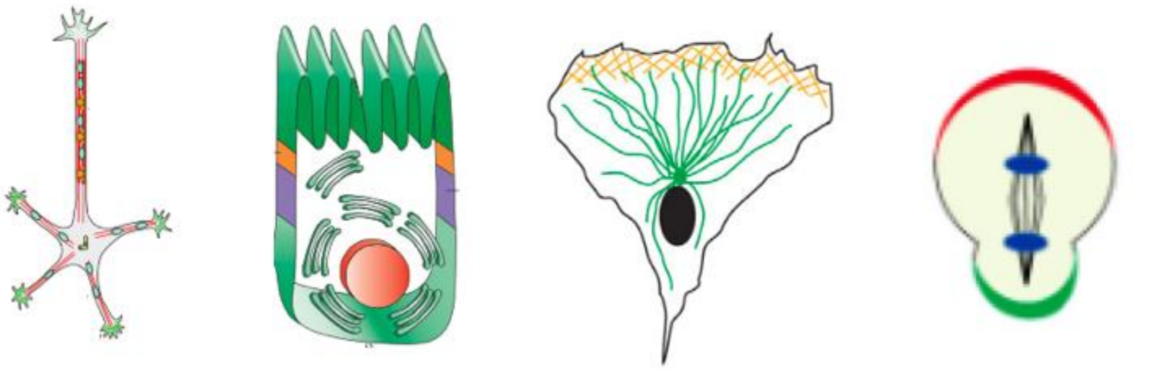
1. Cell polarity

Cell polarity is a spatial asymmetry in shape, structure, and function of cells. Polarity is necessary for coordination of proliferation, differentiation, morphogenesis, motility and signalling processes. Polarized cells are highly organized; they usually possess plasma membrane domains that differ in proteins and lipids composition and in functions. These specialized plasma membrane domains determine cell orientation and function (Mellman & Nelson 2008).

Cell polarity can be permanent or temporary but in both cases the main purpose of polarized organization is to assure a proper function. For example polarization allows (I) fibroblastic cells to migrate in a given direction, (II) neurons to rapidly transduce an electric signal or (III) epithelial cells to control the exchange between two different environments (Mostov et al. 2003; Rodriguez-Boulan, Kreitzer & Musch 2005; Takano et al. 2015; Overeem et al. 2015) (Figure 1).

Cell polarity is governed by interconnected regulations between signaling cascades, that controls membrane trafficking, proteins and lipid sorting, and cytoskeleton organization and dynamics (Mostov et al. 2003; Rodriguez-Boulan, Kreitzer & Musch 2005; Takano et al. 2015; Overeem et al. 2015).

Because cell polarity is often challenged in human diseases (bacterial or virus infection, cancer…), a fundamental question in cell biology is to understand how cells establish and maintain their polarity.



(a) Neuron (b) Epithelial cell (c) Migrating fibroblast (d) Asymmetric cell division

Figure 1. Examples of polarized cells. *Spatially and functionally restricted sub-compartments underlie the function of neurons (a) and epithelial cells (b). The spatial and temporal restriction of morphogen- and cytokine-receptor interactions directs cell migration during embryonic development and immune surveillance (c), while the asymmetric distribution of cell fate determinants enables asymmetric cell division and lead to the differentiation (d).* (Rodriguez-Boulan, Kreitzer & Musch 2005; Tahirovic & Bradke 2009; Neumüller & Knoblich 2009; Kadir et al. 2011).

1.1 Polarized epithelia

The most abundant cell type in animals is epithelial cells with epithelial tissues that line organs through the whole body (O'Brien et al. 2002). In the tissue, several epithelial cells form sheets held together through several intercellular interactions. There are two kinds of epithelial tissues: protective epithelium delimiting the body and internal organs, and glandular epithelium executing secretory function. Epithelial sheets can be composed by one-cell layer in the case of simple epithelia, or of many cells on top of each other for stratified epithelia (Figure 2).

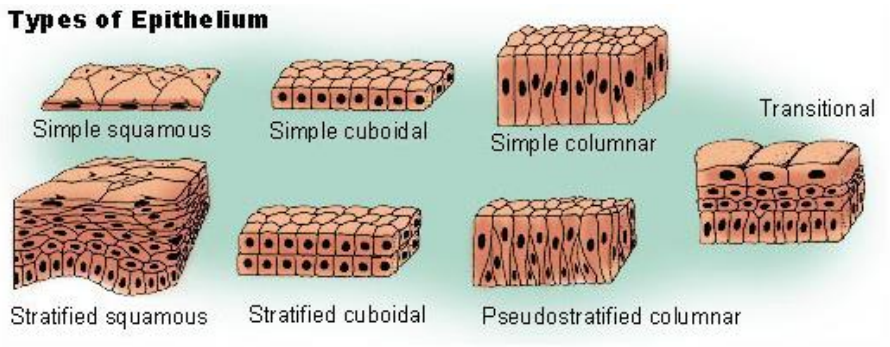


Figure 2. Different types of epithelial cells. from

(https://en.wikipedia.org/wiki/Epithelium#/media/File:Illu_epithelium.jpg)

Polarized epithelial cells are characterized by an asymmetric plasma membrane with an apical and basolateral domains (Figure 3).

The apical domain of epithelial cells is usually in contact with the external surface of an organism or with the body cavities, while the basolateral surface faces basement membrane and adjacent cells. Interactions between adjacent cells are either simple mechanical adhesion via tight junctions, adherent junctions and desmosomes or metabolic cooperation via gap junctions (Citi et al. 2014). The apical and basolateral domains have distinct morphologies, and are composed of different proteins and lipids (Rodriguez-Boulan & Macara 2014) with the apical domain that executes the specialized function (such as barrier, secretion, absorption etc.). For example, depending on the cell function the apical plasma membrane can be enriched in intestinal hydrolases, ion channels, transporters, whereas the basolateral domain in all epithelial cell is enriched in E-cadherin and integrins, which play a role in the formation of cell/cell or cell/ECM (extracellular matrix) contacts. Lipids such as cholesterol and sphingolipids are enriched in the apical domain, whereas phosphotidylcholine is enriched in the basolateral domain (van Meer & Simons 1988; Apodaca et al. 2012).

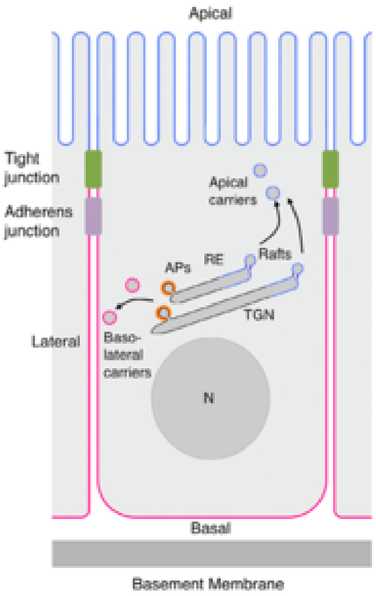


Figure 3. Schematic representation of a polarized epithelial cell. The image shows a polarized epithelial cell flanked by two neighbor cells. Tight junctions and adherens junctions hold cells togheter and block the passage of substancestrough the intracellular space and also is use as a bondary between the apical (blue) and the basolateral (red) domains. Vesicular carriers transport proteins to the plasma membrane from the trans-Golgi network (TGN) and recycling endosomes

(RE). Apical carriers are shown to arise from cholesterol- and lipid-enriched domains, whereas basolateral carriers arise from adaptor protein (AP)-enriched “coat” domains. *(Bonifacino 2014)*

1.2 Molecular mechanisms underlying epithelial polarization

The overall process through which the network of epithelial polarity proteins and lipids mediate the organization of a polarized epithelial cell is called the epithelial polarity program (EPP). Cell polarity involves the spatiotemporal coordination of many processes such as signaling cascades, proteins and lipid sorting, trafficking and endocytosis as well as cytoskeletal dynamics (Mostov et al. 2003; Rodriguez-Boulan, Kreitzer & Musch 2005; Takano et al. 2015; Overeem et al. 2015).

The first step of polarization is the response to extracellular cues, it involves cell–matrix and cell–cell recognition (Manninen 2015). This step is determining how to orientate the cell and where to form the apical surface. The second step of apical-basal polarization required heavy rearrangement of the cytoskeleton, establishment of apical basal axis with formation of intercellular junctions. Importantly, polarization requires the establishment of polarized trafficking machinery.

Studies on model organisms such as yeast, worms and flies have led to the identification of core protein complexes that regulate various aspects of EPP. Three major polarity complexes, the PAR (PAR3-PAR6-aPKC), Crumbs (Crumbs3-PALS1-PATj) and Scribble (Scribble-DLG1-LGL1/2) have been shown to be involved in the epithelial polarization and also in asymmetric cell division (Overeem et al. 2015; Rodriguez-Boulan & Macara 2014; Assemat et al. 2008) (Figure 4). These complexes distribute asymmetrically in the cells, promoting the establishment of apical and basolateral membrane domains.

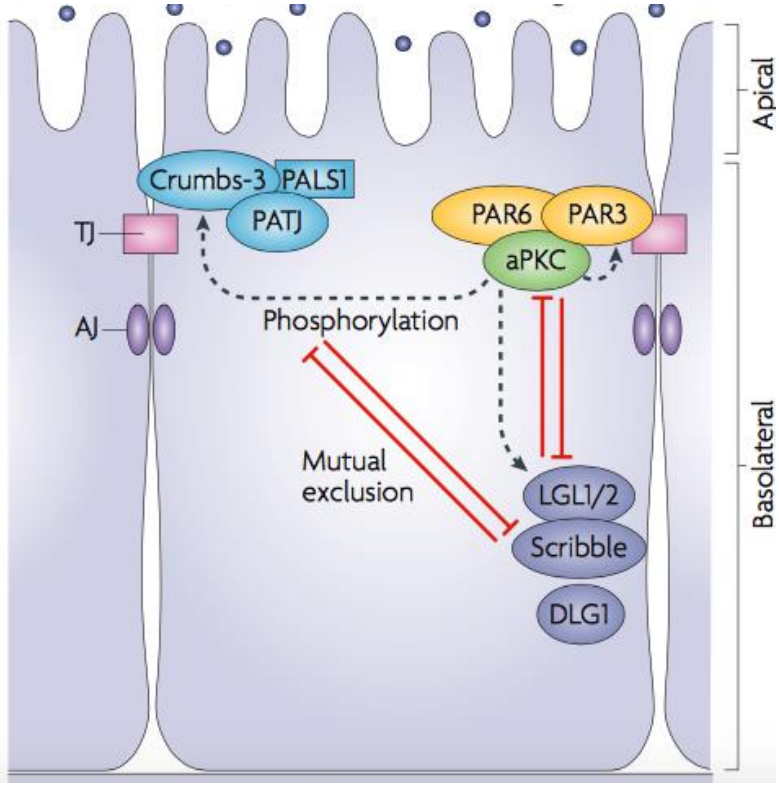


Figure 4. Polarity proteins of the PAR3, Crumbs and Scribble complexes. Three conserved protein complexes − the partitioning defective (PAR), Crumbs and Scribble complexes − control many polarization processes in different organisms. The PDZ-domain proteins PAR-3 and PAR-6 together with the Ser/Thr kinase atypical protein kinase C (aPKC), constitute the PAR complex. The Crumbs complex comprises the transmembrane proteins Crumbs and the cytoplasmatic scaffolding molecules PALS1 (protein associated with LIN-7) and PATJ (PALS1-associated tight-junction protein). The cytoplasmic protein Scribble interacts with two others cytoplasmatic proteins: Discs large (DLG) and Lethal giant larvae (LGL). In polarized mammalian ephithelial cells the PAR3 and Crumbs-3 complexes localize predominantly in tight junctions, whereas components of the Scribble present basolateral localization. Several interactions between the three complexes have been identified. (Iden & Collard 2008*)*

The cell polarity is maintained during the lifetime of an epithelium by constant plasma membrane turnover of lipids and proteins. A continuous sorting of newly synthesized molecules and recycling of membrane components are required to maintain the molecular asymmetry at the cell surface (Rodriguez-Boulan & Macara 2014).

1.3 Polarized protein sorting

Epithelial polarity is established and then maintained due to a polarized exocytosis of lipids and proteins as well as their polarized endocytosis. Polarized cells must specifically address plasma membrane proteins and lipids to the apical or basolateral domains (Yeaman et al. 1999; Mostov et al. 2003; Rodriguez-Boulan, Kreitzer & Musch 2005). Polarized sorting of proteins relies on the recognition of intrinsic signals by the cellular sorting machinery.

1.4 Basolateral sorting signals

Basolateral signals are found in the primary structure of the proteins, as specific amminoacidic sequences located in the cytoplasmic tail of cargo proteins. The two most common types of basolateral signals are tyrosine- and di-leucine-based (Mellman & Nelson 2008; Edeling et al. 2006; Bonifacino & Traub 2003; Rodriguez-Boulan, Kreitzer & Müsch 2005; Stoops & Caplan 2014). Moreover, single leucine patch as in CD147 (Deora et al. 2004) or other sequences as identified in neural cell adhesion molecule (Le Gall et al. 1997), pIgR (Aroeti & Mostov 1994), epidermal growth-factor receptor (He et al. 2002), epidermal growth-factor receptor 2 (Dillon et al. 2002) and transforming growth factor β (Dempsey et al. 2003) have been identified as basolateral sorting signals.

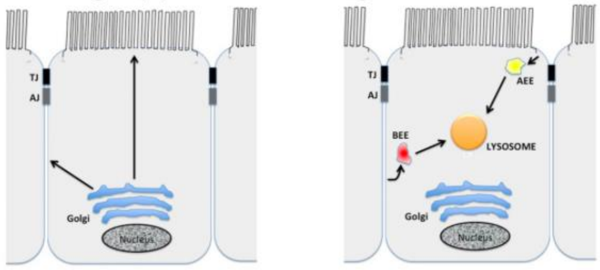
1.5 Apical sorting signals

Regarding the nature of apical sorting signals they appear to be more elaborated compared to BL sorting signals. Apical sorting signals are of variable nature including peptide sequences and post-translational modifications (Stoops & Caplan 2014; Weisz & Rodriguez-Boulan 2009), such as lipid and sugar moieties, and they can be localized in the extracellular, transmembrane or intracellular domains of the cargo proteins (Weisz & Rodriguez-Boulan 2009). Originally one of the first apical signal described was the glycosylphosphotidylinositol (GPI) anchor. It was shown that addition of the GPI anchor of decay-accelerating factor (DAF) to the ectodomain of a basolateral (herpes simplex glycoprotein D) or a secretory (human growth hormone) protein resulted in their apical misorting (Lisanti et al. 1989; Lisanti et al. 1988; Brown et al. 1989).

1.6 Trafficking routes in polarized cells

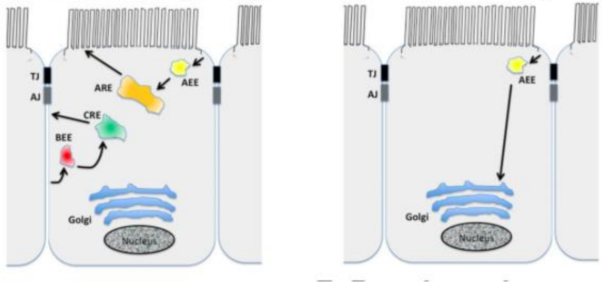
Polarity is maintained by the selective traffic of *de novo* synthesized proteins and by the selective polarized endocytosis and recycling. In general, membrane proteins are synthesized and modified in the Endoplasmatic Reticulum (ER) and then are sorted and further maturated within the Golgi apparatus to their proper destination (Mellman & Nelson 2008; Goldenring et al. 2013; Rindler et al. 1984; Fuller et al. 1985; Griffiths & Simons 1986; Muñiz & Zurzolo 2014; Rodriguez-Boulan, Kreitzer & Musch 2005) (Figure 5). By live imaging and biochemical approaches, it was shown that the Golgi is the main sorting platform although endosomes also constitute a sorting platform for certain proteins. There are several trafficking roads for newly synthesized membrane proteins. In the simplest case proteins leave the Golgi in vesicles and are sorted to the apical or basolateral membranes directly (Figure 5A). Several studies have shown that the biosynthetic route of several membrane proteins includes a post-TGN transit through recycling endosomes (RE) (Ang et al. 2004; Lock 2005; Cancino et al. 2007; Cresawn et al. 2007; Gravotta et al. 2007).

Once protein is located on the plasma membrane, endocytosis can relocate proteins into the cell (Figure 5C). Proteins undergoing endocytosis can be additionally sorted in recycling endosomes, like Transferrin receptor (TfR), and further degraded in lysosome (Figure 5B) or recycled back to the Golgi (Matter & Mellman 1994; Mostov & Cardone 1995; Odorizzi & Trowbridge 1997).



A. De novo synthesis from Golgi apparatus

B. Endocytosis and degradation



C. Apical and basolateral recycling

D Endocytosis and recycling to Golgi

***Figure 5. Paradigms for trafficking in polarized epithelial cells*.** (A) *De novo* trafficking from the Golgi apparatus. (B) Endocytosis and recycling inside of to the same membrane domain. (C) Endocytosis leading to degradation in the lysosome. (D) Endocytosis and trafficking back to the Golgi apparatus. All of these pathways may be operating in polarized epithelial cells. AEE: apical early endosome; ARE: apical recycling endosome; BEE: basolateral early endosome; CRE: common recycling endosome. (Goldenring 2013)

1.7 The GPI-Anchored Proteins

Glycosylphosphatidylinositol-anchored proteins (GPI-APs) are lipid anchored membrane proteins that are ubiquitously expressed at the cell surface. The GPI anchor is highly conserved in evolution and more than hundreds GPI-APs have been characterized to date (Nosjean et al. 1997). The GPI-APs are expressed from yeast to humans (Nosjean et al. 1997) and their functions range from enzymatic to antigenic and adhesion properties (Imjeti et al. 2011). They are composed of an inositol phospholipid, which is anchored into the lipid bilayer of external leaflet of the plasma membrane. It is joined to an oligosaccharide containing glucosamine and three mannoses residues through a glycosidic bond. An ethanolamine phosphate is linked to the C-terminal of the protein by an amide bond, so that it forms a bridge between the protein and the oligosaccharide of the GPI (Sabharanjak & Mayor 2004) (Figure 6).

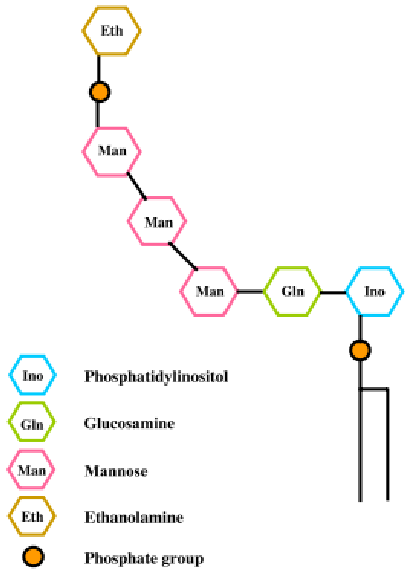


Figure 6. GPI-APs structure. *(*Sabharanjak & Mayor 2004)

All GPI-APs commonly associate with specific membrane domains called raft, which are lipid-ordered membrane microdomains enriched in cholesterol and sphingolipids. The raft concept proposed in 1997 by Simons and Ikonen improved our understanding of membrane organization and the role of proteins and lipids compartmentalization within the membrane. These raft are membrane microdomains highly dynamic that are proposed to be involve in various cellular functions such as protein sorting, cell signaling, endocytosis and virus budding (Harder & Sangani 2009; Parton & Richards 2003; Pike 2003; Simons & Gerl 2010; Simons & Toomre 2000).

GPI-APs are mainly sorted to the apical domain of the plasma membrane in polarized epithelial cells. Apical GPI-APs organization and biological activities are regulated by the selective sorting mechanism that occurs in the Golgi complex of polarized epithelial cells. Thus the functional organization of GPI-APs is directly depending on epithelial polarity (Paladino et al. 2014).

1.8 Mechanisms of sorting and trafficking of GPI-APs

Both apical and basolateral GPI-APs are associated with cholesterol-enriched raft microdomains in the Golgi complex but only apical one form high molecular weight complexes or clusters. Oligomerization is the mechanism that segregates apical and basolateral GPI-APs in the Golgi, leading to their differential sorting (Paladino et al. 2004) (Figure *7*).

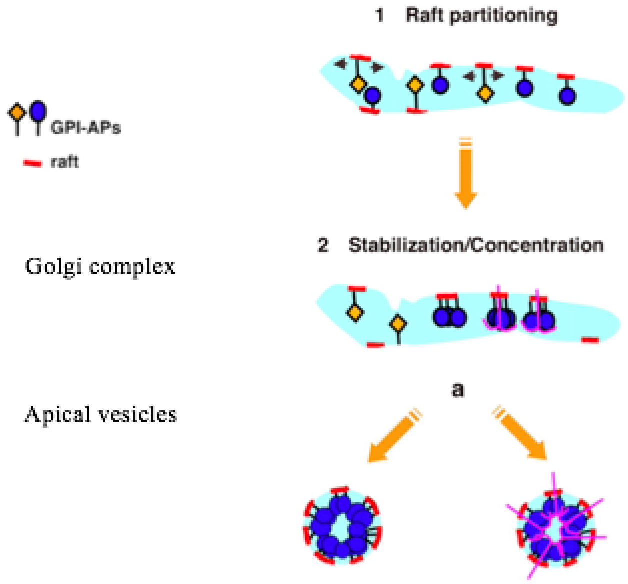


Figure 7. **Apical sorting of GPI-APs in polarized epithelial cells.** (1) Raft partitioning. Both apical and basolateral GPI-APs partition with rafts due to chemical affinity of the GPI-APs for rafts. (2) Stabilization/Concentration. Only apical GPI-APs are stabilized into rafts by protein clustering, increasing their raft affinity. (a) oligomerization in rafts is sufficient to drive apical sorting; Adapted from (Paladino et al. 2004).

This Golgi oligomerization of GPI-APs relies on both a favourable lipid environment and a permissive protein ectodomain (Paladino et al. 2008; Lebreton et al. 2008). Indeed at least two mechanisms exist to determine oligomerization in the Golgi leading to apical sorting of GPI-APs in epithelial cells. In Madin Darby canine kidney (MDCK) cells, cholesterol regulates GPI-APs oligomerization in the Golgi and apical sorting. While in Fisher Rat Thyroid (FRT) cells, this is the N-glycosylation of the GPI-APs that is the critical event for both oligomerization and apical sorting (Imjeti et al. 2011).

Golgi sorting regulates organization and function of GPI-APs at apical membranes. Indeed in polarized MDCK cells, apical GPI-APs form cluster (homocluster) in the Golgi apparatus and reach the apical surface organized in homocluster. Golgi-derived homo-clusters are required for their subsequent plasma membrane organization into larger cholesterol-dependent clusters formed by at least two GPI-APs species (hetero-clusters). Importantly, the functional state of the GPI-APs is regulated by plasma membrane organization with a maximum activity in polarized conditions (Figure 8). In non-polarized MDCK cells, GPI-APs leave the Golgi as monomers and remain un-clustered at the cell surface correlated with low activities (Paladino, Lebreton et al. 2014) (Figure 8). However, in fibroblasts, GPI-APs leave the Golgi as monomers but they are capable of forming nanoclusters (homo- and heteroclusters) at the cell surface. The GPI-APs activities in fibroblasts are independent of Golgi sorting compared to polarized epithelial cells (Paladino, Lebreton et al. 2014) (Lebreton et al. submitted).

Interestingly, in both fibroblasts and epithelial cells GPI-APs are organized in clusters at cell membrane but their mechanisms of formation are drastically different as well as their dependency to actin and cholesterol.

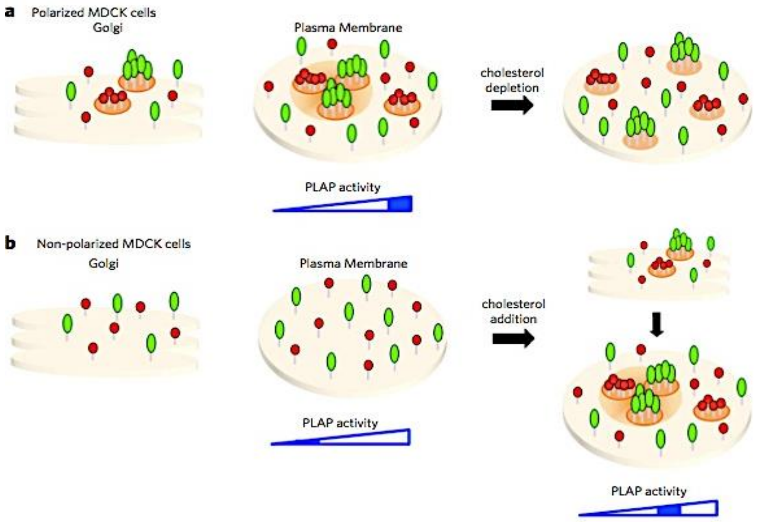


Figure 8. **GPI-APs organization and activity in MDCK cells.** (a) In polarized MDCK cells, apical GPI-APs form homo-cluster in the Golgi complex and at the plasma membrane are organized into homo-clusters (red and green aggregates) and hetero-clusters. Upon cholesterol depletion homo-clusters are unaffected, while the hetero-cluster organization is lost. (b) In non-polarized MDCK cells GPI-APs do not cluster in the Golgi complex and at the plasma membrane remain in the form of monomer and dimer. Upon cholesterol addition GPI-APs cluster in the Golgi and consequently at the plasma membrane assume the same organization found in polarized cells in homo-and hetero-cluster. The clustering organization regulates the activity of PLAP, and in polarized cells this depends on clustering/sorting in the Golgi. *PLAP: Placenta alkaline phosphatase Apical GPI-AP* (Paladino, Lebreton et al. 2014)

Indeed, in polarized epithelial MDCK cells, the GPI-APs Golgi organization is cholesterol dependent since the depletion of cholesterol impairs the association of apical GPI-APs to the raft and its oligomer formation leading to its basolateral missorting. On the reverse the addition of cholesterol leads to the oligomerization of basolateral GPI-APs in Golgi leading to its apical missorting (Lebreton et al. 2008; Paladino et al. 2008). Importantly in non-polarized epithelial MDCK cells, the addition of cholesterol is sufficient to induce re-organization and formation of homoclusters of GPI-APs in Golgi and as consequence promotes their organization into heteroclusters at plasma membrane as in polarized conditions (Paladino, Lebreton et al. 2014) (Figure 8). Therefore cholesterol content in the Golgi is critical for GPI-APs clustering. Moreover, at the apical surface homoclusters are both cholesterol and actin independent while in fibroblasts GPI-APs clustering and organisation rely on actin and cholesterol revealing intrinsic difference between these two cells types (Paladino, Lebreton et al. 2014) (Lebreton et al. submitted).

1.9 Selective Roles Ca^{2+} in Oligomerization of GPI-APs in the Golgi and Plasma Membrane

Beside a role for cholesterol in Golgi clustering of GPI-APs in epithelial cells, the molecular mechanism underlying the critical step of Golgi GPI-APs oligomerization is largely unknown. Because GPI-APs sorting, organization at the apical plasma membrane and biological activity rely on epithelial polarity we investigated whether a calcium chelation by EGTA treatment would alter GPI-APs organization. Upon EGTA treatment apical plasma membrane organization is completely lost (Lebreton et al. submitted) and could be rescue upon replenishment of calcium (chelation followed by re incubation of cells with calcium in the cell culture medium). We further showed that upon EGTA Golgi GPI-APs clustering is affected suggesting that the Calcium would regulate clustering of GPI-APs. The calcium amount within the Golgi is known to regulate protein sorting for some secretor cargo (von Blume et al. 2011) and it was reported that Golgi contain high amount of calcium (Chandra et al. 1994). We recently showed that polarized MDCK cells exhibit higher amount of calcium in the Golgi compared to the Golgi of non polarized MDCK cells (Lebreton et al. Submitted).

The level of calcium in the Golgi is regulated by two main proteins: the Sarco/Endoplasmic Reticulum Ca^{2+}-ATPase (SERCA), known to contribute to the entry of calcium in the *cis* and *medial* Golgi (Aulestia et al. 2015), and the more recently identified the Secretory Pathway Calcium ATPase 1 (SPCA1), that acts at the level of the TGN where GPI-APs cluster.

1.10 The SPCA1 pump

The Secretory Pathway Calcium ATPase 1 (SPCA1) is a Golgi- resident transmembrane protein, encoded by the ATPase, Ca^{2+} transporting type 2C, member 1 (ATP2C1) gene. The SPCA1 uptakes Ca^{2+} into the Golgi in an ATP-dependent manner (Lissandron et al. 2010) (Aulestia et al. 2015). Morevoer SPCA1 has also affinity for magnesium (~20nM).

The SPCA1 pump is activated when Cofilin 1 bounds to the pump via dynamic actin filaments, triggering Ca^{2+} influx (von Blume et al. 2011). The increase of Ca^{2+} levels in the Golgi recruits Cab45, a protein that binds Ca^{2+} with high affinity, and reversibly assembles into oligomers, which specifically binds secretory proteins (von Blume et al. 2012). The secretory proteins, now organized in oligomers, dissociate from Cab45 before packaging into a transport carrier, upon a decrease of the Golgi levels of Ca^{2+} or by a signal such as phosphorylation (von Blume et al. 2012) (Kienzle & von Blume 2014) (Figure 9).

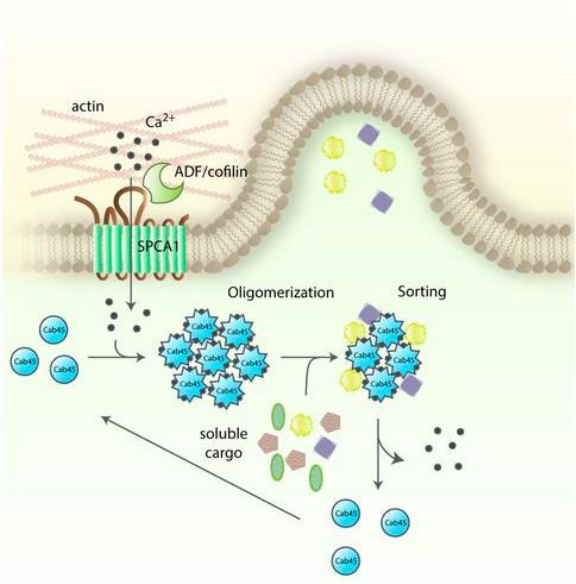


Figure 9. **Schematic of the activation of the SPCA1 pump.** Upon SPCA1 uptake of calcium, the Ca2+ levels in the TGN increase. As consequence, Cab45 binds cargo proteins and separates them from other soluble proteins.(Crevenna et al. 2016)

SPCA1 has been reported to be associated with cholesterol-rich lipid rafts in the Golgi in colon adenocarcinoma HT29 cells. It was further reported that SPCA1 activity is reduced of 50% upon cholesterol depletion indicating that the cholesterol-rich microdomains are essential for the proper function of the SPCA1 (Baron et al. 2010).

SPCA1 pump levels are elevated in colon, prostate and breast cancers (Dang & Rao 2016) (Grice et al. 2010). More precisely SPCA1 in the case of breast cancers is overexpressed in basal-like tumours and downregulated in luminal subtypes (Grice et al. 2010). In MDA-MB-231 cells, a basal-like breast cancer cell line, the knockdown of SPCA1 reduced the cellular proliferation. All these findings reveals that SPCA1 has been correlated with a poor diagnosis in breast cancer, suggesting the potential of SPCA1 as a marker for basal-like breast cancers (Grice et al. 2010).

Inactivation of one allele of ATP2C1 causes Hailey-Hailey disease (HHD), an autosomal dominant skin disorder characterize by a defect in keratinocyte adhesion in the suprabasal layers of epidermis that will lead to a “dilapidated brick wall” (Missiaen et al. 2007). In N2a cells, a mouse neuroblastoma cell line, the depletion of SPCA1 induces disruption of the polarized trafficking leading to impairment of neuronal differentiation (Sepúlveda et al. 2007) and revealing the role of SPCA1 in the regulation of protein trafficking.

GPI-APs oligomerization in the Golgi is depending on calcium levels in this compartment where SPCA1 is the main pump allowing Ca^{2+} uptake. Moreover, SPCA1 is found in the cholesterol- and sphingolipid-rich microdomains of the TGN, the same domains where the clusters of GPI-APs form. Therefore, SPCA1 appears to be an excellent candidate as regulator of the trafficking of GPI-APs.

CHAPTER 2- OBJECTIVES

The focus of this work was to study the role of the SPCA1 pump in the trafficking of GPI-APs in polarized cells.

Accordingly, the main objectives of this study were:

-Characterize the endogenous expression of SPCA1 in polarized and non-polarized conditions in MDCK cells, by western blot and immunofluorescence methodologies;

-Quantify the percentage of SPCA1 associated to the Golgi apparatus, applying Golgi fractionation;

-Characterize stable clones MDCK GFP-FR shscramble (CTRLi) and MDCK GFP-FR shSPCA1 (SPCA1i) by western blot and immunofluorescence methodologies;

- Determine if the trafficking of GPI-APs is altered in SPCA1i cells by performing an exocytosis experiment.

CHAPTER 3- Material and Methods

3.1 Reagents and antibodies

Cell culture media were purchased from ThermoFisher Scientific. Antibodies were purchased from the following companies: polyclonal α–GFP from ThermoFisher Scientific, polyclonal Furin from Thermo scientific (1:1000 for WB and 1:100 for IF), polyclonal SPCA1 from BioRad (1:1000 for WB and 1:500 for IF), polyclonal calnexin from Sigma (1:1000), polyclonal Giantin from Ozyme (1:400). All the secondary antibodies were from Life Technologies (WB and 1:500 for IF). ECL was bought from Amersham. Cyclohexemide was from Sigma.

3.2 Cell culture and transfections

MDCK were grown in DMEM (Dulbecco's modified Eagle's medium) containing 5% of Fetal Bovine Serum（FBS) in an atmosphere of 5% CO_2 at 37^0C.

3.3 Western blot

Cells were grown in 100-mm petri dishes, washed with ice-cold phosphate buffered saline containing $CaCl_2$ and $MgCl_2$ (PBS+/+), and treated with lysis buffer containing 20mM Tris-HCl, 150mM NaCl, 5mM ethylene diamine tetra acetic acid (EDTA), 1% Triton X-100 (pH 7,2), and 3μL protease cocktail inhibitor per mL of Lysis Buffer for 20min on ice under agitation, and further centrifuged at 14000 *rpm* for 5min at 4^0C. Total protein concentration was determined by Bradford assay. Protein samples (40µg) were heated with 2-mercaptoethanol and separated on a 8% denaturing polyacrylamide gel, in order to detect our proteins of interest, followed by transfer to PVDF (Polyvinyl difluoride) membrane. Membranes were blocked with 5% (w/v) non-fat dry milk in Tris-buffered saline (TBS) containing 0.05% (v/v) Tween-20 (TBST) for 1 h at room temperature. Membranes were then incubated overnight at 4^0C with anti-SPCA1 antibody (diluted 1:1000) anti-Furin antibody (diluted 1:1000) or anti-Calnexin (diluted 1:1000) in 1% (w/v) nonfat dry milk in TBST. After several washes, the blots were incubated with secondary antibody linked to horseradish peroxidase diluted 1:5000 in 1% (w/v) nonfat dry milk in TBST. Bound antibody was detected using the ECL detection system and visualized on a chemiluminescence imager for high-resolution imaging of protein in gels and membranes (Amersham Imager 600, GE Healthcare Life Sciences).

3.4 Immunofluorescence

Two different protocols were performed: one for permeabilized and other for non permeabilized conditions.

In non permeabilized conditions, cells were grown on coverslip for 3 days, washed with phosphate-buffered saline containing $CaCl_2$ and $MgCl_2$ (PBS+/+), fixed with PFA 4% for 20min, quenched with NH_4Cl for 10 min and blocked for 1h using blocking solution (PBS+/+ plus 0.2% gelatin). Then cells were incubated with primary antibody diluted in blocking solution for 30 min. Antibody used was α-GFP antibody (1:500). Cells were extensively washed with blocking solution and incubated 30min with the Alexa-conjugated secondary antibody at a 1:500 dilution (Life Technologies). Images were acquired with a Zeiss LSM 700 microscope equipped with a 63x oil-immersion objective lens.
In permeabilized conditions, MDCK cells were grown on coverslips, washed with PBS containing $CaCl_2$ and $MgCl_2$, fixed with Paraformaldehyde (PFA) 4% for 20 min for 20min at room temperature, saturated for 1h in buffer (PBS+/+, 0.2% Triton X-100, 4% BSA) and incubated in PBS+/+ plus 4% BSA solution for 1h with primary antibodies used: Furin (Rabbit/1:100), Giantin (Rabbit/1:400) and SPCA1(Mouse/1:500). Cells were extensively washed and incubated for 1h with the respective Alexa-conjugated secondary antibody at a 1:500 dilution (Life Technologies). Images were acquired with a Zeiss LSM 700 microscope equipped with a 63x oil-immersion objective lens.

3.5 Velocity Gradient

Cells were grown for 1 or 3 days in ten 150-mm dishes, for each condition, washed in phosphate buffered saline (PBS) containing EDTA and incubated on ice for 5min in the same solution. Lysates are scraped from dishes and collected in a 15mL Falcon where they are centrifugated 10min at 1000rpm at 4^0C and the supernatant was discarded and the pellet pooled and ressuspended in a Hepes-Sucrose solution and again centrifugated at 1000rpm at 4^0C being the pellet ressuspended in 400μL of Hepes-Sucrose solution. This suspension was then centifugated 5 minutes at 1000 rpm at 4^0C, in order to remove the nucleus. The supernatant was collected and layered on top of the discontinuous sucrose gradient (60 to 15%) and ultracentrifuge at 45000rpm in SW50 (Beckman counter) for 1h15min at 4°C. Fourteen fractions of 360μL were collected from the top of the tube. Proteins were revealed by western blot using specific antibodies for SPCA1, ER (Calnexin), and Golgi (Giantin/Furin) markers.

3.6 Temperature Block

In order to achieve an almost complete protein block in the TGN, we used a previously published protocol (Paladino et al., 2006). Confluent MDCK cells grown on coverslips were incubated at 19.5°C for 2h in areal medium (F12 Coon's modified medium without NaHCO and with 0.2% BSA and 20mM Hepes, pH 7.4) along with 150mg/mL cycloheximide in the last hour. Cells were fixed with PFA (time 0) or alternatively cells were warmed at 37°C for 30min or 1h, in order to release the protein from the Golgi to the cell surface. Cells were then treated for immunofluorescence and serial confocal images were collected using a Zeiss LSM 700 confocal microscope with a 63x oil immersion objective lens.

3.7 Colocalization assay

After fixation and immunofluorescence Z-stack images were acquired using a Zeiss LSM 700 confocal microscope with a 63x oil immersion objective lens. Colocalization analysis was performed using the Jacop plugin on ImageJ software.

3.8 Heigh and Golgi´s area measurement

After fixation and immunofluorescence images were acquired using a Zeiss LSM 700 confocal microscope with a 63x oil immersion objective lens. Golgi area was obtained by drawing manually the Golgi using the Measure option on the ImageJ software. Height was obtained by counting the stacks on the ImageJ software and multiplying them by 0,47μm.

3.9 Statistical analysis

In all experiments, we used two-tailed Student’s test for statistical analyses.

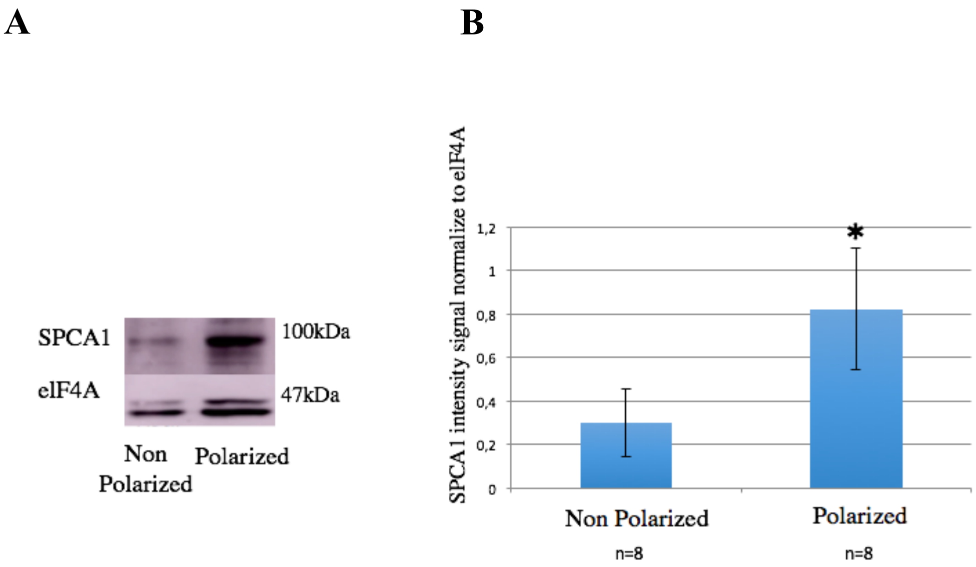
CHAPTER 4- Results

4.1 Characterization of endogenous SPCA1 expression and localization in polarized and non-polarized MDCK cells

In order to investigate the putative involvement of SPCA1 in apical sorting of GPI-APs, we firstly study the endogenous expression and localization of the calcium magnesium pump in MDCK cells, our cellular model.

We firstly consider MDCK cells grown on coverslip for either 1 (non-polarized) or 3 days (polarized) and performed western blot analysis (see material and methods). As loading control we used the transcription factor eIF4A (Achard et al. 2007). As shown in Figure 10A, we detected SPCA1 protein at 100kDa as expected according to the molecular weight. Eight different experiments were performed and in each experiment the intensity signal of SPCA1 was normalized to eIF4A intensity signal. As shown Figure 10B SPCA1 exhibits a higher level of expression in polarized MDCK cells compared to non polarized condition.

Next, we performed immunofluorescence to monitor the endogenous SPCA1 both in polarized and non-polarized MDCK cells (see material and methods). Because SPCA1 is reported to localize in the Golgi apparatus we also monitor Golgi markers as Giantin and Furin in order to define the level of co-localization between SPCA1 and Cis/medial Golgi and trans-Golgi respectively (see material and methods). As shown Figure 10C in both conditions (polarized and non-polarized) SPCA1 is detected in the Golgi with similar Pearson´s coefficient (0,99±0,006 and 0,98±0,008, respectively, n=50 cells) (Van Baelen et al. 2003) (Baron et al. 2010) (Sepúlveda et al. 2009). We can note that although different antibodies and protocol have been used we have an aspecific nucleus staining in MDCK.



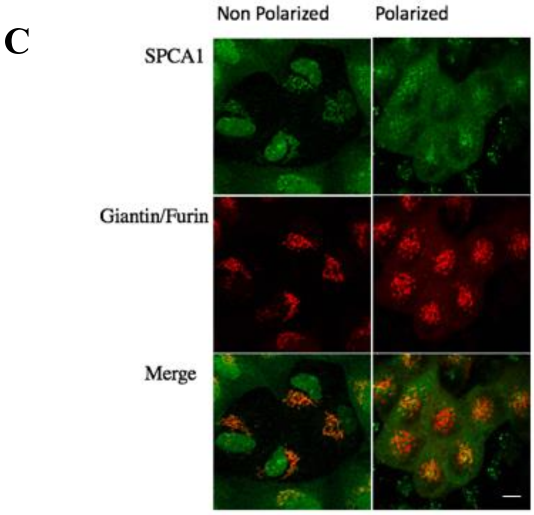


Figure 10. Polarized MDCK cells exhibit higher amount of SPCA1 compared to non-polarized MDCK cells. (**A**) MDCK cells were grown for 1 or 3 days. Cell lysates were analysed by western blot using SPCA1 and elF4A antibodies. The molecular weight of SPCA1 and elF4A are indicated. (**B**) Quantification of endogenous SPCA1 detected by WB. The intensity signal of SPCA1 was normalized to the intensity signal of elF4A (homogenize). Error bars indicate the S.D. *<0,05, Student´s t-test. (**C**) MDCK cells were grown for 1 and 3 days on coverslip Giantin/Furin, fixed, permeabilized and stained with SPCA1 and Giantin/Furin antibodies followed by secondary antibodies (see material and methods). Scale bars 10µm.

4.2 Percentage of SPCA1 associated to the Golgi

The data gathered by immunofluorescence indicate that SPCA1 is associated with the Golgi both in polarized and non polarized conditions nevertheless we cannot precisely quantify the amount of SPCA1 associated with the Golgi. In order to evaluate the amount of SPCA1 associated with the Golgi we performed a cell fractionation and evaluate the percentage of SPCA1 associated with the Golgi fraction (Imjeti et al. 2011).

Therefore, velocity gradients with discontinuous sucrose gradients were performed in both polarized and non-polarized conditions and analysed by western blot using SPCA1, Golgi (Furin) and ER (Calnexin) markers (Figure 11A) (see material and methods).

Quantification analysis revealed that 70% of SPCA1 is associated with the Golgi fraction in both non polarized and polarized conditions (Figure 11C).

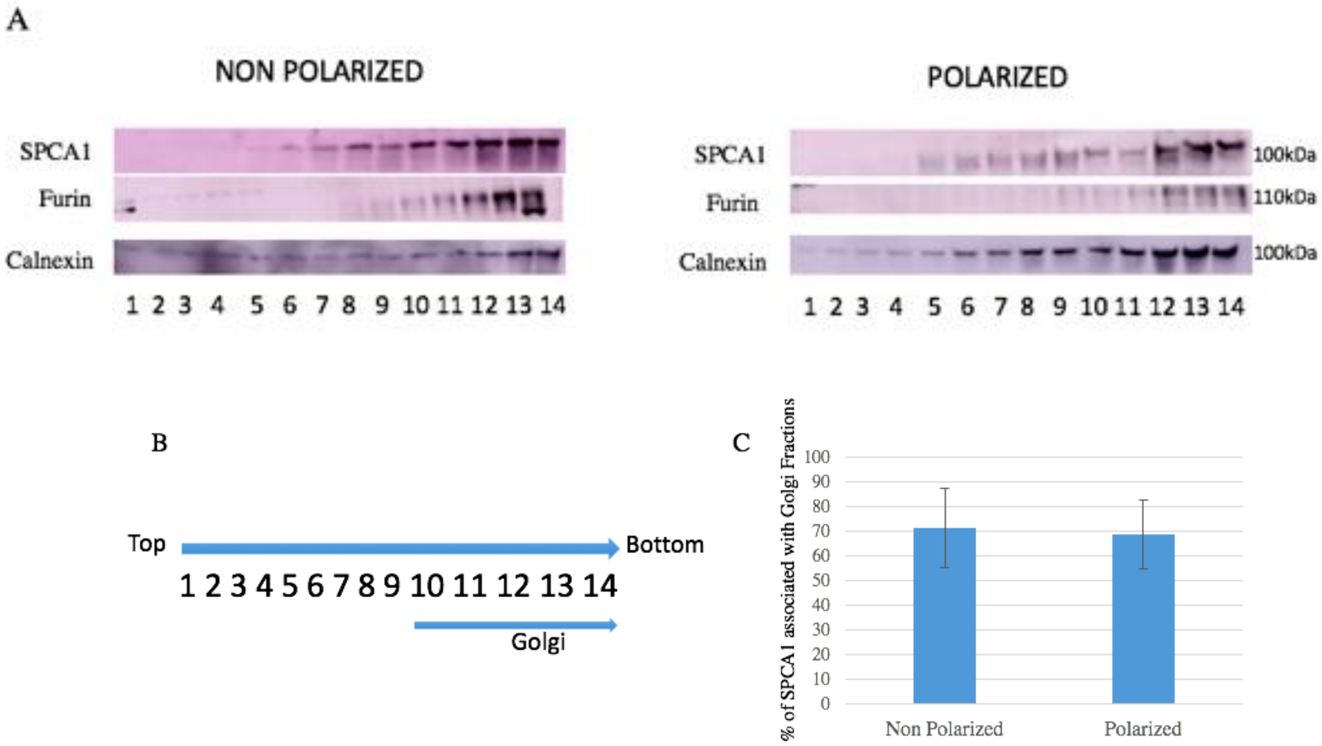


Figure 11. Golgi fractionation of MDCK cells in non polarized and polarized conditions. (A) MDCK cells were grown in 150mm for 1 or 3 days and scraped with a PBS/EDTA solution and lysed with sequential centrifugations (see material and methods). The supernatant obtained was added to the top of the 60-15% sucrose gradient and ultracentrifugated for 1h15min at 4°C. Fractions of 360µL were collected from the top (fraction 1) to the bottom (fraction 14) of the gradient. Proteins were detected by WB using SPCA1, Furin and Calnexin antibodies. The molecular weight of the SPCA1/Furin and Calnexin are indicated. (B) Schematic representation of the Golgi markers along the gradient. (C) Percentage of SPCA1 associated with Golgi fractions. The percentage was obtained by considering the signal intensity of SPCA1 associated with the Golgi fraction (9 to 14) compared to the total intensity signal of SPCA1 in all fractions (1 to 14). This experiment was performed twice. Error bars means S.D.

4.3 Characterization of MDCK GFP-FR shscramble and MDCK GFP-FR shSPCA1

Calcium level in the Golgi appears to be critical for clustering of GPI-APs and SPCA1 has been shown to govern uptake of calcium in this compartment. Furthermore we showed that clustering of GPI-APs in the Golgi directly regulate their trafficking, we therefore decided to investigate the putative role of SPCA1 in the regulation of GPI-APs trafficking. We generated stable MDCK cell lines expressing the apical GPI-AP GFP-FR (the GFP fused to the GPI-anchor attachment signal of Folate Receptor (Paladino et al. 2008) with either shscramble (MDCK GFP-FR shscramble) (CTRLi) or shSPCA1 (MDCK GFP-FR shSPCA1) (SPCA1i). The MDCK GFP-FR shscramble and MDCK GFP-FR shSPCA1 cells were both silenced by using a short hairpin (sh) targeting either specifically SPCA1 or not.

In this project, CTRLi cells are used as internal control since the level of SPCA1 is not altered compared to SPCA1i.

We selected several clones (2, 9, 20) upon transfection of shSPCA1 in MDCK GFP-FR and monitor by western blot whether these clones exhibit less SPCA1 compared to CTRLi. Here as a loading control we used tubulin (Luo et al. 2016) and we found that SPCA1i clone 2 do not exhibit a reduction of SPCA1 (while clone 9 and 20 exhibit 72% and 47% of reduction of SPCA1 level, respectively (Figure 12). Because SPCA1i cl9 is the clone with the lowest SPCA1 expression compared with CTRLi, we consider this clone 9 for further experiments.

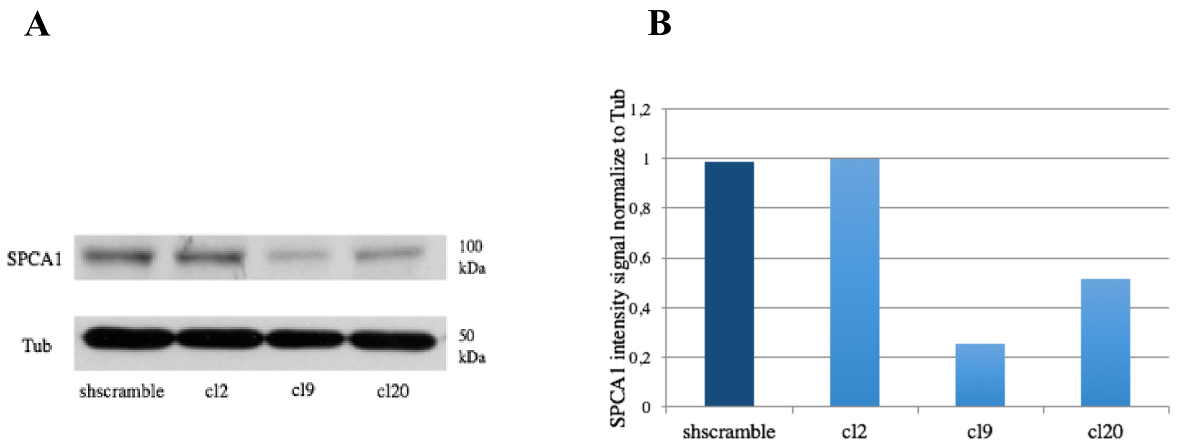


Figure 12. Characterization of MDCK GFP-FR shscramble and MDCK GFP-FR shSPCA1. (A) MDCK GFP-FR shscramble and shSPCA1cells were grown until confluency. Cell lysates were collected and analysed by western blot using SPCA1 and Tubulin (Tub) antibodies. The molecular weight of SPCA1 and Tubulin are indicated. (B) Quantification of endogenous SPCA1 in MDCK: GFP-FR shscramble and in the different MDCK GFP: FR shSPCA1. The amount of endogenous SPCA1 was normalized to the amount of endogenous Tubulin.

We firstly investigated the expression pattern of GFP-FR in confluent CTRLi and SPCA1i cells grown for 3 days on coverslip. In order to discriminate between intracellular GFP-FR and cell surface expression of GFP-FR we performed immunofluorescence in non-permeabilised condition allowing detecting only the cell surface expression of GFP-FR. On the Z projection (Figure 13) we revealed that in CRLi cells, GFP-FR is mostly at the cell surface (shown by the merge signal of GFP-FR and anti-GFP antibody) while in SPCA1i cells GFP-FR is found intracellularly and might be less abundant at the cell surface. This result indicates that lowering SPCA1 expression could affect the trafficking of GFP-FR.

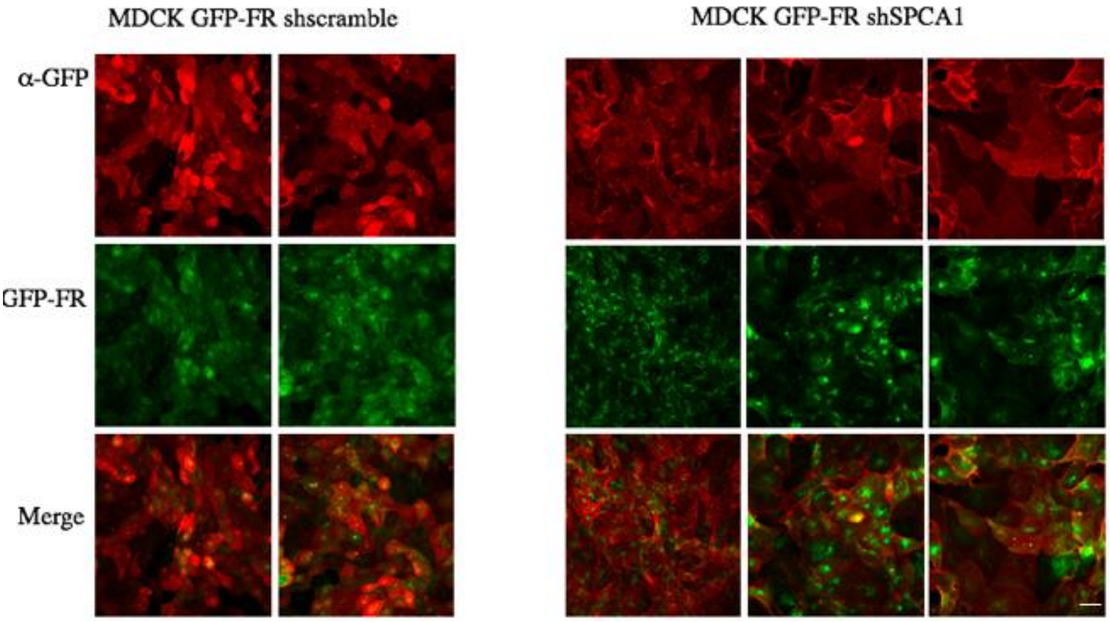


Figure 13. Localization of GFP-FR in polarized MDCK GFP-FR shscramble and MDCK GFP-FR shSPCA1 cells. MDCK cells were plated on coverslips for 3 days. Cells were fixed and stained with α-GFP antibody followed by secondary antibody under non-permeabilized conditions.Scale bars 10μm.

4.4 The trafficking of GFP-FR is delayed in MDCK GFP-FR shSPCA1 cells

The previous experiment in CTRLi and SPCA1i cells indicates that the localization of GFP-FR is affected upon knock down of SPCA1.

In order to test this hypothesis, we performed an exocytosis assay that allows studying the traffic of GFP-FR from the Golgi to the cell surface.

Polarized CTRLi and SPCA1i cells grown on coverslips were incubated at 19.5 degree for 2 hours in presence of protein synthesis inhibitor in the last hour. This first step allows accumulating GFP-FR in the Golgi compartment. Next, upon temperature release GFP-FR is allowed to exit from the Golgi and traffic to the cell surface.

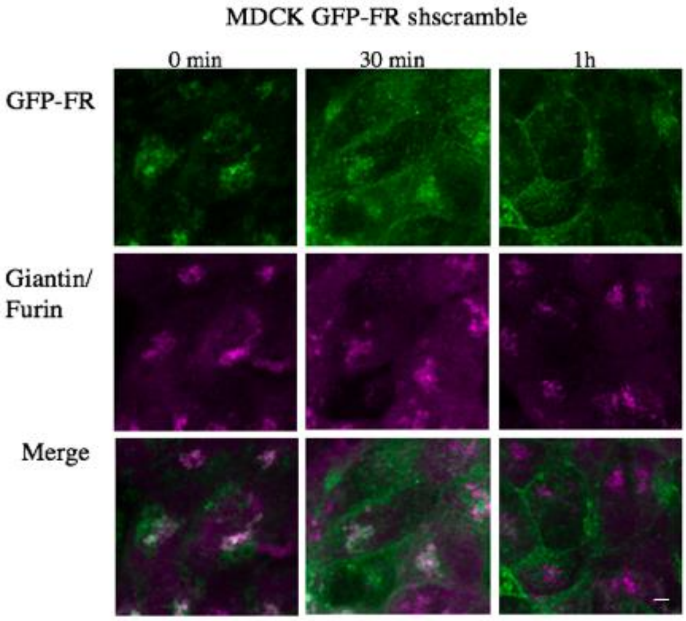
The cells were fixed after temperature block at 19.5°C (t=0min) or upon 30min or 1h of release at 37°c (t=30min and t=1h) and proceed for immunofluorescence (see material and methods). Then Pearson´s coefficient was measured in order to define the percentage of colocalization between GFP-FR and Golgi compartment at the different time points (by considering giantin/ furin as Golgi markers).

As shown in Figure 14A and 14C at t=0min, GFP-FR, colocalizes with the Golgi markers (Giantin and Furin), with a Pearson´s coefficient of 0,87±0,075 and 0,92± 0,026 in MDCK GFP-FR shscramble and MDCK GFP-FR shSPCA1 cells, respectively (Figure 14B, 14D), with no statistical difference between them.

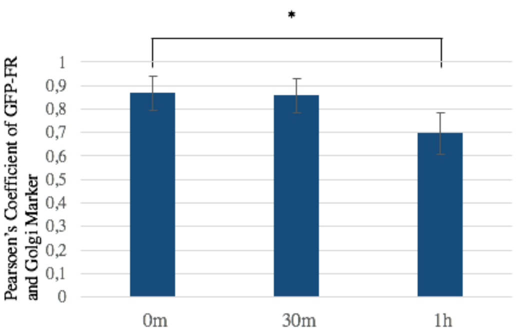
Upon 30 minutes of release in MDCK GFP-FR shscramble and MDCK GFP-FR shSPCA1 cells we see a small amount of GFP-FR at the cell surface, but almost all of it remains intracellularly, more specifically colocalized with Golgi markers (Figure 14A, 14C), with the Pearson´s coefficient between the Golgi markers and the GFP-FR decreasing slightly (CTRLi=0,857±0,07 and SPCA1i=0,898±0,05) (Figure 14B, 14D).

Upon 1h of release in CTRLi cells we recorded a statistical significant decrease of the Pearson´s coefficient between GFP-FR and the Golgi markers compared to t=0min revealing that as expected GFP-FR left the Golgi to reach the cell surface (from 0,87±0.075 to 0,696±0,087) (Figure 14A, 14B). In SPCA1i cells, upon 1h of release GFP-FR is still colocalizing with the Golgi markers as shown by the Pearson´s coefficient (from 0.92±0,026 to 0.855±0.043) suggesting a delay in the trafficking of GFP-FR upon reduction of SPCA1 expression.

A



B



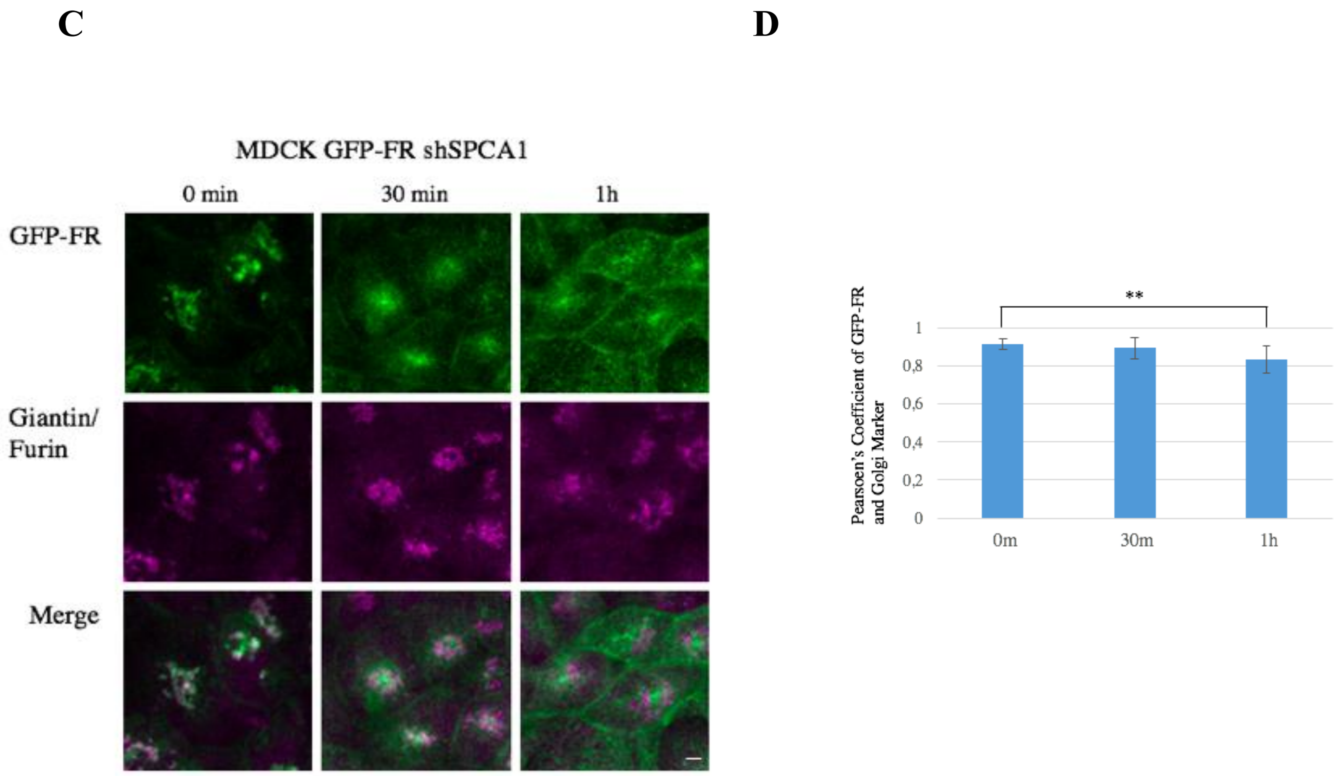


Figure 14. Silencing the SPCA1 pump delays the traffic of the GPI-FR. MDCK GFP-FR shscramble(A) and MDCK GFP-FR shSPCA1 (C) cells were plated on coverslips and grow for 3 days. Confluent MDCK cells were incubated at 19.5°C for 2h in areal medium and in the last hour cycloheximide was added. Cells were fixed (time 0) and alternatively cells were warmed at 37°C for 30min or 1h, in order to release proteins from the Golgi block before fixation. Then cells were permeabilized and stained with Giantin/Furin antibodies followed by secondary antibodies. Pearson´s coefficient of GFP-FR and Golgi markers in MDCK GFP-FR shScramble (B) and MDCK GFP-FR shSPCA1 (D) cells. MDCK cells were growth and treated like described before and analysed by ImajeJ software. Cells were analysed independently in order to avoid aspecificity. Experiments were performed two independent times (n>60 cells). Error bars indicate the S.D (*and ** p-value<0,05 (Student´s t-test)). Scale bars 10μm.

From the same set of exocytosis images we monitor both the heigh of the cells as well as the surface area of the Golgi that we have shown to reveal the polarization status of the cells (Imjeti et al. 2011). As shown in Figure 15 both the heigh and the Golgi surface area are similar in CTRLi and SPCA1i, highlighting that both cells were polarized with the same extent. This is an important control since the polarity status of the cell can affect protein sorting and trafficking.

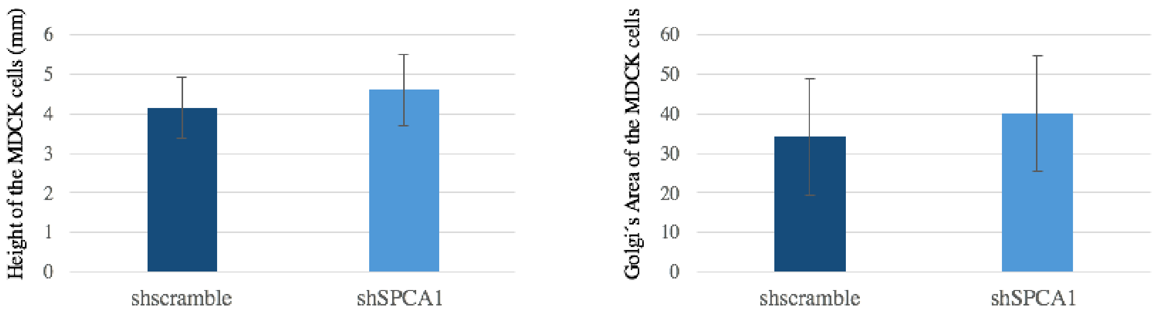


Figure 15. Height and Golgi´s Area of MDCK GFP-FR shSPCA1 and MDCK GFP-FR shscramble cells. MDCK GFP-FR shSPCA1 and MDCK GFP-FR shscramble cells were grown and treated like described in Figure 14 and analysed by ImajeJ software. Cells were analysed independently in order to avoid aspecificity. Experiments were performed two independent times (n>60 cells). Error bars indicate the S.D.

CHAPTER 5- Discussion

Clustering of GPI-APs in the Golgi is crucial for their correct sorting. This oligomerization in homo-clusters in MDCK cells, which is regulated by cholesterol and calcium, is essential to a proper organization and activity of GPI-APs at the plasma membrane. In fact, our lab reported that, in polarized MDCK cells, depletion of cholesterol results in the impairment of oligomerization and missorting of apical GPI-APs to the basolateral surface. Moreover, depletion of calcium affects GPI-APs clustering and its organization at the plasma membrane. Two proteins regulate the calcium level in the Golgi, the well known SERCA and the more recently identify SPCA1. Because SPCA1 is i) active in the TGN where GPI-APs abund and cluster (Lissandron et al. 2010), ii) its activity relies on the amount of cholesterol within the Golgi (Baron et al. 2010) and iii) it is found associated with cholesterol-rich domains (Baron et al. 2010), we hypothesized that SPCA1 could regulate clustering and trafficking of GPI-APs in the Golgi complex of polarized MDCK cells. Put back references

The first evident step of this project was to characterize the endogenous SPCA1 in MDCK cells, a known cellular model system to study protein trafficking on epithelial cell polarity.

As described for other cell lines (van Baelen et al. 2003) (Baron et al. 2010) (Sepúlveda et al. 2009), in MDCK cells, the SPCA1 pump is found colocalised with the Golgi both in polarized and non-polarized conditions. Interestingly, polarized MDCK cells exhibit a higher amount of endogenous SPCA1 compared to non-polarized conditions (Figure 10). We further revealed by cell fractionation that in both non-polarized and polarized conditions, 70% of SPCA1 is associated with the Golgi. These results are in correlation with our finding that polarized MDCK cells exhibit a higher calcium concentration in the Golgi complex compared to non-polarized MDCK cells (Lebreton et al. submitted) and suggest that SPCA1 would be required to regulate the uptake of calcium in the Golgi (see perspective).

By immunofluorescence, we highlighted that GFP-FR is found intracellularly in polarized MDCK cells where the expression of SPCA1 is lowered (SPCA1i) compared to control cells (CTRLi) (Figure 13) suggesting a role of SPCA1 in the trafficking of GFP-FR.

In order to directly tackle this scientific question, we performed an exocytosis assay in polarized MDCK cells transfected with scramble short hairpin (CTRLi) or whit short hairpin directed towars SPCA1 (SPCA1i). Importantly we found that in

SPCA1i (where the levels of SPCA1 was drastically decrease), after 1h of release of the temperature block GFP-FR is still colocalizing with Golgi markers indicating its retention in the Golgi and therefore a delay in its trafficking towards the cell surface.

We can therefore speculate that in cells where SPCA1 expression is lowered, a lower amount of calcium is uptaken in the Golgi leading to an impairement to apical GFP-APs clustering, which results in its Golgi retention. This retention could induce either a delay in its apical trafficking or alternatively to its basolateral sorting. By analysing exocytosis images we can think that in SPCA1i cells, GFP-FR will be basolaterally addressed but this observation has to be address (see perspectives).

CHAPTER 6- Perpectives

As explained earlier the first experiment to perform is to perform the exocytosis experiment in fully polarized CTRLi and SPCA1i cells by polarizing them on filters. This new set of experiment will allow us confirming the retention of GFP-FR in the Golgi and will help to decipher whether GFP-FR in SPCA1i cells is still address to the apical surface or is missorted to the basolateral surface.

Next, it will be important to perform live imaging experiments by using fast spinning disk system couple to 3D tracking analysis in order to follow in live fully polarized cells the speed and rate of trafficking of GFP-FR in CTRLi and SPCA1i cells. It will also be interesting to do it by considering also a model basolateral GPI-AP (Lebreton et al. 2008) to compare with apical GPI-AP.

Finally, it is also essential to directly determine the activity of SPCA1 in CTRLi and SPCA1i cells by using a TGN Ca^{2+} FRET sensor, which will measure the amount of calcium that is uptaken into the TGN. This experiment will allow directly correlating the expression of SPCA1 with the level of Calcium in the Golgi.

My scientific research highlights an unexpected and critical role of SPCA1 in the Golgi by directly regulating the trafficking of GPI-APs in epithelial cells and open new avenues of research to study protein-protein interaction in the context of specific environment (of lipid and ions).

CHAPTER 7- References

Achard, Patrick, et al. "The plant stress hormone ethylene controls floral transition via DELLA-dependent regulation of floral meristem-identity genes." *Proceedings of the National Academy of Sciences* 104.15 (2007): 6484-6489.

Ang, Agnes Lee, et al. "Recycling endosomes can serve as intermediates during transport from the Golgi to the plasma membrane of MDCK cells." *J Cell Biol* 167.3 (2004): 531-543.

Aroeti, Benjamin, and Keith E. Mostov. "Polarized sorting of the polymeric immunoglobulin receptor in the exocytotic and endocytotic pathways is controlled by the same amino acids." *The EMBO Journal* 13.10 (1994): 2297.

Assémat, Emeline, et al. "Polarity complex proteins." *Biochimica et Biophysica Acta (BBA)-Biomembranes* 1778.3 (2008): 614-630.

Aulestia, Francisco J., María Teresa Alonso, and Javier García-Sancho. "Differential calcium handling by the cis and trans regions of the Golgi apparatus." *Biochemical Journal* 466.3 (2015): 455-465.

Baron, Szilvia, et al. "The secretory pathway Ca 2+-ATPase 1 is associated with cholesterol-rich microdomains of human colon adenocarcinoma cells." *Biochimica et Biophysica Acta (BBA)-Biomembranes* 1798.8 (2010): 1512-1521.

Bonifacino, Juan S. "Adaptor proteins involved in polarized sorting." *J Cell Biol*204.1 (2014): 7-17.

Bonifacino, Juan S., and Linton M. Traub. "Signals for sorting of transmembrane proteins to endosomes and lysosomes." *Annual review of biochemistry* 72.1 (2003): 395-447.

Brown, Deborah A., Bruce Crise, and John K. Rose. "Mechanism of membrane anchoring affects polarized expression of two proteins in MDCK cells." *Science*245.4925 (1989): 1499-1502.

Cancino, Jorge, et al. "Antibody to AP1B adaptor blocks biosynthetic and recycling routes of basolateral proteins at recycling endosomes." *Molecular biology of the cell* 18.12 (2007): 4872-4884.

Chandra, Subhash, et al. “Imaging of total intracelular calcium and calcium influx and efflux in individual resting and stimulated tumor mast cells using ion microscopy.” *Journal of BIological Chemistry* 269.21 (1994): 15186-15194.

Citi, Sandra, et al. "Epithelial junctions and Rho family GTPases: the zonular signalosome." *Small GTPases* 5.4 (2014): e973760.

Cresawn, Kerry O., et al. "Differential involvement of endocytic compartments in the biosynthetic traffic of apical proteins." *The EMBO journal* 26.16 (2007): 3737-3748.

Crevenna, Alvaro H., et al. "Secretory cargo sorting by Ca2+-dependent Cab45 oligomerization at the trans-Golgi network." *J Cell Biol* (2016): jcb-201601089.

Dang, Donna, and Rajini Rao. "Calcium-ATPases: gene disorders and dysregulation in cancer." *Biochimica et Biophysica Acta (BBA)-Molecular Cell Research* 1863.6 (2016): 1344-1350.

Dempsey, Peter J., Katherine S. Meise, and Robert J. Coffey. "Basolateral sorting of transforming growth factor-α precursor in polarized epithelial cells: characterization of cytoplasmic domain determinants." *Experimental cell research* 285.2 (2003): 159-174.

Deora, Ami A., et al. "The basolateral targeting signal of CD147 (EMMPRIN) consists of a single leucine and is not recognized by retinal pigment epithelium." *Molecular biology of the cell* 15.9 (2004): 4148-4165.

Dillon, Christian, et al. "Basolateral targeting of ERBB2 is dependent on a novel bipartite juxtamembrane sorting signal but independent of the C-terminal ERBIN-binding domain." *Molecular and cellular biology* 22.18 (2002): 6553-6563.

Edeling, Melissa A., Corinne Smith, and David Owen. "Life of a clathrin coat: insights from clathrin and AP structures." *Nature Reviews Molecular Cell Biology* 7.1 (2006): 32-44.

Fuller, S. D., R. Bravo, and K. Simons. "An enzymatic assay reveals that proteins destined for the apical or basolateral domains of an epithelial cell line share the same late Golgi compartments." *The EMBO journal* 4.2 (1985): 297.

Goldenring, James R. "A central role for vesicle trafficking in epithelial neoplasia: intracellular highways to carcinogenesis." *Nature Reviews Cancer*13.11 (2013): 813-820.

Gravotta, Diego, et al. "AP1B sorts basolateral proteins in recycling and biosynthetic routes of MDCK cells." *Proceedings of the National Academy of Sciences* 104.5 (2007): 1564-1569.

Grice, Desma M., et al. "Golgi calcium pump secretory pathway calcium ATPase 1 (SPCA1) is a key regulator of insulin-like growth factor receptor (IGF1R) processing in the basal-like breast cancer cell line MDA-MB-231." *Journal of Biological Chemistry* 285.48 (2010): 37458-37466.

Griffiths, Gareth, and Kai Simons. "The trans Golgi network: sorting at the exit site of the Golgi complex." *Science* 234.4775 (1986): 438-443.

Harder, Thomas, and Dhaval Sangani. "Plasma membrane rafts engaged in T cell signalling: new developments in an old concept." *Cell Communication and Signaling* 7.1 (2009): 21.

He, Cheng, et al. "The epidermal growth factor receptor juxtamembrane domain has multiple basolateral plasma membrane localization determinants, including a dominant signal with a polyproline core." *Journal of Biological Chemistry*277.41 (2002): 38284-38293.

Iden, Sandra, and John G. Collard. "Crosstalk between small GTPases and polarity proteins in cell polarization." *Nature reviews Molecular cell biology* 9.11 (2008): 846-859.

Imjeti, Naga Salaija, et al. "N-Glycosylation instead of cholesterol mediates oligomerization and apical sorting of GPI-APs in FRT cells." *Molecular biology of the cell* 22.23 (2011): 4621-4634.

Kadir, Shereen, et al. "Microtubule remodelling is required for the front–rear polarity switch during contact inhibition of locomotion." *J Cell Sci* 124.15 (2011): 2642-2653.

Kienzle, Christine, and Julia von Blume. "Secretory cargo sorting at the trans-Golgi network." *Trends in cell biology* 24.10 (2014): 584-593.

Lebreton, Stéphanie, Simona Paladino, and Chiara Zurzolo. "Selective roles for cholesterol and actin in compartmentalization of different proteins in the Golgi and plasma membrane of polarized cells." *Journal of Biological Chemistry*283.43 (2008): 29545-29553.

Le Gall, Annick H., et al. "The neural cell adhesion molecule expresses a tyrosine-independent basolateral sorting signal." *Journal of Biological Chemistry*272.7 (1997): 4559-4567.

Lisanti, Michael P., and Michael A. Davitz. "A glycophospholipid membrane anchor acts as an apical targeting signal in polarized epithelial cells." *J. Cell Biol* 109 (1989): 2145-2156.

Lisanti, Michael P., et al. "Polarized apical distribution of glycosyl-phosphatidylinositol-anchored proteins in a renal epithelial cell line." *Proceedings of the National Academy of Sciences* 85.24 (1988): 9557-9561.

Lissandron, Valentina, et al. "Unique characteristics of Ca2+ homeostasis of the trans-Golgi compartment." *Proceedings of the National Academy of Sciences*107.20 (2010): 9198-9203.

Lock, John G., and Jennifer L. Stow. "Rab11 in recycling endosomes regulates the sorting and basolateral transport of E-cadherin." *Molecular biology of the cell* 16.4 (2005): 1744-1755.

Manninen, Aki. "Epithelial polarity–generating and integrating signals from the ECM with integrins." *Experimental cell research* 334.2 (2015): 337-349.

Matter, Karl, and Ira Mellman. "Mechamisms of cell polarity: sorting and transport in epithelial cells." *Current opinion in cell biology* 6.4 (1994): 545-554.

Mellman, Ira, and W. James Nelson. "Coordinated protein sorting, targeting and distribution in polarized cells." *Nature reviews Molecular cell biology* 9.11 (2008): 833-845.

Missiaen, Ludwig, et al. "Calcium in the Golgi apparatus." *Cell calcium* 41.5 (2007): 405-416.

Mostov, Keith E., and Michael H. Cardone. "Regulation of protein traffic in polarized epithelial cells." *Bioessays* 17.2 (1995): 129-138.

Mostov, Keith, Tao Su, and Martin ter Beest. "Polarized epithelial membrane traffic: conservation and plasticity." *Nature Cell Biology* 5.4 (2003): 287-293.

Muniz, Manuel, and Chiara Zurzolo. "Sorting of GPI-anchored proteins from yeast to mammals–common pathways at different sites?." *J Cell Sci* 127.13 (2014): 2793-2801.

Neumüller, Ralph A., and Juergen A. Knoblich. "Dividing cellular asymmetry: asymmetric cell division and its implications for stem cells and cancer." *Genes & development* 23.23 (2009): 2675-2699.

Nosjean, Olivier, Anne Briolay, and Bernard Roux. "Mammalian GPI proteins: sorting, membrane residence and functions." *Biochimica et Biophysica Acta (BBA)-Reviews on Biomembranes* 1331.2 (1997): 153-186.

O'Brien, Lucy Erin, Mirjam MP Zegers, and Keith E. Mostov. "Building epithelial architecture: insights from three-dimensional culture models." *Nature Reviews Molecular Cell Biology* 3.7 (2002): 531-537.

Odorizzi, Greg, and Ian S. Trowbridge. "Structural requirements for basolateral sorting of the human transferrin receptor in the biosynthetic and endocytic pathways of Madin-Darby canine kidney cells." *The Journal of cell biology* 137.6 (1997): 1255-1264.

Overeem, Arend W., David M. Bryant, and Sven CD van IJzendoorn. "Mechanisms of apical–basal axis orientation and epithelial lumen positioning." *Trends in cell biology* 25.8 (2015): 476-485.

Paladino, Simona, et al. "Different GPI-attachment signals affect the oligomerisation of GPI-anchored proteins and their apical sorting." *J Cell Sci*121.24 (2008): 4001-4007.

Paladino, Simona, et al. "Golgi sorting regulates organization and activity of GPI proteins at apical membranes." *Nature chemical biology* 10.5 (2014): 350-357.

Paladino, Simona, et al. "Protein oligomerization modulates raft partitioning and apical sorting of GPI-anchored proteins." *J Cell Biol* 167.4 (2004): 699-709.

Parton, Robert G., and Ayanthi A. Richards. "Lipid rafts and caveolae as portals for endocytosis: new insights and common mechanisms." *Traffic* 4.11 (2003): 724-738.

Pike, Linda J. "Lipid rafts bringing order to chaos." *Journal of lipid research* 44.4 (2003): 655-667.

Rindler, Michael J., et al. "Viral glycoproteins destined for apical or basolateral plasma membrane domains traverse the same Golgi apparatus during their intracellular transport in doubly infected Madin-Darby canine kidney cells." *J Cell Biol* 98.4 (1984): 1304-1319.

Rodriguez-Boulan, Enrique, Geri Kreitzer, and Anne Müsch. "Organization of vesicular trafficking in epithelia." *Nature reviews Molecular cell biology* 6.3 (2005): 233-247.

Rodriguez-Boulan, Enrique, and Ian G. Macara. "Organization and execution of the epithelial polarity programme." *Nature reviews Molecular cell biology* 15.4 (2014): 225-242.

Sabharanjak, Shefali, and Satyajit Mayor. "Folate receptor endocytosis and trafficking." *Advanced drug delivery reviews* 56.8 (2004): 1099-1109.

Sepúlveda, M. Rosario, et al. "Functional and immunocytochemical evidence for the expression and localization of the secretory pathway Ca2+-ATPase isoform 1 (SPCA1) in cerebellum relative to other Ca2+ pumps." *Journal of neurochemistry* 103.3 (2007): 1009-1018.

Sepúlveda, M. Rosario, et al. "Silencing the SPCA1 (secretory pathway Ca2+-ATPase isoform 1) impairs Ca2+ homeostasis in the Golgi and disturbs neural polarity." *Journal of Neuroscience* 29.39 (2009): 12174-12182.

Simons, Kai, and Derek Toomre. "Lipid rafts and signal transduction." *Nature reviews Molecular cell biology* 1.1 (2000): 31-39.

Simons, Kai, and Elina Ikonen. "Functional rafts in cell membranes." *Nature*387.6633 (1997): 569.

Simons, Kai, and Mathias J. Gerl. "Revitalizing membrane rafts: new tools and insights." *Nature reviews Molecular cell biology* 11.10 (2010): 688-699.

Stoops, Emily H., and Michael J. Caplan. "Trafficking to the apical and basolateral membranes in polarized epithelial cells." *Journal of the American Society of Nephrology* 25.7 (2014): 1375-1386.

Tahirovic, Sabina, and Frank Bradke. "Neuronal polarity." *Cold Spring Harbor perspectives in biology* 1.3 (2009): a001644.

Takano, Tetsuya, et al. "Neuronal polarization." *Development* 142.12 (2015): 2088-2093.

Van Baelen, Kurt, et al. "The contribution of the SPCA1 Ca 2+ pump to the Ca 2+ accumulation in the Golgi apparatus of HeLa cells assessed via RNA-mediated interference." *Biochemical and biophysical research communications*306.2 (2003): 430-436.

van Meer, Gerrit, and Kai Simons. "Lipid polarity and sorting in epithelial cells." *Journal of cellular biochemistry* 36.1 (1988): 51-58.

von Blume, Julia, et al. "ADF/cofilin regulates secretory cargo sorting at the TGN via the Ca 2+ ATPase SPCA1." *Developmental cell* 20.5 (2011): 652-662.

von Blume, Julia, et al. "Cab45 is required for Ca2+-dependent secretory cargo sorting at the trans-Golgi network." *J Cell Biol* 199.7 (2012): 1057-1066.

Weisz, Ora A., and Enrique Rodriguez-Boulan. "Apical trafficking in epithelial cells: signals, clusters and motors." *Journal of cell science* 122.23 (2009): 4253-4266.

Yeaman, Charles, Kent K. Grindstaff, and W. James Nelson. "New perspectives on mechanisms involved in generating epithelial cell polarity." *Physiological reviews* 79.1 (1999): 73-98.