The role of Cab45 in the apical sorting of GPI-APs and soluble proteins in polarized MDCK cells

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Le rôle de Cab45 dans le tri apical des GPI-APs et des protéines solubles dans les cellules MDCK polarisées

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ÉCOLE DOCTORALE
Structure et dynamique des systèmes vivants (SDSV)

Titre : Le rôle de Cab45 dans le tri apical des GPI-APs et des protéines solubles dans les cellules MDCK polarisées

Mots clés : Polarité ; Épithélium ; Golgi ; Calcium ; Cab45

Résumé : Les cellules eucaryotes disposent de la capacité de se polariser de façon transitoire ou permanente. La polarité cellulaire est fondamentale pour la fonction cellulaire et repose notamment sur l'établissement d'un trafic intracellulaire polarisé. Les cellules polarisées ont donc développé des mécanismes cellulaires hautement régulés afin d'établir et maintenir ce trafic intracellulaire polarisé essentiel à cette polarité cellulaire. L'appareil de Golgi et plus précisément le TGN est la plateforme majeure de tri des protéines et lipides au sein des cellules. Les cellules épithéliales polarisées se caractérisent par leur asymétrie de la membrane plasmique divisée en deux domaines, apical et basolatéral, distincts en composition protéique et lipidique mais aussi en fonction. Bien qu'étudiés depuis des décennies certains mécanismes sous-tendant l'établissement et le maintien de la polarité épithéliale sont toujours méconnus. Alors que les mécanismes de tri des protéines membranaires à la surface apicale ou basolaterale ont été bien identifié, ceux régissant le tri apical des protéines glypiées ou solubles sont toujours peu élucidés. Au cours de ces dernières années, nos études ont montré que le tri apical des protéines glypiées dans les cellules épithéliales polarisées reposent sur leur capacité à former des complexes de haut poids moléculaires ou oligomères ou cluster au niveau de l'appareil de Golgi. Plus récemment nous avons révélé que la formation de cluster de protéines glypiées au sein de l'appareil de Golgi régule le tri apical de ces protéines à la surface cellulaire mais également leur organisation à la surface membranaire apicale ainsi que leurs activités biologiques. Néanmoins, à part le rôle essential du contenu en cholestérol des membranes Golgiennes, les facteurs moléculaires régulant l'oligomérisation des protéines glypiées dans le Golgi sont inconnus. Ici on revèle pour la première fois le rôle critique des ions calcium au niveau du Golgi dans le tri apical des protéines glypiées. Plus précisément, une pompe à calcium et manganèse, SPCA1, située au niveau du TGN permet l'entrée d'ions calcium dans ce compartiment conduisant à l'oligomérisation de Cab45, une protéine Golgienne capable de lier le calcium, qui stabiliserait les clusters de protéines glypiées afin de permettre leur tri apical. Une diminution de l'expression de SPCA1 ou Cab45 réduit la capacité des protéines glypiées à former des clusters dans le Golgi conduisant à leur tri aberrant à la surface basolatérale. De plus, nous avons montré que cette même protéine Cab45 régule également le tri apical d'une protéine soluble secrétée depuis la membrane apicale dans les conditions contrôles PLAP-sec qui est une forme tronquée de la protéine glypiée PLAP à laquelle l'ancre GPI a été enlevé. En conclusion, ce travail révèle un rôle inattendu du calcium dans le tri apical des protéines glypiées et identifie SPCA1 et Cab45 comme étant les régulateurs de l'organisation de ces protéines glypiées en oligomère dans l'appareil de Golgi. Enfin, il est fascinant que la même protéine Cab45 puisse gouverner à la fois le tri apical 1) de protéines glypiées associées aux domaines membranaires enrichies en cholestérol et sphingolipides mais également 2) d'une protéine soluble reportée comme non-associée à ces mêmes domaines membranaires. Nos résultats nous permettent de mieux appréhender les mécanismes d'exocytoses dans les cellules épithéliales polarisées et confirment l'existence de nombreuses voies d'exocytoses différentes pour des protéines transmembranaires ou glypiées ou solubles.

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ÉCOLE DOCTORALE
Structure et dynamique des systèmes vivants (SDSV)

Title: The role of Cab45 in the apical sorting of GPI-APs and soluble proteins in polarized MDCK cells

Keywords: Polarized ; Epithelial ; Golgi ; Calcium ; Cab45

Abstracts: Eukaryotic cells exhibit the capacity to polarize either transiently or permanently. In all cases cell polarity is fundamental for proper cellular functions and relies of polarized intracellular protein trafficking and secretion. Therefore, nature evolved highly elaborated and finely tuned machineries to sort and transport proteins to their correct destinations. Within the cell, the Trans Golgi network (TGN) is the main sorting hub allowing to selectively sort lipids and proteins. Polarized epithelial cells are characterized by the asymmetry of their plasma membrane divided into apical and basolateral domains that differ in protein, lipid composition and in function. Although studied since decades, how epithelial cell establish and maintain their polarity is still an open question in cell biology. While the sorting and transport of either apical or basolateral transmembrane proteins has well been studied, our knowledge regarding the mechanisms regulating the apical sorting of glycosylphosphatidylinositol-anchored proteins (GPI-APs) or soluble cargoes in polarized epithelial cells is more fragmented. In recent years, our group reported that cholesterol-dependent clustering of GPI-APs in the Golgi is the key step driving their apical sorting and their further plasma membrane organization and biological activities. However, the specific molecular factor regulating the formation of GPI-APs clustering in the Golgi is unknown. Here, we show that the clustering of GPI-APs in the Golgi relies directly on the levels of calcium within this organelle. Specifically, we further demonstrate that SPCA1, the TGN calcium/manganese pump that regulates Calcium entry in the TGN, and Cab45, a calcium-binding Golgi luminal resident protein, are essential for Golgi clustering of GPI-APs and therefore for their apical sorting. Silencing of SPCA1 or Cab45 in polarized MDCK cells impairs Golgi GPI-APs clustering leading to their basolateral mis-sorting. Intriguingly, we also found that Cab45 regulates the apical secretion of the soluble PLAP-sec protein (a truncated PLAP, apical GPI-AP, devoid of its GPI-anchor). Silencing of Cab45 results in the mis-secretion of PLAP -sec from the basolateral cell surface. In conclusion, this thesis reveals for the first time an unexpected role of calcium ions in the apical exocytosis of GPI-APs and identifies SPCA1 and Cab45 as master regulators of Golgi GPI-APs clustering. Additionally, it is fascinating that the same calcium binding Golgi resident protein Cab45 regulates the apical sorting of i) raft-associated GPI-AP and ii) soluble PLAP-sec protein. While our discovery represents a fundamental advance in our general understanding of the exocytosis in polarized epithelial cells, they also greatly improve our knowledge on the machinery regulating polarized trafficking of GPI-APs and soluble cargoes.

不积跬步，无以至千里；不积小流，无以成江海。---荀子
A journey of a thousand miles may not be achieved without accumulation of each single step, just as the enormous ocean may not be formed without gathering every brook or stream.

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Synthèse en français

Les cellules eucaryotes présentent la capacité de se polariser de manière transitoire ou permanente. Dans tous les cas, la polarité cellulaire est fondamentale pour le bon fonctionnement cellulaire et repose sur le trafic intracellulaire polarisée et la sécrétion de protéines. Par conséquent, les cellules ont développé des mécanismes moléculaires hautement élaborés et finement régulés pour trier et transporter les protéines vers leurs destinations finales. La plupart des cancers humains ont une origine épithéliale et la perte de cette polarité épithéliale est une étape critique vers la malignité. De ce fait, comprendre les mécanismes moléculaires permettant l'établissement et le maintien de la polarité épithéliale demeure d'un intérêt fondamental.

Les cellules épithéliales polarisées sont caractérisées par l'asymétrie de leur membrane plasmique divisée en domaines apical et basolatéral qui diffèrent par leurs composition protéiques et lipidiques mais également de par leurs fonctions. Au sein de la cellule, l'appareil de Golgi est le principal pôle de tri des protéines et lipides. Cet organelle gère les flux membranaires des voies sécrétoires et endocytiques, triant des cargoes très diverses en topologie et en taille, telles que les protéines transmembranaires, les protéines glypiées (GPI-AP) et les cargoes solubles. Environ 1/3 des protéines humaines passent par l'appareil de Golgi avant leur destination finale. Dans les cellules épithéliales, les protéines et les lipides atteignent leur destination finale soit par une voie directe où les protéines transitent directement du Golgi/ Trans Golgi network (TGN) vers la membrane apicale ou basolatérale (Mogelsvang et al. 2004) soit par une voie indirecte. Dans la voie indirecte également appelée transcytose, les protéines sont d'abord ciblées vers la membrane basolatérale avant d'être endocytosées et redirigées vers la surface apicale. Alors que le tri et le transport des protéines transmembranaires apicales ou basolatérales ont été bien étudiés, nos connaissances concernant les mécanismes régulant le tri apical des

protéines GPI-AP ou des cargoes solubles dans les cellules épithéliales polarisées sont plus fragmentées.

Les GPI-AP sont des protéines sécrétoires luminales associées aux lipides triées sélectivement à la surface apicale de l'épithélium, où elles résident et jouent diverses fonctions vitales. Ces dernières années, notre groupe a montré que seuls les GPI-AP apicaux forment des complexes de hauts poids moléculaires (HMW) ou oligomers ou clusters dans l'appareil de Golgi de cellules entièrement polarisées (et non dans des cellules non polarisées). L'altération de l'oligomérisation des GPI-APs conduit à leur mauvais tri vers la membrane plasmique basolatérale. Cela signifie que les HMW ou clusters des GPI-AP dans l'appareil de Golgi est essentiel pour leur tri apical dans les cellules épithéliales polarisées. Ils ont également révélé que les taux de cholestérol dans l'appareil de Golgi régulent la formation de HMW ou oligomers des GPI-APs et donc leur tri apical mais également leurs organisations ainsi que leurs activités biologiques à la surface apicale. Cependant, outre le cholestérol, quels sont les facteurs moléculaires régulant le clustering des GPI-APs au niveau du complexe Golgien est encore mal compris.

L'appareil de Golgi présente des niveaux élevés de calcium qui se sont révélés essentiels pour le le tri de certaines protéines solubles sécrétées dans des cellules HeLa (Chandra et al. 1991; Kienzle et von Blume 2014; Micaroni 2012; Pinton, Pozzan et Rizzuto 1998). Sur la base de ces preuves, nous avons analysé le rôle possible des ions calcium dans le complexe de Golgi dans la formation d'oligomers des GPI-AP et leur tri à la surface apicale.

Dans cette étude, nous avons mesuré la concentration de calcium dans l'appareil de Golgi en utilisant une photoprotéine sensible au calcium (chimère de Golgi-aequorine) et avons constaté que dans les cellules Madin-Darby Canine Kidney (MDCK), les niveaux de calcium dans l'appareil de Golgi des cellules polarisées sont plus élevés que dans les cellules non polarisées où les GPI-APs sont organisés en

monomères/ dimères. Afin d'analyser le rôle putatif des ions calcium comme régulateur de la formation des clusters GPI-APs, nous avons spécifiquement réduit la concentration du calcium en traitant des cellules MDCK polarisées avec de l'ionomycine, un ionophore qui permet de vider efficacement et rapidement le calcium contenu dans les différents compartiments intracellulaires dont le Golgi. Dans ces conditions, nous avons observé une réduction de la formation des oligomères de GFP-FR au niveau de l'appareil de Golgi ce qui indique que la concentration de calcium dans la lumière de Golgi régule la formation des clusters de GPI-AP dans les cellules épithéliales polarisées.

La concentration de calcium dans l'appareil de Golgi est régulée par deux groupes de pompes à calcium, les SERCA bien connus (sarcoendoplasmic-reticulum Ca-ATPases) et les SPCA plus récemment découvertes (Missiaen et al. 2007). Il est intéressant de noter que l'activité de la SPCA1 a été montrée comme étant dépendante du cholestérol et de la sphingomyéline (Baron et al. 2010). Ainsi, nous avons d'abord évalué si SPCA1 régule la concentration de calcium dans l'appareil de Golgi des cellules épithéliales polarisées. Fait intéressant, le Western blot a montré que les cellules MDCK entièrement polarisées présentent des quantités relativement plus élevées de SPCA1 par rapport aux cellules MDCK non polarisées, suggérant que les niveaux d'expression de SPCA1 peuvent augmenter avec l'établissement de la polarité. Après avoir réduit l'expression de SPCA1 par knockdown, il y a une forte réduction de la concentration de calcium dans le Golgi de ces cellules, indiquant que la pompe SPCA1 régule l'absorption de calcium dans l'appareil de Golgi des cellules MDCK. Dans ces cellules MDCK GFP-FR où l'expression de SPCA1 est réduite, nous avons révélé la diminution des clusters de GFP-Fr dans l'appareil de Golgi conduisant à son tri à la surface basolatéral. Dans l'ensemble, ces résultats montrent que le tri apical des protéines GPI-APs est dépendant des ions calcium dans le Golgi dont la concentration est régulée par SPCA1.

Pour tester si SPCA1 avait un rôle spécifique dans le tri apical des GPI-APs, nous avons analysé la distribution membranaire de protéines transmembranaires apicale et basolatérale endogène, GP114, et E-cadhérine respectivement dans les cellules de contrôle et dans les cellules knockdown pour SPCA1. Nos données n'ont montré aucun changement dans la localisation polarisée de ces protéines transmembranaires. Comme contrôle supplémentaire, nous avons également analysé l'intégrité de la protéine jonctionnelle ZO1, montrant que le knockdown de SPCA1 n'altère pas l'assemblage des complexes jonctionnels et donc l'intégrité des monocouches de cellules épithéliales. Dans l'ensemble, ces données révèlent que SPCA1 joue un rôle spécifique dans la régulation des clusters de GPI-APs dépendant du calcium dans le Golgi et leur tri vers la surface apicale.

Il a déjà été démontré que les niveaux de calcium dépendant de SPCA1 dans l'appareil de Golgi régulent la ségrégation d'un sous-ensemble de protéines sécrétoires Cartilage Oligomeric Matrix Protein (COMP) dans les cellules HeLa (Blank et von Blume 2011; von Blume et al. 2009). La sécrétion de ces cargoes est dépendante de Cab45, une protéine de liaison au calcium luminale de Golgi qui oligomérise avec sa liaison au calcium et interagit sélectivement avec ces cargoes sécrétoires solubles, permettant leur exportation (Blank et von Blume 2017; von Blume et al. 2012; Crevenna et al. 2016 ; Pakdel et von Blume 2018, Scherer et al. 1996; von Blume et al. 2011). Ces données nous ont incité à rechercher si Cab45 pourrait être impliqué dans la régulation du tri apical des GPI-AP dans les cellules polarisées.

Nous avons d'abord analysé l'expression et la localisation de Cab45 dans les cellules MDCK, et nous avons constaté que, comme les cellules HeLa, Cab45 est enrichi au niveau du TGN. Les analyses qRT-PCR et Western blot ont montré que les niveaux d'ARNm et de protéines de Cab45 sont plus élevés dans les cellules MDCK non polarisées par rapport aux conditions polarisées. Il est intéressant de noter que Cab45 est principalement monomérique dans les cellules MDCK non polarisées, alors qu'elle forme des complexes HMW dans les cellules MDCK polarisées soutenant une

corrélation entre l'oligomérisation de Cab45 et les niveaux plus élevés de calcium dans l'appareil de Golgi des cellules MDCK polarisées comparés aux cellules MDCK non polarisées.

Pour comprendre la fonction de Cab45 dans le tri des protéines dans les cellules MDCK polarisées, nous avons généré une lignée cellulaire Knockdown Cab45 MDCK : GFP-FR stable et analysé l'état d'agrégation de GFP-FR au niveau de l'appareil de Golgi et nous avons constaté une réduction des oligomers de GFP-FR conduisant à son tri à la surface basolatéral. Nous avons également montré que toutes les protéines glypiées endogènes dans les cellules MDCK polarisées sont triées basolatéralement dans les cellules où l'expression de Cab45 est réduite.

Nous avons également détecté la distribution polarisée des protéines transmembranaires apicales et basolatérales, GP114 et E-cadhérine, respectivement. Les deux n'ont pas été affectés dans les cellules knockdown Cab45, ce qui confirme la spécificité du rôle de Cab45 dans le tri apical des GPI-AP. En conclusion, Cab45 est un modulateur calcium-dépendant du clustering GPI-AP et de leur tri apical.

Dans l'ensemble, pour la première fois, nos données révèlent un rôle inattendu du calcium dans le mécanisme de tri apical des GPI-AP dans les cellules épithéliales polarisées et identifient la machinerie moléculaire impliquée dans le regroupement des GPI-AP dans l'appareil de Golgi. Nos résultats représentent une avancée fondamentale dans la compréhension générale des mécanismes de l'exocytose dans les cellules épithéliales polarisées qui sont cruciales pour l'établissement et le maintien de la polarité des cellules épithéliales. En outre, nos données améliorent également les connaissances sur la machinerie régulant le trafic polarisé des protéines à ancrage GPI, une classe de protéines associées aux lipides jouant diverses fonctions vitales, révélant un rôle inattendu du calcium dans leur tri apical dans les cellules épithéliales polarisées.

Par rapport aux GPI-AP, nos connaissances sur les mécanismes régulant le tri apical des cargaisons solubles dans les cellules épithéliales polarisées sont plus fragmentées. La sécrétion de protéines solubles, selon les types cellulaires, se fait par les voies sécrétoires constitutives ou régulées. Dans le cas de la voie sécrétoire constitutive, les protéines sont sécrétées aussi vite qu'elles sont synthétisées : après tri de Golgi elles sont incorporées dans des vésicules de sécrétion qui par fusion avec la membrane plasmique permettent la libération de leur contenu vésiculaire par exocytose. Dans les cellules sécrétoires professionnelles telles que les neurones ou les cellules endocrines, l'exocytose des protéines est régie par le stockage de protéines nouvellement synthétisées dans des vésicules intracellulaires, appelées granules de stockage sécrétoires, qui fusionnent avec la membrane plasmique pour libérer leur contenu dans l'espace extracellulaire uniquement à l'arrivée d'un stimulus extracellulaire spécifique.

Jusqu'à présent, différents mécanismes de tri ont été décrits pour les cargoes solubles reposant soit sur l'agrégation des protéines, soit sur un mécanisme à médiation par les récepteurs au niveau du TGN. Dans les cellules exocrines ou endocrines professionnelles, les protéines solubles telles que les granines s'agrègent dans le TGN pour favoriser leur incorporation dans les granulés immatures qui mûrissent davantage de manière intracellulaire avant leur exocytose lors d'un stimulus extracellulaire. Ce modèle d'agrégation de protéines TGN nécessite une concentration élevée en calcium et une acidification du pH dans le TGN. Récemment, une voie de sécrétion de sphingomyéline (SMS) a été décrite dans les cellules HeLa impliquant une interaction entre l'actine, le calcium et la sphingomyéline. Plus précisément, il a été démontré que la cofiline recrute le cytosquelette d'actine qui se lie et favorise l'activation de SPCA1 permettant l'absorption des ions calcium dans le TGN qui à son tour conduit à l'oligomérisation de Cab45, ce qui favorise l'exocytose d'un sous-ensemble de cargoes solubles telles que COMP ou lysozyme C (LyzC) (Blank and

von Blume 2017; von Blume et al. 2012; Crevenna et al. 2016; Pakdel and von Blume 2018; Scherer et al. 1996; von Blume et al. 2011).

Comme nous avons révélé un rôle inattendu des ions calcium, SPCA1 et Cab45 dans le tri apical des GPI-AP dans les cellules MDCK polarisées. Ici, nous nous sommes donc demandé si le Calcium et Cab45 pouvaient réguler la sécrétion apicale dans les cellules épithéliales polarisées.

À cette fin, nous avons utilisé comme protéine modèle une forme tronquée de la phosphatase alcaline placentaire GPI-AP native (PLAP) dépourvue de son signal d'attachement GPI, PLAP-sec, dont nous avons précédemment démontré qu'elle est sécrétée apicale dans les cellules polarisées de la thyroïde. (Lipardi et al, 2000; Lipadi et al, 2002). De plus, notre choix est également dicté pour sa pertinence physiopathologique puisque l'augmentation de la sécrétion de phosphatase alcaline est la principale caractéristique phénotypique du syndrome de retard mental hyperphosphatasie (HPMR), une maladie autosomique récessive. (Murakami et al. 2012).

Afin de déchiffrer si un mécanisme dépendant du calcium, Cab45, régule la sécrétion apicale de de PLAP-sec dans les cellules MDCK polarisées, nous avons observé que PLAP-sec intracellulaire co-localise partiellement avec le KDEL un marqueur du réticulum endoplasmique RE par immunofluorescence. Nous avons ensuite évalué le taux de sécrétion de PLAP-sec pendant la polarisation épithéliale en analysant la quantité de PLAP-sec dans les milieux collectés après 1 jour ou 4 jours de culture sur boîte, des conditions qui imitent l'état non polarisé ou totalement polarisé, respectivement (Paladino et al, 2006). Après normalisation à la tubuline, les niveaux de sécrétion de PLAP-sec observés sont comparables à 1 jour ou 4 jours, indiquant que le taux de sécrétion de PLAP-sec est indépendant de l'acquisition du phénotype polarisé. Nous avons également analysé la polarité de sécrétion de PLAP-sec dans des

cellules MDCK cultivées pendant 4 jours dans des filtres et évalué la sécrétion polarisée de PLAP-sec. Nous avons constaté que la protéine PLAP-sec est en grande partie sécrétée à la surface cellulaire apicale (73 % +/- 5).

Ensuite, nous avons évalué si Cab45 joue un rôle dans la sécrétion apicale de PLAP-sec. Dans ce but, nous avons évalué tout d'abord la distribution intracellulaire de PLAP-sec dans les cellules CTRLi et Cab45i par des tests d'immunofluorescence et des analyses western blot, et nous avons constaté que le knockdown Cab45 n'affecte pas la distribution intracellulaire de PLAP-sec ainsi que le niveau de sécrétion de cette protéine. De plus, nous avons détecté si le knockdown de Cab45 pouvait altérer la polarité apicale de la sécrétion la PLAP-sec et de façon très intéressante nous avons trouvé que PLAP-sec devient mis-sécrétée à la surface basolatérale dans le cas des cellules knockdown pour cab45 soulignant ainsi le rôle critique de Cab45 dans sa sécrétion polarisée.

Afin d'évaluer si Cab45 régule spécifiquement la sécrétion apicale de PLAP-sec, nous avons surveillé la sécrétion de la protéine soluble Wnt11, dont la sécrétion apicale dans les cellules MDCK polarisées nécessite la liaison à la galectine-3 (Yamamoto et al 2013). Fait intéressant, la sécrétion apicale de Wnt11 n'est pas affectée dans les cellules MDCK knockdown pour Cab45. Ces données mettent en évidence la spécificité de Cab45 en tant que régulateur de la sécrétion apicale de PLAP-sec et suggèrent que la sécrétion apicale de PLAP-sec ne repose pas sur la liaison à la galectine-3.

Nous commençons donc à déchiffrer comment Cab45 régit la sécrétion apicale de PLAP-sec. Puisqu'un mécanisme dépendant de l'agrégation s'est avéré crucial pour le tri des cargoes sécrétoires, et que Cab45 est essentiel pour le clustering des GPI-AP dans l'appareil de Golgi des cellules MDCK polarisées (Lebreton et al, 2021), une caractéristique clé pour leur tri apical (Paladino et al, 2004; Paladino et al, 2007;

Paladino et al; 2015; Lebreton et al, 2019). Ainsi, nous avons proposé que Cab45 stabilise les clusters GPI-AP en favorisant leur tri apicale (Lebreton et al, 2021). Nous avons analysé l'état de cluster de la PLAP-sec sécrétée. À cette fin, nous avons évalué l'état d'agrégation non seulement de la PLAP-sec sécrétée dans le milieu mais aussi de la PLAP-sec intracellulaire dans les cellules CTRLi et Cab45i. Nous avons constaté que la PLAP-sec sécrétée est organisée en monomères, dimères et trimères, la PLAP-sec intracellulaire est formée de clusters. Cependant, aucun d'eux n'a été affecté par Cab45i, ce qui indique que Cab45 n'affecte pas le clustering de PLAP-sec.

Ensuite, nous avons analysé si Cab45 pouvait interagir directement avec PLAP-sec comme indiqué dans les cellules HeLa pour son client COMP et LysC (von Blume et al. 2012). Pour cela, nous avons réalisé des tests de co-immunoprécipitation et bien que nous ayons pu révéler un enrichissement en PLAP-sec dans les immunoprécipités élués, nous n'avons pas pu détecter d'intéraction entre PLAP-sec et Cab45 endogène. Ces données peuvent impliquer que i) Cab45 n'interagit pas avec PLAP-sec ou, alternativement, ii) leur interaction est transitoire de sorte qu'elle est indétectable ou indirecte.

Comme les glucides/glycanes ont été précédemment montrés comme étant un signal de tri apical pour les protéines sécrétoires dans les cellules MDCK polarisées (Kitagawa et al. 1994; Schelffele, Perinen, and Simons 1995; Urban et al. 1987). Comme PLAP a deux sites de N-glycosylation (Catino et al, 2008), nous avons émis l'hypothèse que la N-glycosylation peut réguler la sécrétion apicale de PLAP-sec dans les cellules MDCK totalement polarisées. Pour tester cette hypothèse, nous avons traité les cellules avec de la tunicamycine pour inhiber la N-glycosylation (Catino et al. 2008) et détecter la polarité de la sécrétion de PLAP-sec. De manière frappante, le traitement à la tunicamycine diminue considérablement la sécrétion de PLAP-sec, en effet avec une PLAP-sec à peine révélée dans le milieu apical ou basolatéral. L'effet est spécifique car nous avons détecté la présence de protéines à la fois dans le milieu

cellulaire apical et basolatéral par coloration S-Ponceau, suggérant que l'exocytose totale n'est pas complètement altérée. Conformément à la diminution drastique de la sécrétion de PLAP-sec, nous avons observé une augmentation statistiquement significative du pool intracellulaire de PLAP-sec lors du traitement à la tunicamycine. En conséquence, le signal d'intensité de fluorescence de PLAP-sec est plus élevé dans les cellules traitées à la tunicamycine par rapport aux cellules non traitées, indiquant en outre que l'exocytose de PLAP-sec est bloquée lors d'une altération de la N-glycosylation.

En résumé, nous montrons que le knockdown de l'expression Cab45 conduit à la mauvaise sécrétion de PLAP-sec à la surface basolatérale révélant le rôle essentiel de Cab45 dans sa sécrétion polarisée apicale. Il est intéressant de noter que le tri apical de Wnt11 reposant sur la N-glycosylation et la galectine-3 est inchangé lors de l'extinction de Cab45. Ensuite, nous rapportons en outre que les clusters intracellulaires de PLAP-sec ainsi que la forme sécrétée de PLAP-sec sont indépendants de Cab45. Deplus, la N-glycosylation de PLAP-sec affecte leur exocytose mais pas la sécrétion polarisée. Jusqu'à présent, nous ne savons toujours pas comment Cab45 régule la sécrétion apicale de PLAP-sec. Étant donné que la PLAP possède des sites de liaison au calcium (Millán 2006), la détection du rôle du calcium en utilisant l'ionomycine et la perturbation de la fonction de SPCA1 sont les prochaines questions à adresser.

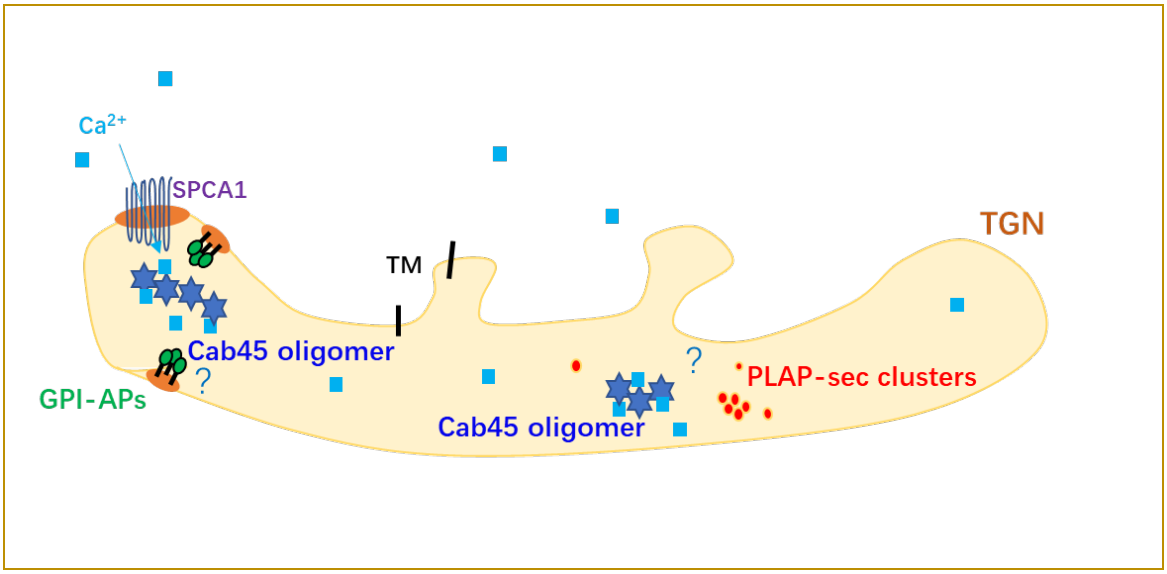

Figure 1 Cab45 régule le tri apical des GPI-AP et PLAP-sec soluble mais pas les protéines transmembranaires

En conclusion, comme l'illustre le schéma, le calcium a été absorbé par le luminal de l'appareil de Golgi par SPCA1, une pompe à calcium et Cab45, une protéine de liaison au calcium luminale de Golgi, se liant au calcium pour former des oligomères et régulant le tri apical de la membrane attachée. GPI-APs en affectant leur regroupement dépendant du cholestérol. Curieusement, Cab45 régule également la sécrétion apicale de la protéine PLAP-sec soluble et de la PLAP-sec soluble, mais pas des protéines transmembranaires. Même si la façon dont le même facteur moléculaire Cab45 régule l'exocytose apicale des GPI-AP et des cargaisons solubles n'est toujours pas résolue. Ces résultats suggèrent qu'il existe une variété de différentes voies antérogrades dans les cellules épithéliales polarisées.

Il est évident qu'une meilleure compréhension de la variété des différentes voies antérogrades est importante car elle est nécessaire à l'établissement et au maintien de la polarité épithéliale, elle est altérée dans de nombreuses maladies humaines et pourrait aider à identifier des composés sélectifs pour bloquer l'arrivée de récepteurs spécifiques. à la surface cellulaire par exemple en cas de surexpression dans les cellules cancéreuses. Par conséquent, le prochain travail consistera à utiliser

dans des cellules MDCK entièrement polarisées le test RUSH (Rétention sur crochet sélectif) (Boncompain et al. 2012) caractériser la cinétique de différentes protéines TM, GPI-APs, solubles apicale ou basolatéralement triées pour décrypter le rôle des facteurs moléculaires identifiés jusqu'à présent comme les lipides (cholestérol, sphingomyéline), l'actine, le calcium, SPCA1 et Cab45. En fin de compte, nous devrons analyser si ces machineries moléculaires sont altérées dans les cellules MDCK soumises à l'EMT, ou en comparant des cellules épithéliales saines et des cellules cancéreuses du sein par exemple.

LIST OF ABBREVIATIONS

1D: 1 day	KDEL: His-Asp-Glu-Leu ER retention
4D: 4 days	KRB: Modified Krebs-Ringer buffer
aa: Amino acid	LDL: Low density lipoprotein
AC-LL: Acidic cluster dileucine	LyzC: Lysozyme C
ADF/cofilin: Actin-filament severing protein	M: Molar
ALP: Alkaline phosphatase	M6P: Mannose-6-Phosphate
AP: Adaptor protein	M6PR: M6P receptor
Ap: Autophagosome	Man: Mannose
AP-1: Activator protein 1	MCSs: Membrane contact sites
APN: Aminopeptidase N antigen	MDCK: Madin-Darby Canine Kidney
ASSP: Aerolysin mutant, carrying a double mutation (residues 202 and 445 are changed to cysteines)	MPRs: Mannose-6-phosphate receptors
ATP2C1: (gene name of SPCA1) forward 5'-GAGGCGGGTTGTGTATGCAATG-3', reverse 5'-GATATTCAGCTTTTCTGACATAGTCC-3';	mRNA: Messenger RNA
Ca^{2+}: Calcium	MSGs: Mature secretory granules
Cab45: A calcium-binding luminal Golgi resident protein,	MVB: Multivesicular bodies
Caco2: Colorectal cancer cells	N&B: Number & Brightness
CFTR: Cystic fibrosis transmembrane conductor	N: Nucleus
CLICs: Clathrin-independent carriers	NA: Influenza virus neuraminidase
COMP: Cartilage Oligomeric Matrix Protein	nm: Nanometer
COPI: Coat protein complex I	NP: Native PAGE maker.
COPII: Coat protein complex II	NTR-PLAP: Neurotrophin Receptor-Placental Alkaline Phosphatase
CPS: Conventional protein secretion	p75: Neurotrophin receptor
CPY: Carboxypeptidase Y	P75NTR: Neurotrophin receptor p75
CTRL: Control	PCR: Polymerase chain reaction
CTRLi: Scrambled interfered cells	phospholipase C
CUPS: Compartment for unconventional protein secretion	PI: Phosphatidylinositol
DAF: Decay-accelerating factor	pIgR: Polymeric immunoglobulin receptor
DMEM: Dulbecco's Modified Eagle Medium	PIP: Phosphoinositol-phosphates

DNA: deoxyribonucleic acid	PI-PLC: Phosphatidylinositol-specific
DPPIV: Dipeptidyl peptidase	PLAP: Placental alkaline phosphatase
DRM: Detergent resistant membrane	PLAP-sec: secretory form of PLAP
ECL: Enchanced chemiluminescence	PM: Plasma membrane
ECM: Extracellular matrix	PNGase: peptide-N-glycosidase F
EE/RE: Early endosome/recycling endosome	PrP: Prion protein
EMT: Epithelial-to-mesenchymal transition	REs: Recycling endosomes4
EPP: Epithelial polarity programme	rGH: Rat growth hormone
ER: Endoplasmic Reticulum	RNA: Ribonucleic acid
ERES: ER-exit sites	SAP: Sphingolipid activator proteins
ERGIC: Endoplasmic-reticulum-Golgi intermediate compartment	SDF4: (gene name for Cab45) forward 5'-CCATGATCCAGTGCTGCATC-3'; reverse 5'-AGGAGCAGGCGGAAGCTGAT-3';
EtNP: Ethanolamine phosphate	SDS: Sodium dodecyl sulfate
FAPP2: Four-phosphate-adaptor protein 2	SDS-PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis
FBI: Fumonisin B1	SERCAs: Sarcoendoplasmic-reticulum Ca-ATPases
FCS: Fetal Bovine Serum	ShRNA: Short hairpin RNA
FGF2: Fibroblast growth factor 2	SI: Sucrase isomaltase
FPV: Fowl plague virus	SL: Secretory lysosomes
FR: Folate receptor	SL: Sphingolipides
FRT: Fisher rat thyroid cells	SNARE: Soluble NSF attachment protein receptor
GA: Golgi apparatus	SPCA1: Secretory pathway Ca (2+) -ATPase pump type 1
GAP: GTPase-activating protein	SRP: Signal recognition particle
gD1: Herpes simplex glycoprotein D	SSG: Secretory storage granules
GFP: Green fluorescent protein	SVs: Secretory vesicles
GFP-FR: GFP fuse with FR GPI attachment signal	TCA: Trichloro acetic acid
GFP-PrP: GFP fuse with PrP GPI attachment signal	TfR: Transferrin receptor
GlcN: Glucosamine	TGN: Trans Golgi Network
GoAEQ: A chimeric photoprotein Golgi-aequorin	TJ: Tight junctions
GPI: Glycosyl phosphatidyl inositol	TM: Transmembrane
GPI-APs: Glycosylphosphatidylinositol-anchored proteins	Tuni: Tunicamycin
GSL: Glycosphingolipid	UBCH5: forward 5'-TGAAGAGAATCCACAAGGAATTGA-3'; reverse 5'-

	CAACAGGACCTGCTGAACACTG-3’
GSLs: glycosphingolipides	UPS: Unconventional protein secretion
GTPase: Guanosine-5'-triphosphate	Vps10: Vacuolar protein sorting 10
HA: Hemagglutinin A	VSV: Vesicular stomatitis virus
HLA-I: Human leukocyte antigen 1	VSVG: Envelope glycoprotein G of the vesicular stomatitis virus
HMW: High molecular weight	WB: Western blot
IF: Immunofluorescence	Wls: Wntless
Ino: Inositol	Wnt: Wingless/int conserved family of secreted proteins
IP: Immunoprecipitation	Wnt11: Wnt family member 11
ISGs: Immature secretory granules	WT: Wildtype
KD: Kilodalton	

INTRODUCTION

1. Cell polarity

Prokaryotic and eukaryotic cells have the property to polarize. Cell polarity, essential for proper cellular function, is characterized by spatial asymmetry in shape and structure. Cell polarity can either be (1) temporary as in case of activated and migrating immune cells or migrating glial and fibroblastic cells (Kadir et al. 2011) and also for some dividing cells (Neumüller and Knoblich 2009) or (2) permanent as in case of neuronal and epithelial cells (Rodriguez-Boulan et al. 2005). Polarized cells are characterized by a strictly organized and asymmetric plasma membrane domains differing in proteins and lipids composition and thus in functions (Eaton and Martin-Belmonte 2014; Mellman and Nelson 2008; Rodriguez-Boulan and Macara 2014).

Cell polarity, permanent or transient, is essential for the cellular function for instance for neurons to transduce rapidly an electric signal or the continuous exchange between two different environments in case of epithelial cells (Mostov et al, 2003; Overeem et al, IJzendoorn 2015; Rodriguez-Boulan et al. 2005; Takano et al. 2015).

The mechanisms regulating establishment and maintenance of cell polarity are studied since decades (Rodriguez-Boulan et al. 2005). However, there are still many open questions regarding the molecular mechanisms and components governing this cellular process. Cell polarity relies on the interconnected cellular events such as signaling cascades regulating membrane trafficking and endocytosis, protein and lipid sorting as well as cytoskeleton organization and dynamics (Mostov et al. 2003; Overeem et al. 2015; Rodriguez-Boulan et al. 2005; Takano et al. 2015).

1.1 Cell polarity in epithelial cells

Epithelial tissues are divided into protective epithelium that cover all the surfaces of the body (skin) exposed to the outside world and lining the internal organs and the glandular epithelium that is responsible for the secretion of hormones for instance. The polarity of epithelial cells is essential for the whole tissue integrity and the normal physiological function (Delacour and Jacob 2006), therefore understanding the molecular mechanisms regulating establishment and maintenance of epithelial polarity is critical. During the lifetime of the epithelium, cell polarity has to be maintained therefore requiring a constant plasma membrane turnover of membrane components and a tight regulation of continuous sorting of newly synthesized proteins and lipids and their recycling. Polarized epithelial cells constitute a protective barrier against the external world but also serve as exchange interfaces with the outside word. This paradigm results from the asymmetrical plasma membrane that is compartmentalized into apical and basolateral domains. Establishment of cell polarization required several polarization events including as initial step an influx of information from the extracellular milieu (Delacour and Jacob 2006) followed by the establishment of cell-cell and cell-extracellular matrix contacts, which in turn leads to the formation of tight junctions (TJs), cytoskeleton reorganization and vectorial intracellular trafficking (Rodriguez-Boulan *et al.* 2005; Yeaman et al, 1999). A continuous sorting of newly synthesized proteins and lipids and their recycling are required to maintain the molecular asymmetry at the cell surface (Rodriguez-Boulan and Macara 2014). Thus, polarized epithelial cells are characterized by an asymmetrical plasma membrane divided into two distinct domains: 1) the apical surface facing the outer lumen and 2) the basolateral surface referring to lateral membranes where cell-cell junctions connect neighboring cells and to basal membranes which face to the extracellular matrix (ECM) (**Figure 1**). The apical surface is normally characterized by membrane extensions that increase surface area as in intestinal enterocytes and tubule or duct epithelia. One notable exception in

mucosal epithelia is M cells, which display a unique apical membrane lacking microvilli (Bennett, Walker, and Lo 2014). Apical and basolateral membrane domains are distinct not only in their protein and lipid composition but also in their functions. In case of absorptive polarized epithelial cells as enterocytes the presence of the apical brush border composed of microvilli allows absorption; While in hepatocytes the apical surface face the bile canaliculi where the secretion of the bile occurs (Gissen and Arias 2015; Treyer and Müsch 2013).

Because epithelial polarity is essential for the homeostasis of the whole tissue and organism; it is not surprising that epithelial polarity is often challenged in human disease such as upon viral or bacterial infection or in case of cancer that mostly results from the loss of epithelial polarity (Partanen et al, 2013).

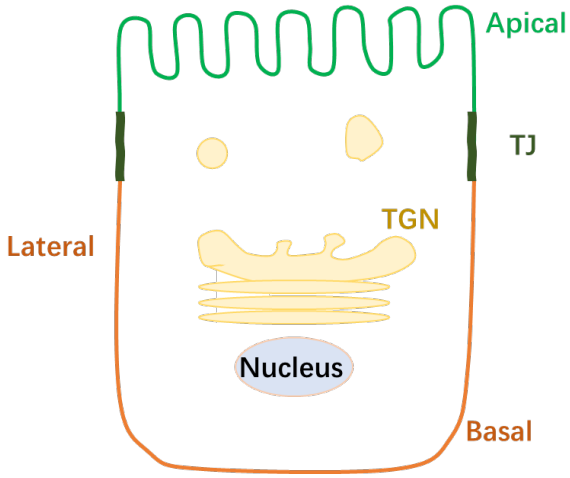

Figure 1. Polarized epithelial cells.

Polarized epithelial cells are characterized by an apical membrane domain (in green) and a basolateral domain (in orange) physically separated by Tight Junctions. Establishment and maintenance of this polarity require a tight regulated protein and lipids intracellular trafficking. Modified from (Bergstralh and St Johnston 2012).

1.2 Molecular mechanisms underlying epithelial polarization

1.2.1 The asymmetric distribution of protein

The organization of a polarized epithelial cell is mediated through a network of epithelial polarity proteins and lipids, which is called the epithelial polarity program (EPP). EPP is regulated by a core of three protein modules, identified in model organisms such as yeast, worms and flies, the PAR (PAR3-PAR6-aPKC), Crumbs (Crumbs3-Sdt-PATj) (the mammalian homologue of Sdt is PALS1) and Scribble (Scribble-DLG1- LGL1/2) (Scrib in mammalian). PAR and Crumbs modules are localized at the apical surface where they co-operate in the establishment of the apical domain and the assembly of tight junctions, while Scribble defines the basolateral plasma membrane domain. The PAR module is a well-known master regulator of polarity (Munro 2006) with Par-3 exhibiting diverse protein-interaction domains allowing its association with Par-6 and atypical protein kinase C (aPKC) (Izumi et al. 1998; Joberty et al. 2000; Lin et al. 2000). Par-3 or Bazooka in *Drosophila* is essential for epithelial cell polarization in *Drosophila* while its mammalian counterpart localizes to tight junctions at the apical/lateral boundary (Izumi et al. 1998) where it is implicated in the assembly of tight junctions (Chen and Macara 2005). Par6/ aPKC can interact with aPKC (Rodriguez-Boulan and Macara 2014) but also recruits Crumbs modules (Hurd et al. 2003) and is involved in the maintenance of the integrity of the apical domain (Martin-Belmonte et al. 2007). Drosophila Scribble localized to the basolateral membrane in epithelial cells and function as a molecular scaffold to establish the basolateral membrane and overall epithelial polarity since its mutation leads to loss of polarity. However, in mammals, the loss of Scrib does not drive significant defects in the establishment of the epithelial polarity but has been shown to regulate cell survival, protein trafficking, adhesion and migration (Bilder 2004; Humbert et al. 2008; Nelson 2009) and to be necessary for epithelial morphogenesis and tubulogenesis (Eastburn et al. 2012). Moreover, these modules, in MDCK cells,

contribute to the biogenesis of the primary cilium; an organelle essential for tissue homeostasis and during development (Fan et al. 2004; Weisz and Rodriguez-Boulan 2009) but also in asymmetric cell division (Assémat et al. 2008; Overeem et al. 2015; Rodriguez-Boulan and Macara 2014). Of interest, these polarity modules regulate also the organization of the microtubule network and the synthesis of the phosphoinositol phosphate that specify membrane polarity (Bryant and Mostov 2008). These modules, distributed asymmetrically in the cells, promote the establishment of apical and basolateral membrane domains.

Another role of membrane protein involved in the organization of epithelial polarity in Madin-Darby canine kidney cell (MDCK) cyst regards podocalyxin and small GTPase Rab35. It was recently reported that Rab35 is involved in polarity initiation during the first cell division of epithelial cells cyst development by its direct interaction with the cytoplasmic tail of podocalyxin allowing the tethering of intracellular vesicles containing key apical determinants at the cleavage site. This novel and unconventional mode of Rab-dependent vesicle targeting triggers initiation of apico-basal polarity and lumen opening at the center of cysts (Klinkert et al. 2016).

1.2.2 The asymmetric distribution of lipid

The establishment of epithelial polarity involved as mentioned earlier proteins but also lipids as exemplified by the asymmetrical distribution of lipids in apical or basolateral domains. A pioneer study from Kai Simons and his colleagues (van Meer and Simons 1982) examined the compositional differences of membrane lipids in the apical and basolateral membranes by taking advantage of the enveloped virus. They extracted lipids from the fowl plague virus (FPV) that selectively sprouts from the apical membrane and the vesicular stomatitis virus (VSV) that emerges from the basolateral membrane in MDCK cells. By comparing the lipid composition of these enveloped viruses. They found that, the apical and basolateral plasma membrane

domains of MDCK cells have different phospholipid compositions (van Meer and Simons 1982).

An important lipid family involved in the epithelial polarity regards phosphatidylinositol one of the most well-characterized phospholipid species among membrane lipids. Phosphatidylinositol exists in eight different phosphorylation states depending on the phosphorylation of the hydroxyl groups at the 3, 4, 5 positions of the inositol ring. Among them, PI(3,4,5)P3 and PI(4,5)P2 are crucial for the establishment of cell polarity. PI(4,5)P2 is transformed to PI(3,4,5)P3 by PI3-kinases (PI3K), and PI(3,4,5)P3 can be transformed to PI(4,5)P2 by a 3-phosphatase (PTEN) inversely. PtdIns(4,5)P2 exists mainly in apical membrane in MDCK II cells whereas PtdIns(3,4,5)P3 is distributed in basolateral membrane (Martin-Belmonte et al. 2007). In particular, PI(4,5)P2 is reported to play a role in the endocytosis by modulating the activity of AP-2 and Epsin involved in the assembly of clathrin-coated vesicles. Importantly, upon ectopical supply of PtdIns(4,5)P2 to the basolateral membrane, apical marker membrane proteins are re-localized to the basolateral membrane (Martin-Belmonte et al. 2007) highlighting that the asymmetric distribution of PtdIns(4,5)P2 is crucial for the asymmetric distribution of membrane proteins in epithelial cells.

It was shown that PtdIns(3,4,5)P3 of the lateral membrane is necessary to recruit β II spectrin, an essential component of submembranous cytoskeletal networks, to the lateral membrane and this is critical to the formation of the lateral membrane (He et al, 2014). PI(3,4,5)P3 is also showed to regulate transcytosis of basolateral membrane components and the presence of PI(3,4,5)P3 is able to transform an apical membrane into a basolateral membrane by dragging basolateral proteins (Gassama-Diagne et al. 2006).

The asymmetric distribution of these two phosphoinositol-phosphates is regulated by specific recruitment of kinases PI3K to the basolateral domain and

exclusion of the phosphatase PTEN from the basolateral domain and it's enrichment at the apical domain (Rodriguez-Boulan and Macara 2014). In addition, evidences suggest that phosphoinositides can control the activity of PAR modules, thereby regulating epithelial polarity (Gassama-Diagne and Payrastre 2009).

Kai Simons and his colleagues also firstly described that sphingolipids that are biosynthesized in the Golgi apparatus can together with cholesterol in the trans-Golgi network form lipid raft (Simons and Ikonen 1997). These lipid raft, highly dynamic, exhibit a size below the classical optical resolution. These membrane microdomains can accommodate different type of proteins and therefore regulate protein function by including or excluding them from these cholesterol-and sphingolipid enriched membrane microdomains. These rafts membrane microdomains are lipid ordered (Hao, Mukherjee, and Maxfield 2001; Madore et al. 1999; K. Simons and Toomre 2000). Apical sphingolipid rafts are involved in the polarized sorting of newly synthesized apical resident proteins from the TGN to the apical membrane (Röper, Corbeil, and Huttner 2000; Simons and Ikonen 1997). Cholesterol enriched membrane microdomains increase the thickness of lipid bilayers in apical membrane of polarized epithelial cells (Kaiser et al. 2011), and is necessary for surface transport of influenza virus hemagglutinin (Keller and Simons 1998).

Compared with the abundance of phospholipids and cholesterol, glycosphingolipids (GSLs) are found in relatively minor level (less than 5%) in eukaryotic cell membranes. GSLs are highly enriched in the outer leaflet of the apical plasma membrane domain of polarized epithelial cells. GSLs also appear to be organized in clusters or microdomains called rafts with or without cholesterol (Hoekstra et al. 2003). With cholesterol and saturated phospholipids, GSLs form a unique liquid-ordered phase which is the state of most of the PM surface area. Göbel and colleagues showed that GSLs are critical to maintain the epithelial polarity in the nematode C. elegans (Zhang et al. 2011). GSLs can also stabilize and protect the

certain membranes, such as the apical bile canalicular membrane of liver hepatocytes (Hoekstra et al. 2003).

Lipid raft direct regulates the polarized sorting of newly synthesized apical resident protein such as GPI anchors proteins and vesicular integral membrane proteins (VIPs) from the TGN to the apical membrane (Simons and Ikonen 1997). The perturbation of the association of the apical proteins placental alkaline phosphatase (PLAP) with lipid raft perturbed its apical sorting for instance, (Röper, Corbeil, and Huttner 2000). Thus, lipid rafts were proposed to be involved in the establishment of apico-basal polarity.

The protein and lipid differences between apical and basolateral domains revealed the essential role of protein and lipids sorting from the Golgi apparatus that is the major sorting hub in polarized epithelial cells.

1.3 MDCK cells: Model system to investigate molecular mechanisms underlying cell polarity

How epithelial cells establish and maintain their polarity remains a fundamental question although studied since decades. These cellular processes could be addressed thanks to the establishment of MDCK cell line in 1966 (Gaush et al, 1966). Once plated on a permeable substratum these cells develop a tight epithelial monolayer. A pioneer study justifying the use of polarized MDCK cells to analyze epithelial polarity comes from Rodriguez-Boulan and colleagues who could monitor that influenza virus assembles from the apical surface while vesicular stomatitis virus (VSV) assembles from the basolateral surface of MDCK cells. Indeed, their envelope glycoproteins influenza hemagglutinin (HA) and VSVG protein (VSVG) exhibit in MDCK cells a polarized distribution of (Rodriguez Boulan and Pendergast 1980; Rodriguez Boulan and Sabatini 1978; Rodriguez-Boulan et al. 2005). These studies therefore indicated the existence of different biosynthetic routes from the Golgi, the main hub of protein and lipid sorting, to the apical and basolateral surface of polarized MDCK cells.

Interestingly, these two proteins are still widely used as apical and basolateral markers to study polarized protein trafficking.

MDCK cells rapidly became the cellular model use to investigate epithelial polarity protein exocytosis and endocytosis. These cells can be plated at low density for few hours where MDCK cells are non-polarized but once they are plated on permeable filter, they exhibit all characteristic of fully polarized epithelial cells (Bacallao et al. 1989; Cereijido et al. 1978; Hanzel et al. 1991) (**Figure 2**) with an apical and basolateral membrane domain physically separated by tight junctions (van Meer and Simons 1986; Tanos and Rodriguez-Boulan 2008). The unusual robustness of the apical membrane is largely attributable to its peculiar lipid composition (Brasitus and Schachter 1980; Kawai, Fujita, and Nakao 1974; Simons and van Meer 1988). When MDCK cells are plated in or on top of Matrigel or another ECM, they form 3D cyst (O'Brien et al. 2002), providing a physiological model recapitulating numerous features of an *in vivo* epithelia in 3D system (Debnath and Brugge 2005; Leighton et al. 1970; Lever 1979). In humans, more than 80% of all tumors are carcinomas that originate from epithelial tissues (Jemal et al. 2008). A characteristic hallmark for almost carcinomas is the loss of epithelial morphology and the acquisition of a mesenchymal-like phenotype, through a process called epithelial-to-mesenchymal transition (EMT) (Coradini et al, 2011; Thiery 2002; Thompson et al, 2005). EMT is a central driver of epithelial-derived tumor malignancies (Cano et al. 2000; Thiery 2002). EMT has since been shown to trigger the dissociation of carcinoma cells from primary carcinomas, which subsequently migrate and disseminate to distant sites (Nieto et al. 2016).

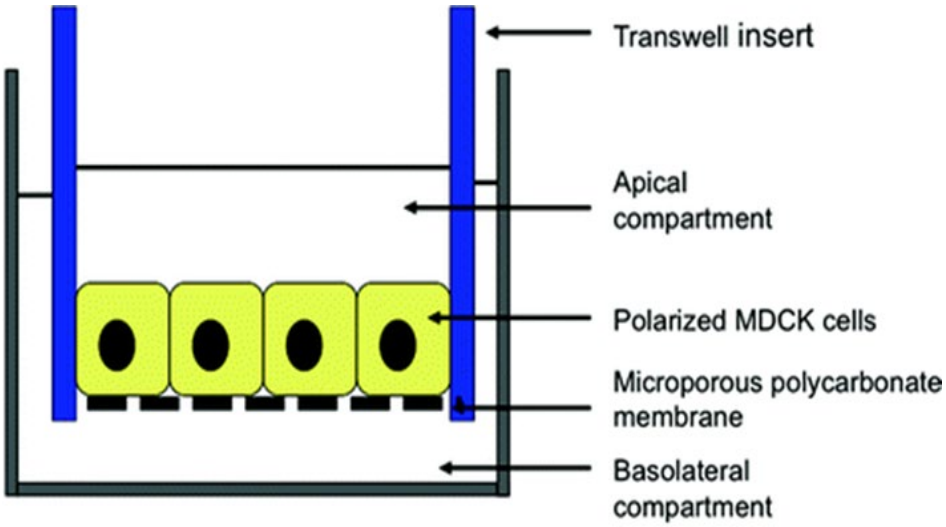

Figure 2. Culturing MDCK cells grown on Transwell polycarbonate filters. *MDCK cells cultured on Transwell insert filters are able to form fully polarized epithelial monolayers with functional tight junctions. The pores of the filter allow cell to uptake nutrients from the basolateral surface. With this system both the apical and basolateral chambers are accessible to collect secreted proteins from these two distinct compartments mimicking the in vivo situation in epithelia (Kausalya and Hunziker 2011). 2. Membrane protein intracellular trafficking in polarized epithelial cells.*

2. Trafficking routes of exocytosis

The secretory and endocytic pathways of eukaryotic organelles consist of multiple functionally membrane-enclosed compartments exhibiting specific protein and lipid composition conferring unique identity. It is fascinating that each individual organelle maintains its own biochemical identity while they are highly interconnected, highlighting a tight regulation of protein and lipids intracellular trafficking. Forward transport of newly synthesized proteins and lipids are initiated at the endoplasmic reticulum (ER). The ER to Golgi to plasma membrane (PM) transport represents a vital gateway to the membrane-enclosed compartments system (Tiklová et al. 2010) (**Figure 3**).

The biosynthetic-secretory pathway leads outward from the ER toward the Golgi apparatus and plasma membrane, with a side route leading to lysosomes (or via endosomes). For example, proteins that are transported within the secretory pathway are either secreted from the cell, bound to the plasma membrane, sorted to lysosomes, or are retained as residents in any of the organelles. In human 1/3 of proteins go through the secretory pathway for their exocytosis (Lodish et al. 2000). Within the ER, newly synthesized proteins are scrutinized to ensure they are correctly folded before being packaged into transport intermediates or vesicles, and then moved forward to the entry site of the Golgi apparatus. Secretory proteins are then transported through the Golgi cisternae to the trans-Golgi network (TGN) where they are further matured. Biochemistry and live cell imaging revealed that the Golgi is main sorting platform in cells. From the Golgi they are selectively sorted to reach their final destination.

Contrary to exocytosis, the endocytic pathway leads inward from the plasma membrane to early endosomes and then (via late endosomes) to lysosomes. Many endocytosed molecules are retrieved from early endosomes and returned to the cell surface for reuse; similarly, some molecules are retrieved from the early and late endosomes and returned to the Golgi apparatus, and some are retrieved from the Golgi apparatus and returned to the ER (**Figure 3**). In each case, the flow of membrane between compartments is balanced, with retrieval pathways balancing the flow in the opposite direction, bringing membrane and selected proteins back to the compartment of origin.

Membrane contact sites (MCSs) are the areas where the membranes of two different organelles come into close apposition (10–30 nm) but without any fusion between the two organelles (Venditti et al, 2020). MCSs exist between any known intracellular organelles, including endoplasmic reticulum (ER), mitochondria, Golgi, endosomes, peroxisomes, lysosomes, lipid droplets, and the PM (Leung et al. 2021). MCS play critical roles in exchanging intracellular lipids and calcium. MCS must

fulfill function such as bidirectional transport of calcium, lipids or amino acids for instance; transmission of signaling information or force important for remodeling activities, including regulate organelle biogenesis, position, inheritance (English and Voeltz 2013; Helle et al. 2013; Prinz 2014; Scorrano et al. 2019).

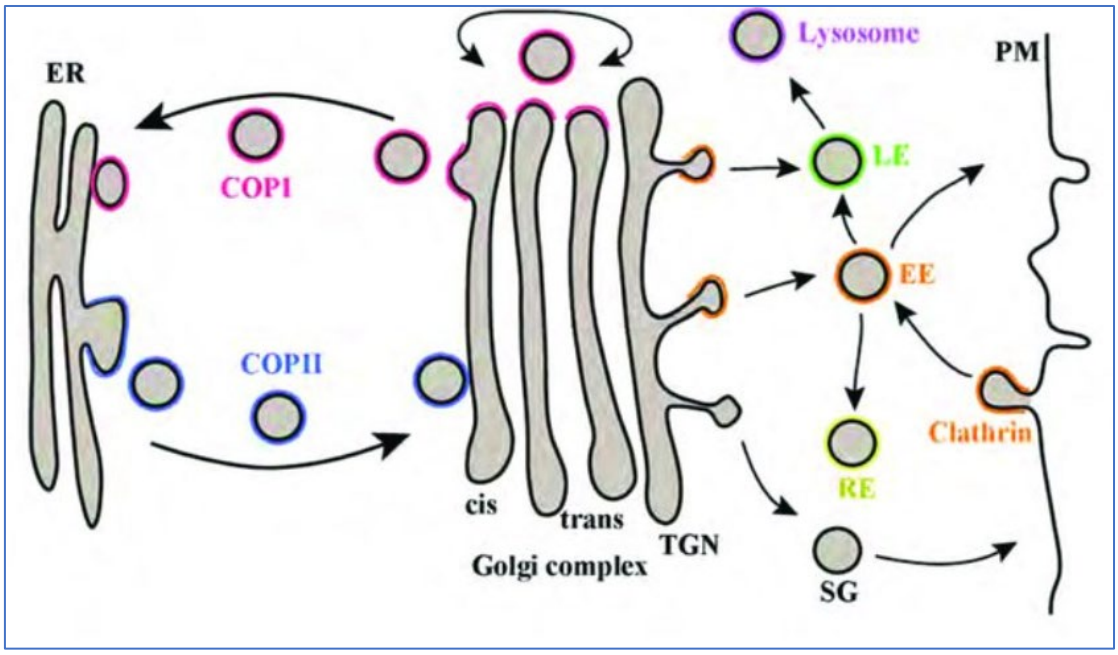

Figure 3. Different coats involved in vesicular traffic in the biosynthetic-secretory and endocytic pathways.

Different coat proteins select different cargo and shape the transport vesicles that mediate the various steps in the biosynthetic-secretory and endocytic pathways. In the former pathway, the vesical exit from ER mediated by the COPII (coat protein complex II) (blue). The COPII assembles on specific locations of the ER membrane where COPII-coated vesicles bud off. The later pathway is mediated by (coat protein complex I) (purple). COPI-coated vesicles that traffic from Golgi back to the ER. Clathrin-coated (orange) buds at the TGN and transport material from the plasma membrane and between endosomal and Golgi compartments. EE: early endosome, RE: recycling endosome, LE: late endosome, SG: secretory granule, PM: plasma membrane (Tiklová et al. 2010).

2.1 Transport from ER to the Golgi

Membrane proteins and nascent secretory proteins are translocated in the ER upon the interaction between the signal recognition particle (SRP) and its receptor SR by signal sequence recognition; then the proteins are transported into the ER lumen through the translocon. Thanks to the action of a multitude of molecular chaperons and cofactors, these proteins are eventually glycosylated, and properly folded in order to adopt the appropriate confirmation for their exit (Johnson et al. 1995).

Membrane traffic between the ER and the Golgi is bidirectional from the ER to the Golgi it is the anterograde route while the reverse is the retrograde route. In both carriers these vesicular pathways rely on formation of a carrier on the donor organelle that then tethers to and fuse with the target organelle (brandizini and barlowe 2013). For eukaryotic cells, the best characterized mechanism of ER exit is the COPII (coat protein complex II)-mediated transport. The COPII assembles on specific locations of the ER membrane named ER-exit sites (ERES), where COPII-coated vesicles bud off (**Figure 3**) (Barlowe et al. 1994). The directionality and fidelity of COPII vesicle transport and fusion with either the ERGIC or the cis-Golgi (depending on the organism) are mediated by the concerted action of RAB GTPases, tethering factors, and integral membrane SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor).

Regarding COPII cage, its outer layer is composed of secretion associated RAS-related 1 (SAR1) GTPase and the two subcomplexes SEC23-SEC24 and SEC13-SEC31 (Brandizzi and Barlowe 2013). SEC24, the main COPII adaptor, can recognize specific ER export signals on membrane proteins for selective uptake (Miller et al. 2003; Wendeler et al, 2007). For instance, transmembrane cargo proteins are recognized and bound by Sec24, whereas soluble cargoes bind specific receptors that span the ER membrane (Miller et al. 2003; Sato and Nakano 2005). Polymerized SEC13–SEC31 subcomplexes imposes curvature to the nascent vesicle as it buds

from the ER (Copic et al. 2012; Stagg et al. 2006). The vesicle keeps the coat subunits until they reach their target membrane (Lord et al. 2011).

The retrograde pathway requires COPI-coated vesicles that traffic from Golgi back to the ER (**Figure 3**).

COPI mediates retrograde transport of receptors and soluble proteins from the ERGIC back to the ER along microtubules. The retrograde pathway ensures not only the retrieval of resident proteins (Pelham 1988) that escape the ER but also facilitates the recycling of lipids (Wieland et al. 1987). Importantly, inhibition of the COPI mediated retrograde trafficking route leads to the collapse of anterograde trafficking (Brandizzi and Barlowe 2013) revealing that the two anterograde and retrograde pathways are tightly connected for the overall cellular homeostasis.

2.2 Transport through the Golgi

The Golgi complex is the main sorting hub in cells, it is an essential biosynthetic center for both proteins and lipids. This organelle is composed of cisternae whose numbers vary depending on the cellular system. Roughly 1/3 of human proteins are going through the Golgi before their final destinations. Protein processing and maturation, sorting, lipid synthesis and cargo packaging are the main functions of this complex intracellular organelle. Proteins and lipids enter in the cis Golgi cisternae then go through medial Golgi before reaching the TGN (Mellman and Warren, 2000). It is fascinating how the Golgi complex at the cross between the exocytosis and endocytosis can handle sorting of cargoes so diverse in size, structure, morphology suggesting that this organelle could be modulated dynamically by the ingoing and outgoing cargoes.

Although studied since many decades, how proteins traverse the Golgi, is still highly debated (Glick and Luini 2011). None of the proposed models of Golgi allow to reconcile all the observations made in several diverse organisms: (1) The vesicular

transport model. A newly synthesized secretory cargo would be delivered through anterograde vesicular transport between stable compartments as I describe in last Section. Cargo moves from one Golgi compartment to the next thanks to COPI vesicles and encounter different enzymes in each subsequent compartment, until it reaches the trans cisterna. This model does not explain the mechanism of large secretory cargoes transport that cannot fit within COPI vesicles. (Farquhar and Palade 1981; Rothman 1981; Dunphy and Rothman 1985; Farquhar 1985; Orci et al. 2000; Pelham and Rothman 2000). (2) Cisternal progression/maturation model (**Figure 4**). In this model, cargo remains in a compartment that is remodeled thanks to new enzymes composition. In this model a cis cisterna will be convert to a medial one and then mature to a TGN which would dissociate in anterograde and retrograde transport carriers (Grasse 1957; Morre and Ovtracht 1977) (Emr et al. 2009; Glick and Nakano 2009; Pelham and Rothman 2000). This model cannot easily explain the existence of heterotypic tubular connections between cisternae, the fused Golgi network in microsporidia, the exponential kinetics of secretory cargo exit from the Golgi region or the rapid intra-Golgi traffic of small secretory cargoes (Glick and Nakano 2009; Patterson et al. 2008). (3) Another "cisternal progenitor" model is proposed by Suzanne R. Pfeffer's lab. The stable compartments fission into subsequent compartments as cisternal progenitors and "homotypic" fusion with other cisternal to transport large cargo, such as procollagen, without leaving the lumen of the Golgi cisternae (Bonfanti et al. 1998; Emr et al. 2009; Glick and Luini 2011). However, this model is most suitable for animal cells only, but not for plants, algae, and fungi Golgi cisternae (Melkonian, Becker, and Becker 1991; Staehelin and Kang 2008; Yelinek, He, and Warren 2009). (4) Cisternal progression/maturation with heterotypic tubular transport. Golgi cisternae within a stack is connected by tubular continuities (Mellman and Simons 1992; Weidman 1995; Mironov et al. 1997), allowing small secretory cargoes fast anterograde traffic or retrograde traffic of resident Golgi proteins, or both (Rambourg and Clermont 1990; San Pietro et al. 2009). This model cannot easily explain the exit of the large secretory cargo procollagen which is too large to diffuse

through heterotypic tubular connections and seems to traverse Golgi stacks by cisternal progression (Bonfanti et al. 1998; Patterson et al. 2008). (5) On top of heterotypic tubular model, rapid partitioning in a mixed Golgi was proposed. In this model, the Golgi is regarded as a single compartment contains processing domains and export domains. Secretory cargoes equilibrate across the stack via intercisternal continuities and would exit from every level of the Golgi to their final destinations. There are also weaknesses in this model. For instance, the distinct Golgi compartments and existence of discrete cisternae in most eukaryotes, the polarized distribution of Golgi glycosylation enzymes, the existence of secretory cargo waves for procollagen, the apparent formation and peeling off of Golgi cisternae, or the transient nature of yeast Golgi cisternae, cannot easily be explained by this model. Moreover, this model does not provide the role of COPI vesicles. (Emr et al. 2009; Lippincott-Schwartz and Phair 2010; Patterson et al. 2008).

Considering all these five models each of them or two of them in combination could explain a set of key observations of multiple cell types (Glick and Luini 2011). Importantly, the Golgi is a very complex organelle with an incredible plasticity allowing to handle the sorting of cargoes so diverse in size, morphology or to reposition in response to specific physiological needs (Ayala, Crispino, and Colanzi 2019; Jasmin et al. 1995).

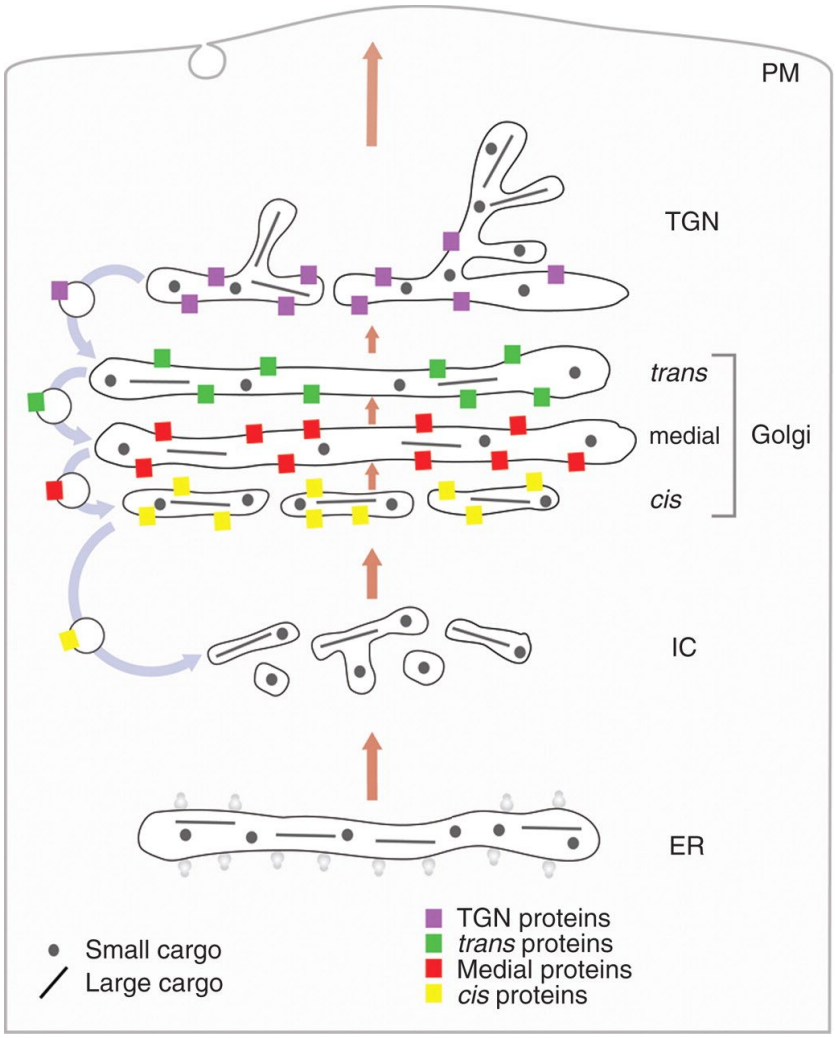

Figure 4. Cisternal progression/maturation model.

Secretory cargoes exit the ER in COPI vesicles form the intermediate compartment. It coalesces in turn to form a new cis-cisterna. The new cisterna matures by receiving proteins from older medial Golgi cisternae while exporting cis and then medial-Golgi proteins to younger cisternae. With rounds of COPI-mediated recycling, the cisterna progresses through the stack while carrying small and large secretory cargoes. The cisterna become a TGN at the final stage of maturation and is able to break down into anterograde and retrograde transport carriers (Glick and Luini 2011).

2.3 Transport from the Golgi to PM (direct and indirect delivery)

The Golgi stack is compartmentalized into cis, medial, and trans compartments which are enriched with specific Golgi enzymes. The cis face of the Golgi complex is where the newly synthesized secretory proteins enter and the TGN is where they exit. Conventionally, the TGN is viewed as the main cargo sorting station where proteins and lipids are sorted into distinct transport carriers which target to various downstream destinations. These destinations include endosomal compartments, basolateral or apical plasma membrane, and additional compartments in some cells, such as secretory vesicles (De Matteis and Luini 2008) (**Figure 5**). At the TGN, proteins are sorted into different vesicles by certain sorting motifs and cytoplasmic adaptor complexes, and are transported along cytoskeletal elements to different compartments or the plasma membrane. Different kinds of coats or coat-like complexes are identified to transport protein to the plasma membrane and between endosomal and Golgi compartments. Among them, clathrin coats are an important protein group which are heterogeneous and contain different adaptor and accessory proteins at different membranes (**Figure 5**).

In epithelial cells, proteins and lipids are sorted from the TGN to their final destination either via a direct or an indirect route. In the direct route proteins traffic from the TGN to the apical or basolateral membrane directly although they can pass through intermediate sorting station as endosome on their way (Mogelsvang et al. 2004). The indirect route is also called transcytosis, where proteins are firstly targeted to the basolateral membrane before being endocytosed and re-direct to the apical surface.

Importantly, the use of the direct or indirect pathways seems to be cells specific (Rodriguez-Boulan et al 2005). In MDCK and FRT cells, the direct pathway is predominantly used (Apodaca and Mostov 1993; Sarnataro et al. 2000), whereas, all proteins follow the transcytosis pathway in hepatocytes. For instance, the apical

proteins dipeptidyl peptidase (DPPIV) and Hemagglutinin A (HA), follow direct pathway in MDCK while they follow the transcytosis route in intestinal cells and hepatocytes (Bonilha et al. 1997; Casanova et al. 1991).

However, there are exceptions. It was shown that in FRT cells, the apical proteins DPPIV changed their secretory pathway from indirect to direct during the establishment of the epithelial polarity (Zurzolo et al. 1992). In MDCK cells, unlike other GPI-APs, the GPI-AP Prion protein (PrP), follows transcytosis pathway in fully polarized MDCK cells in both 2D and 3D cultures (Arkhipenko et al. 2016).

Of interest, the use of the direct or indirect pathways seems also to be protein specific (Rodriguez-Boulan et al. 2005). Compare to MDCK cells and hepatocyte, Caco-2 cells have an intermediate sorting phenotype by sorting basolateral protein direct pathway and the apical protein either by a direct or indirect pathway. In caco-2 cells, for instance alkaline phosphatase (ALP) is targeted to the apical surface via the direct pathway while aminopeptidase N (APN) is directed to the apical surface via transcytosis, indirect pathway. Regarding the basolateral proteins antigen 525, human leukocyte antigen 1 (HLA-I), or transferrin receptor (TfR) are targeted directly to the basolateral membrane (Le Bivic et al. 1990).

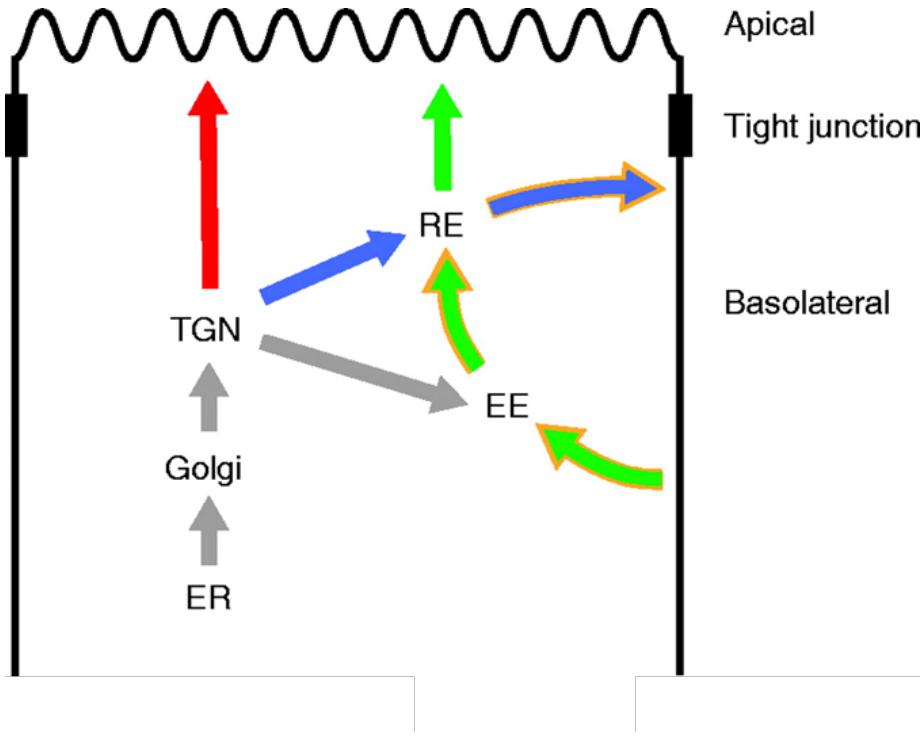

Figure 5. Two ways of sorting plasma membrane proteins in a polarized epithelial cell.

Newly synthesized proteins can reach their proper plasma membrane domain by either a direct specific pathway (red and blue arrows) or an indirect pathway (grey and green arrows). In the indirect pathway, a protein is retrieved from the inappropriate plasma membrane domain by endocytosis and then transported to the correct domain via early endosomes (EE) that is, by transcytosis. The indirect pathway is used in liver hepatocytes to deliver proteins to the apical domain that lines bile ducts. However, in other cases, the direct pathway is used, as in the case of the lipid-raft-dependent mechanism in epithelial cells described in the text. Modified from (Schuck and Simons 2004).

2.4 Polarized sorting of membrane protein

The establishment and maintenance of epithelial polarity require polarized sorting, recycling and endocytosis of lipids and proteins (Mostov 2003; Rodriguez-Boulan and Musch 2005; Yeaman et al. 1999).

The mechanisms of polarized sorting of membrane protein have been studied in greater details in epithelial cells and reveal the essential role of several factors such as clathrin adaptor proteins (AP). AP-1 is a member of the AP complex family, which includes the ubiquitously expressed AP-1A and the epithelium-specific AP-1B. AP-1 regulates polarized sorting at the trans-Golgi network and/or at the recycling endosomes.

Once the AP-1 was mutated in C. elegans, not only the basolateral membrane proteins, including SLCF-1 (a putative monocarboxylate transporter) are mislocalized (Shafaq-Zadah et al. 2012; Zhang et al. 2012), but also the apical molecules, including actin and PAR-6, and the apical lipids, including glycosphingolipids, are also mislocalized. This indicated that AP-1 is indispensable for both apical and basolateral sorting in the intestinal epithelium of C. elegans. cells (Nakatsu, Hase, and Ohno 2014). Recently a study in MDCK cells showed that the silencing of AP-1A, AP-1B independently or both, alter protein trafficking by inducing the redistribution of basolateral proteins and also a large number of apical proteins. All this data expanded the traditional notion that clathrin adaptors mediate only basolateral polarity (Caceres et al. 2019).

AP-1 has also been reported to regulate the polarized sorting in Fruit Fly (Drosophila melanogaster). For instance, AP-1 regulates the basolateral sorting of Sanpodo in the sensory organ cells (Benhra et al. 2011). Moreover, AP-1 controlling the basolateral sorting of the Na+ /K+ -ATPase is reported in zebrafish (Grisham et al.

2013). These demonstrate that the AP-1 as master regulators of polarized sorting in multi- organisms.

Galectins are a family of animal lectins comprising 15 members in vertebrates (Viguier et al. 2014). Galectin-3, -4 and -9, were described as key molecular factor of polarized transport of epithelial cells (Delacour et al. 2005; Delacour et al. 2006; Delacour and Jacob 2006; Mishra et al. 2010; Mo et al. 2012). For instance, Galectin-3 was reported to interacts with newly synthesized gp114 and p75NTR and regulate their apical sorting within the biosynthetic pathway (Delacour et al. 2007; Le Bivic, Garcia, and Rodriguez-Boulan 1993; Straube et al. 2013). Galectin-4 interacts with sulphatides in lipid raft microdomains in the HT29 enterocytic cell line and modulates their apical sorting (Delacour et al. 2005). Galectin-9 can bind the Forssman glycosphingo and interfere with apico-basal polarity in MDCK cells (Mishra et al. 2010). Thus, intracellular Galectins can directly modulate protein transport.

Lipid raft was considered to be sorting factor for apical sorted proteins as mentioned in chapter 1.2.2. However, many open questions remain such as their pre-existence or de novo formation upon protein arrival at the cell surface or their lifetime at the apical surface of epithelial cells.

At the level of the TGN, the apical sorted proteins must be selectively incorporated into specific transport carriers which are thought to be driven by clustering of lipid raft (Bieberich 2018; Cao, Surma, and Simons 2012). Nanoscale rafts are usually dispersed in a continuous non-raft phase (Ian A. Prior 2003; Kai Simons and Toomre 2000). In other words, lipid rafts form distinct liquid-ordered phases which dispersed in a liquid-disordered phases matrix of unsaturated glycerolipids (Brown and London 1998; Schroeder, London, and Brown 1994). Therefore, creating the line tension at the boundary of these two phases. Clustering of nanoscale rafts can further increase the line tension, then generates the membrane curvature, followed by fission and budding at the phase boundaries to form cargo-

containing membrane buds or tubules, finally, release the transport carrier. (Cao et al. 2012; Schuck and Simons 2004) (**Figure 6**). Galectins, annexins and VIP17/MAL proteins, are possible mediators of lipid clustering upon the exit from the TGN. For the raft-mediated pathway, galectin-9 is the strongest candidate in MDCK cells for a clustering function (Lisanti et al. 1989) and also the epithelial polarity because of its binding to the Forssman glycolipid (Mishra et al. 2010).

Vacuolar H^+-ATPase activity was another factor reported in zebrafish to regulate the sorting of O-glycosylated proteins at the TGN, and also the Rab8-dependent post-Golgi trafficking of different classes of apical membrane proteins. Thus, luminal acidification plays distinct and specific roles in apical membrane biogenesis (Levic et al. 2020).

Last but not least, there are also precisely basolateral and apical sorting "signals" could regulate the membrane protein sorting. I will describe below the identified sorting signals driving selective apical or basolateral protein sorting.

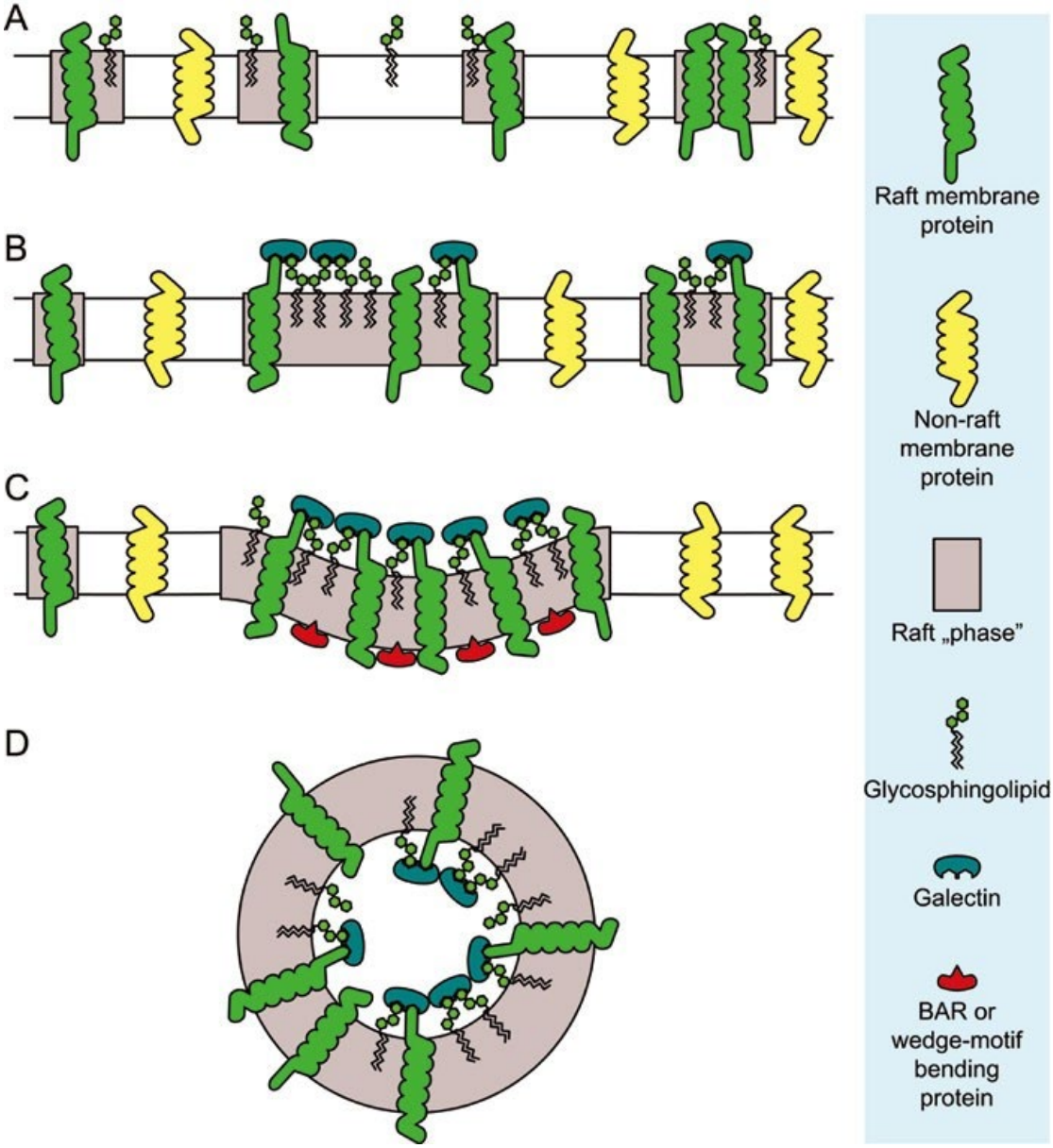

Figure 6 A scheme for apical transport carrier formation by lipid raft-induced budding.

(A) Nanoscale dynamic rafts surrounded by non-raft membrane. (B) lipid rafts form distinct liquid-ordered phases in the lipid bilayer and while non-raft components are excluded. Raft clustering results in increased line tension. (C) Growing rafts are selectively induced by galectin-glycolipid-glycoprotein (blue) interactions into a budding domain. Insertion of hydrophobic or amphipathic protein domains (red)

promotes membrane bending. (D) Fission at the domain boundary results in the release of an apical transport carrier (Cao et al. 2012).

2.4.1 Basolateral sorting signals

The basolateral delivery of protein trafficking was proposed as a default mechanism in the early works (Simons and Wandinger-Ness 1990). Then it was identified later that the basolateral sorting of protein relies on intrinsic basolateral sorting signals that can be found in the cytoplasmic domain of the proteins (Brewer and Roth 1991; Hunziker et al. 1991) as summarized in (**Table 1)**.

Evidences from several laboratories suggested that tyrosine-containing, 4-amino-acid motifs that can function as (or resemble) coated pit localization sequences are sufficient to ensure basolateral targeting in MDCK cells (Thomas and Roth 1994). Di-leucine-based endocytosis signals can also serve to target proteins basolaterally (Matter, Yamamoto, and Mellman 1994). Last but not least, the short cytosolic tails of polymeric immunoglobulin receptor (pIgR) (Casanova et al, 1991; Okamoto et al. 1992) and the low-density lipoprotein (LDL) receptor (Hunziker et al. 1991) are recognized as basolateral sorting signals of MDCK cells.

Table 1. Signals and mechanisms for sorting to the basolateral membrane.

Sorting Signal	Protein	Presumed Sorting Mechanism	References
Tyrosine-based	Low-density lipoprotein receptor	Recycling, but not delivery, is µ1b-dependent	(Klopfenstein et al. 2002; Matter et al, 1992)
	Vesicular stomatitus virus G protein	µ1b-dependent	(Folsch et al. 2003; Thomas and Roth 1994)
	lgp120	µ3a-dependent	(Hunziker et al. 1991; Stephens and Banting 1998)
Dileucine	Fc receptor FcRII-B2	µ1b-independent	(Hunziker and Fumey 1994; Matter et al. 1994; Roush et al. 1998)
	Mannose 6-phosphate receptor	µ1b-independent	(Distel et al. 1998; Johnson and Kornfeld 1992)
	E-cadherin	Rab11-mediated	(Lock and Stow 2005; Miranda et al. 2001)
Monoleucine	Stem cell facto	?	(Wehrle-Haller and Imhof 2001)
	CD147	Clathrin-mediated; µ1b-dependent	(Deora et al. 2005)
	Amphiregulin	Recycling, but not delivery, is µ1b-dependent	(Gephart et al. 2011)

Table from (Stoops and Caplan 2014)

2.4.2 Apical sorting signals

Signals driving the apical sorting of proteins are more heterogeneous and less well understood (Rodriguez-Boulan and Musch 2005). Remarkably, differently from basolateral sorting signals driving protein basolateral sorting, apical sorting signals have been localized to all the portions of apical proteins: extracellular, transmembrane and cytoplasmic domains as show in (**Table 2**) (Cao et al, 2012). **Table 2.** Signals and mechanisms for sorting to the apical membrane.

Sorting Signal	Protein	Presumed Sorting Mechanism	References
GPI-anchor	Decay accelerating factor	Lipid raft–associated	(Lisanti et al. 1989; Paladino et al, 2002)
	PLAP	Lipid raft–associated	(Paladino et al. 2004)
N-Glycans	Clusterin (gp80)	Raft-independent	(Graichen et al. 1996; Urban et al. 1987)
	gp114 Galectin-3–mediated,	raft independent	(Delacour et al. 2006; Le Bivic et al, 1993)
	Growth hormone	Galectins 3 & 4 independent	(Scheiffele et al, 1995)
	Erythropoietin	Cholesterol-dependent	(Kitagawa et al. 1994; Maruyama et al. 2005)
	Endolyn	Raft-independent	(Ihrke et al. 2001)
O-Glycans	p75 neurotrophin receptor	Galectin-3–mediated, raft independent	(Delacour et al. 2007; Delacour and Jacob 2006; Straube et al. 2013)
	Lactase phlorizin hydrolase	Galectin-3–mediated, raft independent	(Delphine Delacour et al. 2006; Delacour and Jacob 2006)
	MUC1	Raft-independent	(Huet et al. 1998; Kinlough et al. 2006; Mattila et al. 2009)
	Podocalyxin	Transient lipid raft association	(Yu et al. 2007)

	Dipeptidyl peptidase IV	Lipid raft–associated	(Naim et al. 1999; Slimane et al. 2000)
	Sucrase isomaltase	Lipid raft–associated	(Alfalah et al. 1999; Naim et al. 1999)
Transmembrane domain	Neuraminidase	Lipid raft–associated	(Barman and Nayak 2000; Kundu et al. 1996)
	Influenza hemagglutinin	Lipid raft–associated	(Lin et al. 1998; Scheiffele et al, 1997)
	Respiratory syncytial virus F protein	Lipid raft–associated	(Brock et al. 2005; Brown et al. 2004)
	Sucrase isomaltase	Lipid raft–associated	(Jacob et al. 2000)
	H, K-ATPase	Raft-independent	(Dunbar et al, 2000)

Modified from (Stoops and Caplan 2014; Weisz and Rodriguez-Boulan 2009)

2.4.2.1 The GPI-anchor

Originally the GPI-anchor *per se* was proposed to act as an apical sorting signal. The simple addition of 37 aa of the GPI-signal attachment sequence of decay accelerating factor (DAF) to the ectodomain of i) herpes simplex glycoprotein D (gD1), a basolateral antigen, or ii) to the human growth hormone, a regulated secretory protein secreted from both apical and basolateral domain, is sufficient to re-target these two chimeric proteins to the apical surface of polarized MDCK cells (Lisanti et al. 1989) therefore suggesting that the GPI anchor *per se* is the apical sorting signal. The GPI- anchor would act as apical sorting signal by mediating protein incorporation into the cholesterol- and sphingolipid- enriched membrane microdomain raft (Simons and Ikonen 1997; see below section 3.2.1). However, this hypothesis has been challenged by the finding that in Fisher Rat Thyroid (FRT) cells the chimeric GPI-APs gD1-DAF, is basolateral sorted (Zurzolo et al. 1993).

2.4.2.2 The role of N-and O-glycosylation and other apical sorting signals

Regarding the role of glycans in the apical sorting of proteins, there are many studies that depending on the cell types and proteins sorting analyzed could appeared contradictory.

N-glycans is essential for the apical sorting of soluble proteins in epithelial cells (Scheiffele et al. 1995). For example, the apically secreted secretory protein gp80 in MDCK cells has been shown to be secreted to both apical and basolateral domains of polarized MDCK cells upon tunicamycin treatment, an inhibitor of the first steps of glycosylation (Heifetz, Keenan, and Elbein 1979; Urban et al. 1987).

Regarding the TM protein, N-glycosylation was also found to be essential for the apical sorting of TM endolyn (Ihrke et al. 2001; Potter et al. 2006) and the TM glycine transporter (Martínez-Maza et al. 2001) in MDCK cells. Another study using Glycosylation-deficient MDCK cell lines showed that the apical glycoprotein TM gp114 was mainly basolateral sorted (>70%) (Le Bivic et al. 1993).

In contrast to these data showing the role of N-glycosylation as apical sorting signal for TM and secreted proteins, other studies revealed that N-glycosylation is not required for apical sorting of TM p75NTR (Yeaman et al. 1997) or secretory hepatitis B antigen (Marzolo et al, 1997) in MDCK cells.

O-glycosylation is another possible mechanism of apical protein sorting (Yeaman et al. 1997). The TM p75NTR and TM sucrase-isomaltase (SI), both apical model proteins, are characterized by the presence of heavily O-glycosylated stalk domains that if deleted lead to the non-polarized sorting to both membrane domains of these two aforementioned proteins (Jacob et al. 2000; Yeaman et al. 1997). Furthermore, addition of the O-glycosylated stalk domain of SI to rGH (rat Growth hormone), is sufficient to modulate the secretion of this chimeric protein from a non-polarized secretion in control Caco-2 cells to its apical sorting (Spodsberg et al, 2001)

Concerning the GPI-APs, N-glycosylation was found to be essential for the apical sorting of the GPI-AP dipeptidase in both MDCK and FRT cells (Pang et al, 2004). In addition, the rat Growth hormone, a non-glycosylated cargo, exhibiting an unpolarized secretion become predominant apical delivery once added N-glycan motif to the GPI-anchored form of growth hormone (Benting et al, 1999; Scheiffele et al. 1995). However, N-glycans are not required for the apical sorting of the GPI form of endolyn in MDCK cells (Potter et al, 2004). Moreover, mutagenesis of putative N-glycosylation sites of PLAP does not affect its oligomerization and apical sorting in polarized MDCK cells (Catino et al., 2008). At least N-glycans of the protein ectodomain are not directly involved in apical GPI-AP sorting in MDCK cells. However, in FRT cells the same mutant of PLAP lacking both N-glycosylation sites does not oligomerize and is missorted to the basolateral surface (Imjeti et al., 2011) (**Figure 9**). This difference might be caused by that the level of cholesterol contained in the Golgi of FRT cells that is higher than MDCK cells. Therefore, suggesting at least two apical sorting mechanisms exist for GPI-APs: one cholesterol-dependent and one rely on glycosylation depending on the epithelial cell lines (Imjeti et al. 2011).

In addition, apical sorting signals can also be found in transmembrane domains as in the case of the transmembrane domain of influenza virus neuraminidase (NA) that is associated to lipid rafts domains (Barman and Nayak 2000; Delacour and Jacob 2006; Kundu et al. 1996).

Interestingly, the cytoplasmic tail, which was described as containing basolateral sorting signals for basolateral proteins, was also reported as a putative apical sorting signal in case of rhodopsin (Chuang and Sung 1998; Tai et al, 2001), Na-dependent bile acid transporter (Sun et al. 1998) and megalin (Takeda et al, 2003).

3. GPI-anchored proteins

Glycosylphosphatidylinositol (GPI)-anchored proteins (GPI-APs) are a class of membrane proteins expressed widely in various species from the protozoa, fungi, to human. In eukaryotes GPI-APs represent 0.5% of total proteins. Importantly, more than 150 GPI-APs have been identified in mammalian cells and the loss of GPI-anchoring results in lethality (Lebreton et al, 2018). The GPI anchor is highly conserved among species and during evolution and GPI-APs exhibit diverse physiological roles including enzymes, cell surface antigens, signaling receptors, cell adhesion, migration molecules and protease inhibitors (Arkhipenko et al. 2016; Kinoshita et al, 2008; Lebreton et al,2018, 2019; Paladino et al, 2015).

GPI-APs are a class of integral membrane proteins containing a soluble protein attached by a conserved posttranslational glycolipid modification, the GPI anchor, to the external leaflet of the plasma membrane. The GPI anchor, Glycosylphosphatidylinositol, is a phosphoglyceride that can be attached to the C-terminus of a protein during posttranslational modification (Caras et al. 1987) (**Figure 7**). Briefly, GPI-APs are synthesized in the ER through about 20 sequential reactions as precursors transferred en bloc by the multi enzymatic complex, GPI-transamidase, to the C-terminal part of the protein after recognition of the GPI attachment signal sequence (Lebreton et al. 2019). One of the signal sequences is composed of the cleavable, hydrophobic amino- terminal and targets the protein to the lumen of the ER. The other one is composed of the cleavable, carboxy- terminal and directs GPI anchoring. The GPI moiety of GPI-APs consists of the conserved core glycan, phosphatidylinositol (PI) and glycan side chains. The structure of the core glycan is EtNP-6Manα2-Manα6-(EtNP)2Manα4-GlNα6-myoIno-P-lipid (EtNP, ethanolamine phosphate; Man, mannose; GlcN, glucosamine; Ino, inositol). The GPI is linked to the C-terminus via an amide bond generated between the C-terminal carboxyl group and an amino group of the terminal EtNP (Kinoshita 2020) (**Figure 7**).

The GPI-anchoring signal contains a hydrophobic region, which is separated from the GPI-attachment site (ω-site) by a hydrophilic spacer 58 region (**Figure 8**). The amino acids with small side chains are highly preferred for the two amino acids, ω + 1, ω + 2 that follow the ω-site. The action of a GPI transamidase in the lumen of the endoplasmic reticulum (ER) governs the glyco-lipid anchor attachment to the ω-site (Mayor and Riezman 2004) (**Figure 8**).

Besides providing a stable anchor to the plasma membrane, the presence of both lipid anchor and protein portion confers unique trafficking features and biological functions to these proteins (Lebreton et al. 2019, 2018). Moreover, GPI-APs partition with cholesterol and sphingolipids (SPLs) enriched membrane microdomains that could influence both their sorting, trafficking, surface organization (Lebreton et al. 2019; Lingwood and Simons 2010; Paladino et al. 2008, 2015; Sharma et al.(Harder and Sangani 2009; Ian A. Prior 2003; Simons and Gerl 2010; Simons and Toomre 2000, 2000)2004; Simons and Gerl 2010; Varma and Mayor 1998; Zurzolo and Simons 2016).

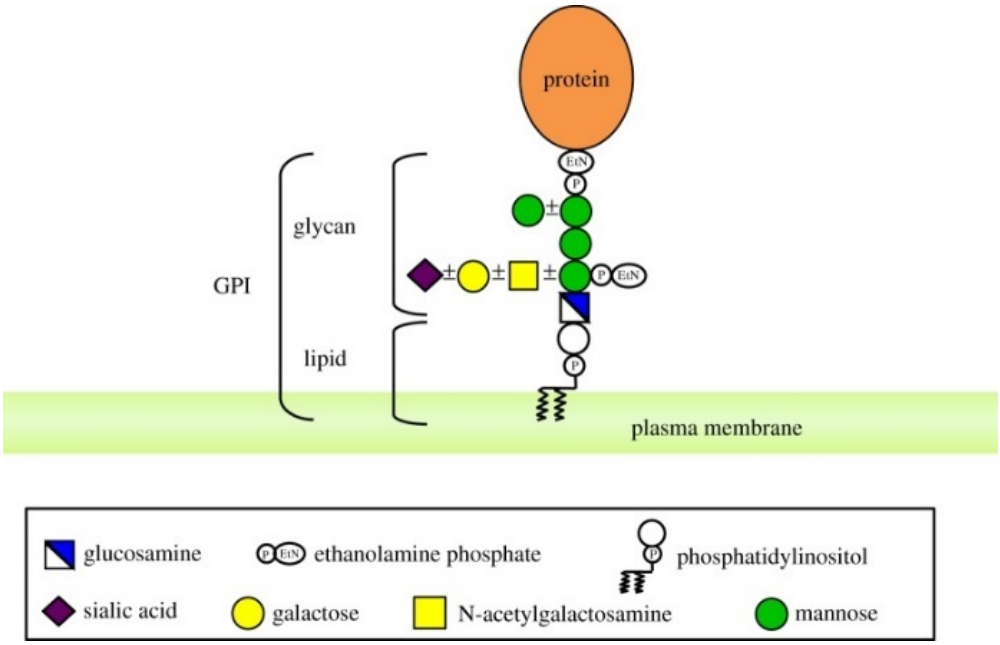

Figure 7. Mammalian GPI-APs.

The conserved core glycan of mammalian GPI, which consists of EtNP attached to the protein, three Mans, EtNP attached to Man1, and GlcN, is linked to the lipid moiety, which is PI. In some GPI-APs, the core glycan is modified by Man4 and/or GalNAc side chains. The GalNAc side chain can be elongated by Gal and Sia. The entire GPI-AP is anchored to the outer leaflet of PM only by hydrocarbon chains of PI (Kinoshita 2020).

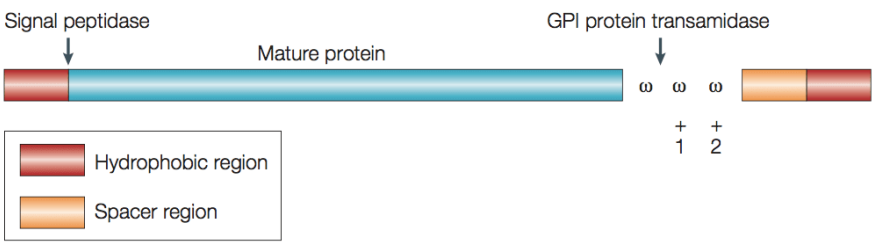

Figure 8. Structure of the GPI-anchored protein precursor.

GPI-APs are synthesized as precursors with a cleavable, hydrophobic amino-terminal signal sequence that targets the protein to the lumen of the ER and a cleavable, carboxy-

terminal signal sequence that directs GPI anchoring. The GPI-anchoring signal consists of a hydrophobic region separated from the GPI-attachment site (ω-site) by a hydrophilic spacer region. Amino-acid residues with small side chains are highly preferred for the two amino acids that follow the ω-site (Mayor and Riezman 2004).

3.1 Mechanism of GPI-APs apical sorting in polarized MDCK cells

As mentioned earlier GPI-anchor itself is not sufficient to drive their apical sorting. Moreover, GPI-APs attached to the external leaflet of the plasma membrane do not have any sorting signals that could be recognized by the cellular machinery as described earlier in section 2.4.2.

However commonly, all GPI-APs are associated with cholesterol-sphingolipid membrane microdomains originally called raft and describe as lipidic platform (Muniz and Zurzolo 2014; Simons and Ikonen 1997).

3.1.1 Lipid raft

The raft concept has enabled to better understand the spatiotemporal regulation of protein function within the plasma membrane, by assigning additional roles to lipids. Raft domains have been well studied in fibroblasts and immune cells, where they have been shown to be involved in the regulation of many cellular processes such as protein sorting, endocytosis, virus and bacteria infection, and cell signaling (Harder and Sangani 2009; Ian A. Prior 2003; Simons and Gerl 2010; Simons and Toomre 2000). Of interest, the size of raft domains is below the classical optical resolution of fluorescence microscopy. This study of raft domains is tightly relying on the existing and available methodologies in order to analyze their dynamics and visualize these membrane microdomains. For many years, their existence has been questioned

because of lack of detection using conventional fluorescence microscopy approaches. However open questions remain to be addressed such as whether lipid microdomains preexist in a form similar to that observed in model membranes during phase separation of liquid-ordered and liquid-disordered domains, and/or whether they are created by mutual interactions with proteins. Recent evidence supports this latter hypothesis, assigning to proteins an important role (Kusumi et al. 2011; Suzuki et al. 2012).

For GPI-anchored proteins, raft association is determined by the lipid anchor with its two (usually) saturated fatty acyl chains (Casey 1995). Particularly, the apical sorting of GPI-APs has been postulated to be mediated by their association with lipid rafts (Lebreton et al. 2019; Paladino et al. 2004; Simons and Ikonen 1997). However, the roles of the GPI anchor and the lipid rafts as apical determinants have been questioned by the findings that not only Fisher rat thyroid cells (Lipardi et al, 2000; Zurzolo et al. 1993) but also MDCK cells can sort GPI-APs both to the apical and basolateral domains (Benting et al. 1999; McGwire et al, 1999; Paladino et al. 2007; Sarnataro et al. 2002).

This indicate that although all GPI-APs are characterized by their affinity for the cholesterol- and sphingolipid- membrane domains, the association with lipid rafts is not sufficient to drive their apical sorting and additional factors must be involved.

3.1.2 Clusters formation in the Golgi

Work from our and other laboratories revealed that formation of high molecular weight (HMW) complexes or oligomer or cluster of GPI-APs concomitantly to their raft-association in the Golgi is the key step driving their apical sorting in polarized epithelial cells (Lebreton et al. 2019; Paladino et al. 2004, 2007, 2014; Schuck and Simons 2004).

Studies performed in our laboratory showed that impairment of Golgi GPI-APs oligomerization leads to their mis-sorting to the basolateral domain (Paladino et al. 2004, 2007a). This selective Golgi clustering mechanism is also functional in FRT cells. Although in FRT cells, the majority of endogenous and transfected GPI-APs are sorted to the basolateral domain (Lipardi et al. 2000; Paladino et al. 2007; Zurzolo et al. 1993) the few apical sorted GPI-APs have to form HMW complexes in the Golgi prior to their apical sorting. Importantly, the oligomerization is a specific requirement for apical sorting of GPI-APs but not for apical transmembrane protein (raft or non-raft associated) therefore suggesting the existence of several sorting mechanisms regulating the apical protein sorting (Paladino et al. 2007) (**Figure 9**).

More recently, thanks to the new biophysical approaches (Number and Brightness), studies from our laboratory showed that GPI-APs organize as homoclusters of 3 to 4 molecules (single species of GPI-AP) in the TGN prior to their apical sorting (Paladino et al. 2014). Importantly, they further reported that Golgi clustering of GPI-APs drives their apical sorting but also their further apical plasma membrane organization and their biological activities (**Figure 9**) (Lebreton et al. 2019; Paladino et al. 2014).

The lipid raft association would promote the stabilization of GPI-APs into rafts and therefore facilitate their apical sorting (Cunningham et al. 2003; Fivaz 2002; Helms and Zurzolo 2004; Paladino et al. 2004; Simons and Vaz 2004). Protein

oligomers could have a higher affinity for rafts than their monomer counterpart therefore resulting in different residency time in these membrane domains. Alternatively, protein oligomerization could drive the coalescence of small rafts into a larger raft which would increase the curvature of the membrane (Harder et al. 1998; Huttner and Zimmerberg 2001; Ikonen 2001; Kwik et al. 2003; Schutz et al. 2000) and result in the budding of an apical vesicle (Cunningham et al. 2003; Fivaz 2002; Paladino et al. 2004).

In conclusion, the clustering of GPI-APs in the Golgi is critical for their apical sorting and also govern their apical plasma membrane organization and their biological activities in polarized epithelial cells.

3.1.3 Mechanism of GPI-APs clustering in the Golgi

Although oligomerization of GPI-APs in the Golgi is the mechanism driving their apical sorting, the molecular factors regulating their Golgi clustering is still unknown. Therefore, we aim to unravel the molecular components that regulate Golgi GPI-AP clustering in polarized epithelial cells.

3.1.3.1 Cholesterol

As I mentioned previously the Golgi membranes are enriched in cholesterol. It has been shown that the reduction of cholesterol levels decreases the exocytosis of protein to the apical surface in MDCK cells. Specifically, it was reported in MDCK cells that Influenza Virus Hemagglutinin (HA) is not properly sorted to the apical surface upon cholesterol depletion and that the apical secretion of the soluble cargoes gp80 relies on cholesterol levels also, while the sorting of the basolateral marker vesicular stomatitis virus glycoprotein (VSVG) in fully polarized MDCK cells is

unaffected (Keller and Simons 1998). Therefore, suggesting a selective role of cholesterol in the apical sorting of membrane and soluble proteins.

Regarding the apical sorting of GPI-APs and the contribution of cholesterol, it was reported that selective Golgi GPI-APs clustering relies on cholesterol levels in MDCK cells but not in FRT cells.

In polarized MDCK cells, depletion of cholesterol is sufficient to selectively impair Golgi clustering of GPI-APs that therefore become basolaterally missorted (Paladino et al. 2008, 2014). On the contrary, the exogenous addition of cholesterol is sufficient to induce GFP-PrP anchor only, (where GFP is fused to the GPI attachment signal of the prion protein) a chimeric basolateral sorted GPI-AP in control condition, to oligomerize in the Golgi leading to its apical missorting (Paladino et al. 2008; Lebreton et al. 2008). Interestingly, the addition of cholesterol results in lower diffusional mobility in the Golgi membranes of GFP-PrP anchor that would stabilize the protein for further oligomerization in Golgi membrane (Lebreton et al. 2008). Therefore, a specific membrane environment enriched in cholesterol is proposed to be required to facilitate the formation of GPI-APs (Lebreton et al. 2008). Precisely, it is hypothesized that cholesterol addition changes the lipid environment by increasing ordering that would favor clustering of the “basolateral” GFP-PrP anchor only in the Golgi. Alternatively, cholesterol might stabilize the interaction of basolateral GPI-APs with lipid domains by acting more directly on a differently remodeled anchor, therefore, inducing the budding into apical vesicles (Campana, Sarnataro, and Zurzolo 2005). Overall cholesterol level is a master regulator of Golgi GPI-APs clustering and therefore of their apical sorting (**Figure 9**).

Interestingly, in FRT cells the selective GPI-APs Golgi clustering mechanism is also regulating their apical sorting. Previously, analyses from our laboratory reported that the higher level of cholesterol in the Golgi of FRT cells compared to MDCK cells is not a master regulator of Golgi GPI-APs clustering but instead the N-glycosylation

of GPI-APs ectodomain is the critical event for oligomerization and apical sorting of GPI-APs in FRT cells (Imjeti et al. 2011).

These data indicate the existence of at least two mechanisms driving Golgi oligomerization of GPI-APS in epithelial cells, one relying on cholesterol levels as in MDCK cells and another one relying on N-glycosylation as in FRT cells.

3.1.3.2 Protein ectodomain

As I mentioned in previous part, GPI-APs have to form homocluster in the Golgi in order to be apically sorted. Intriguingly, once the homoclusters are formed, they become insensitive to cholesterol depletion (Lebreton et al. 2019; Paladino et al. 2014). This suggests that not only protein–lipid but also protein–protein interactions or non-covalent interactions between protein ectodomains are involved in the formation and stabilization of GPI-AP clusters.

Regarding the role of the ectodomain in the oligomerization formation of GPI-APs. One evidence is coming from the single point mutation experiment where our lab members considered GFP S49/71 fused to the GPI attachment signal of the folate receptor (FR) whereby this mutation abolishes the capacity of GFP to dimerize via disulfide bonds. In this case GFPS49/71 GFP-FR (GPI) is basolaterally missorted and this basolateral sorting cannot be reverted by the exogenous addition of cholesterol in MDCK cells (Jain et al. 2001; Paladino et al. 2008)) therefore revealing that 1) the ectodomain of GPI-APs has to be permissive to allow clustering of protein and that 2) dimer of GPI-APs might be the minimal organization unit to further organize in cluster as also suggested by Suzuki in 2012 showing that GPI-APs homodimers will be the units for raft organization and function (Suzuki et al. 2012).

Even though they considerably improved the knowledge on GPI-APs clustering mechanism driving their apical sorting, the molecular machinery regulating this process is still unknown.

3.1.4 Putative interactors, galectins

Besides the role of factors mentioned above in oligomerization formation, other factors could also promote and regulate the clustering of GPI-APs. It is also unknown whether the budding and formation of the apical GPI-AP-enriched vesicle requires cytosolic proteins (Lebreton et al. 2019); whether a putative receptor would be required to interact with GPI-AP oligomers to form or stabilize in the TGN to favor their incorporation into an apical secretory vesicle (Paladino et al. 2004, 2007).

So far, several proteins have been reported to regulate the apical transport of raft-associated TM and GPI-APs such as Vesicular integral protein 17 (VIP17/MAL) and galectins. However, none of them seem to be specific for GPI-APs and their mechanistic role is not completely understood (Cao et al. 2012; Cheong et al. 1999; Delacour, Koch, and Jacob 2009; Lebreton et al. 2019; Martin-Belmonte et al. 2000; Paladino et al. 2015; Zurzolo and Simons 2016). For instance, it was revealed that VIP17/MAL associated to lipid microdomains is involved in the apical sorting of both apical transmembrane p75NTR and some GPI-APs as PLAP in epithelial cells (Cheong et al. 1999; Lebreton et al. 2019; Paladino et al. 2015). The galectins are a kind of interesting candidate which has been reported to be involved in facilitating the clustering of the glycosphingolipid sulfatide specifically enriched in lipid rafts, therefore, promoting the segregation of lipids and proteins in apical exocytosis (Viguier et al. 2014). For instance, although galectin-4 is not a specific factor for GPI-APs, it could help the coalescence of GPI-AP carcinoembryonic antigen (CEA) homoclusters and their subsequent incorporation in the apical GPI-AP-enriched vesicle and affect apical trafficking of GPI-AP raft-associated proteins in HT-29

5M12 cells (Delacour et al. 2005). It is also reported the depletion of Galectin-9 drives the GPI-AP CEA mislocalize to lateral surface of MDCK cells (Mishra et al. 2010).

It has been shown in a recent study that clathrin and AP1 are required for apical sorting of GPI-APs. In MDCK cells, either clathrin or AP1 silencing leads to the basolateral mis-sorting of the GPI-AP CD59 while it does not affect the apical and basolateral transport of transmembrane proteins (Castillon et al. 2018). However, whether the clustering of GPI-APs is affected by the reduced expression of these two aforementioned proteins remains unknown.

Now many open questions remain: How AP-1 is recruited specifically in the GPI-AP–enriched domains? Is there any putative receptor for GPI-AP cargoes? To address these questions, further studies need to be performed.

In summary, the apical sorting of GPI-APs in epithelial cells relies on their capacity to form HMW or oligomer or cluster in the Golgi apparatus. This Golgi selective sorting mechanism regulates their apical sorting, their plasma membrane organization at the apical surface and their biological activities. Overall epithelial cells develop a way to finely regulates GPI-APs activities at the apical surface only when the cells are fully polarized. Overall, this work revealed that both protein-protein interaction and cholesterol levels in the Golgi are essential. However, the molecular machinery regulating Golgi clustering of GPI-APs are not clear and is one of the questions I am going to address.

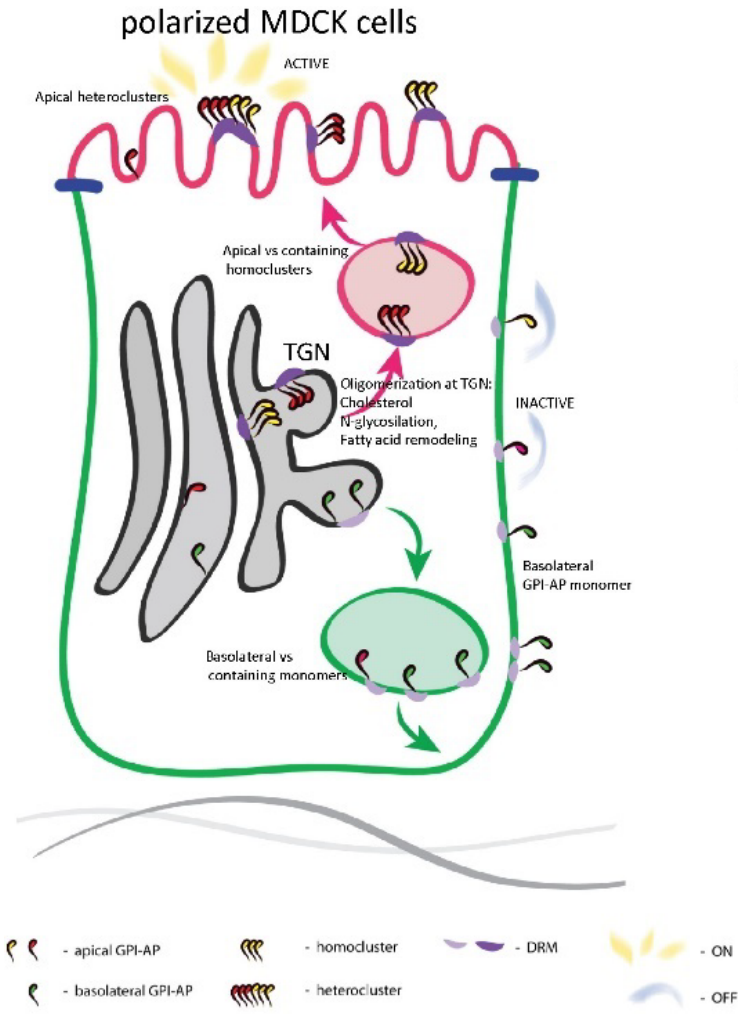

Figure 9. A schematic model of sorting mechanism of GPI-APs in the TGN of polarized MDCK cells.

Upon GPI lipid remodeling with saturated fatty acid chains in the Golgi, GPI-APs are segregated from other proteins into detergent resistant membranes (DRMs) which are enriched in sphingolipids and cholesterol. Further segregation would then occur as a consequence of the oligomerization process that might involve putative luminal receptors binding either the ectodomain or the lipid anchor (Muniz and Zurzolo 2014) for details. Vesicle formation and budding might derive from the coalescence of lipid domains driven by protein oligomerization. Apical GPI-APs form homoclusters in the Golgi of polarized MDCK cells driving their apical sorting that might promote raft coalescence "per se" and reinforce the segregation among different lipid phases, consequently favoring the formation of apical vesicles. Different GPI-APs reach the

apical plasma membrane in homoclusters and coalescence into heteroclusters defined as containing at least two distinct types of GPI-APs. Importantly oligomerization of GPI-APs was shown to regulate their plasma membrane organization and biological activity (yellow aura) (Paladino et al. 2014). (Modified from (Zurzolo and Simons 2016). Note that the mechanism of basolateral sorting is not detailed for clarity.

4. Soluble protein

4.1 secretory pathway of soluble protein

4.1.1 Conventional pathway

In eukaryotes, conventional protein secretion (CPS) is the trafficking route that secretory proteins undertake when they are transported from the ER to the Golgi, and subsequently to the plasma membrane. More precisely, secretory proteins are translocated in the ER upon the interaction between the signal recognition particle (SRP) and its receptor SR by signal sequence recognition; then the proteins are transported into the ER lumen through the translocon. The transport between ER and Golgi is described previously in 2.2. Then the proteins destined to be secreted are sorted from TGN toward the PM in secretory vesicles (SVs) or immature secretory granules (ISGs) (**Figure 10**). Depending on the cell types and cell functions proteins can be sorted via either the constitutive or regulated exocytosis.

Regarding the constitutive pathway, proteins are secreted as fast as they are synthesized. Secretory vesicles fuse with the plasma membrane to release their contents by exocytosis. Constitutive secretory cells are probably the most common class of cells and include liver cells, fibroblasts, muscle cells (**Figure 10**).

The regulated secretion is characterized by the storage of newly synthesized proteins in secretory storage granules that in response to an extracellular stimulus

would fuse with the plasma membrane and be released into the extracellular space. Proteins are sorted from the TGN into an immature secretory storage granule that then further mature before being release in the extracellular space upon signal reception. Professional regulated exocytosis cells are therefore able to release in a short-time scale large amounts of protein and are characterized by the accumulation of secretory granules intracellularly that would be released only upon reception of the signal (Kelly 1985) (**Figure 10**).

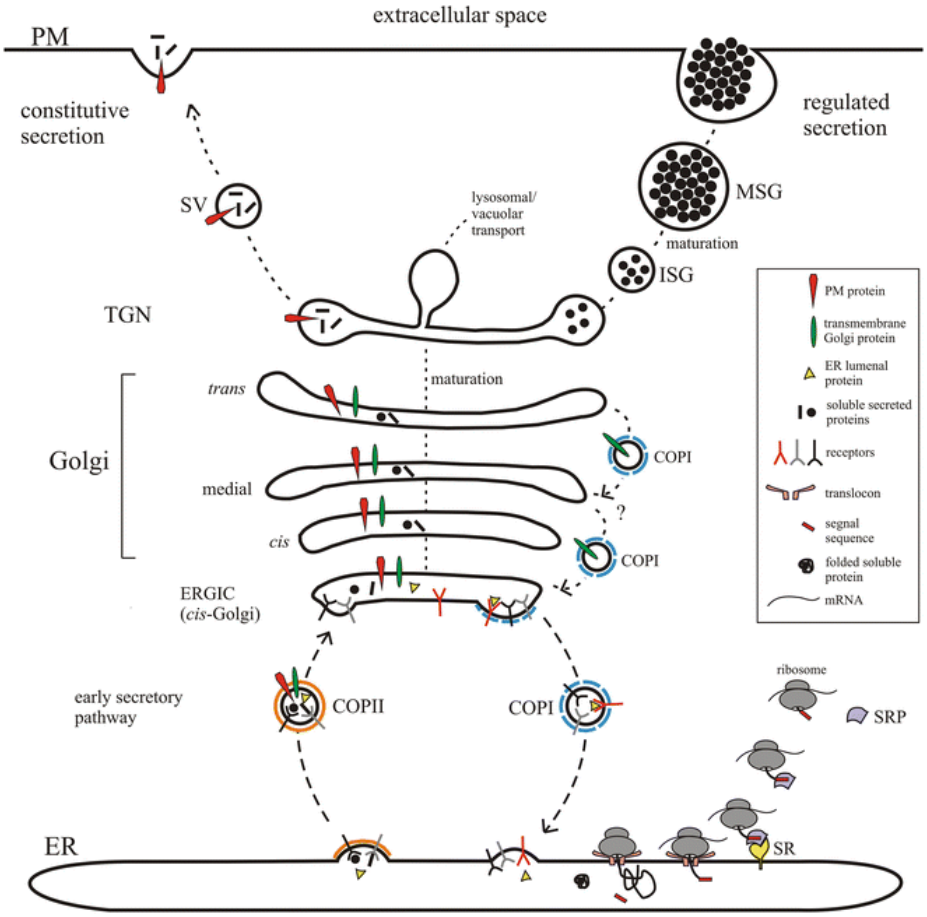

Figure 10. Schematic representation of conventional protein secretion in eukaryotes.

More precisely, secretory proteins are translocated in the ER upon the interaction between the signal recognition particle (SRP) and its receptor SR by signal sequence recognition; then the proteins are transported into the ER lumen through the

translocon. In the ER, the signal sequence is cleaved off and packed in COPII vesicles. COPII vesicles are delivered to the ERGIC. Escaped ER luminal proteins are retrotransported from the ERGIC or from the cis -Golgi to the ER via COPI vesicles. PM proteins and secreted proteins are transported via cisternal maturation to the TGN, whereas integral Golgi proteins are retrieved via intra-Golgi COPI-mediated transport, although another model has been proposed. Then the proteins destined to be secreted are sorted from TGN toward the PM in secretory vesicles (SVs) or immature secretory granules (ISGs). SVs are delivered constitutively toward the PM, whereas ISGs are accumulated in the cytoplasm until the specific stimulation arrived, ISGs form mature secretory granules (MSGs) and transport to the PM (Pompa and De Marchis 2016).

In endocrine cells the secreted proteins are concentrated as much as 200-fold (Eder 2009; Nickel 2011; Salpeter and Farquhar 1981) during their passage from the last cisterna of the Golgi to the mature secretory vesicle. In exocrine cells, concentration of about nine folds relative to RER level occurs in specialized condensing vacuoles (Bendayan 1984; Bendayan et al. 1980; Edgerton et al. 2021; Slot and Geuze 1983).

Cells can regulate their sites of exocytosis as well as the rate. Cells such as fibroblasts do not regulate their site of secretion but may secrete components of the extracellular matrix anywhere on their cell surface. Mast cells or neutrophils secrete the contents of their secretory vesicles toward any part of the plasma membrane that is stimulated. Such cell types can be considered as nonpolarized secretory cells.

Epithelial cells, like liver, endocrine, or exocrine cells, secrete some of their products through a specialized domain of their plasma membrane. Neurosecretory cells, such as the hypothalamic neurons that release hormones only from nerve terminals in the posterior pituitary, are extreme examples of this type of directed or

polarized secretion. Such cells can be considered as polarized secretory cells. A secretory cell, therefore, can be regulated or constitutive, polarized or nonpolarized (**Table 3**).

Table 3

Regulated	Constitutive
Polarized	
Neurons	Liver parenchymal
Endocrine	
Spermatocytes (acrosome reaction)	
Nonpolarized	
Neutrophils	Fibroblasts
Mast cells and basophils	Chondrocytes
Egg cells during fertilization	Macrophages
	B Lymphocytes

4.1.2 Unconventional pathway

A number of proteins are secreted in an unconventional manner, which use a route that bypasses the Golgi apparatus, named unconventional protein secretion (UPS) (**Figure 11**). For instance cytosolic proteins such as fibroblast growth factor 2 (FGF2) (Eder 2009; Nickel 2011) and membrane proteins that are known to also traverse to the plasma membrane by a conventional process of exocytosis, such as α integrin (Schotman, Karhinen, and Rabouille 2008), the cystic fibrosis transmembrane conductor (CFTR) (Gee et al. 2011) or the extracellular matrix proteins like galectins (Seelenmeyer et al. 2005) and the neuropathogenic protein α-synuclein (Ejlerskov et al. 2013; Lee et al, 2005) appear independent of the conventional or canonical secretory pathway. Evidently, both soluble and membrane-bound proteins located at various cellular compartments can undergo UPS.

There are at least three different transport modes in the UPS pathway, which depend on the nature and cellular location of the cargoes involved. Firstly, for proteins that are absolutely cytosolic without any membranous vesicles (such as FGF2) secretion would require some specific membrane translocation processes to bring them across the plasma membrane (Schäfer et al. 2004). Secondly, cytoplasmic proteins could be encased by membrane prior to secretion, and these processes may involve the generation of exosomes or ectosomes (Camussi et al. 2010; Lee et al. 2011; Mathivanan, Ji, and Simpson 2010; Nickel and Rabouille 2009; Record et al. 2011; Sadallah et al, 2011). Thirdly, some soluble and membrane bound cargoes could initially enter the canonical secretory pathway through ER translocation as they possess ER-targeting signals. However, subsequently, they could be transported to the cell surface or be secreted in a manner that is independent of COPII-mediated ER budding, and by bypassing the Golgi apparatus (**Figure 11**).

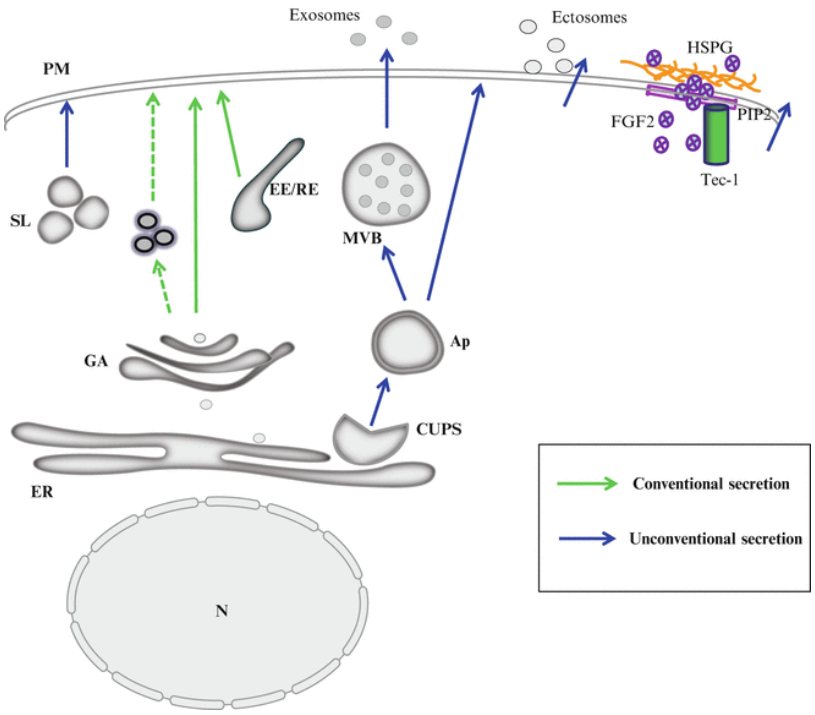

Figure 11. Conventional versus unconventional secretion. A schematic of pathways and compartments involved in UPS.

The conventional secretory pathways are marked by green arrows and the unconventional pathways by blue arrows. FGF2 UPS is regulated by the Tec-1 kinase. phosphatidylinositol 4,5-bisphosphate (PIP2) and extracellular heparin sulfate proteoglycan (HSPG) are also required. While the molecular factor involved in other modes of UPS are less clear. N nucleus, ER endoplasmic reticulum, CUPS compartment for unconventional protein secretion, Ap: autophagosome, GA: Golgi apparatus, MVB: multivesicular bodies, EE/RE: early endosome/recycling endosome, SL: secretory lysosomes, PM: plasma membrane (Pompa et al. 2017).

4.2 Mechanisms for sorting of soluble cargo proteins

Sorting of soluble cargoes remains elusive although three distinct mechanisms have been described so far: either sorting of soluble cargoes into specific storage granules, receptor-mediated sorting and the newest Cofilin/SPCA1/Calcium/Cab45 machinery. I will briefly below present these distinct sorting mechanisms.

4.2.1 Sorting into secretory storage granules

This sorting mechanism of soluble cargoes is taking place in professional secretory cells, exocrine or endocrine cells for instance, where enzymes and hormones such as Chromogranin A and B, secretogranins are aggregated in the TGN in several hundred nanometers vesicles. Then, importantly there is maturation of secretory storage granules that become mature upon removal of the mis-localized proteins. A feature of this sorting mechanism allows monitoring dense core in electron microscope of aggregated proteins inside the TGN then in immature and then mature secretory storage granules. Since this sorting mechanism relies on the efficient aggregation of soluble cargoes in the TGN, an intriguing question is how proteins can

aggregate in this compartment? To date it would either be based on intrinsic characteristic of the proteins or receptor-mediated (see below). In all cases high calcium concentration is required as well as acidification of the compartment. These aggregated proteins in the TGN would interact with cholesterol-sphingolipid enriched domains before being sorting in immature storage secretory granules that then mature before being release upon reception of the external stimuli (**Figure 12**).

4.2.2 Sorting by receptors

Another mechanism of soluble cargo sorting relies on receptors that would link the cargoes to cytosolic clathrin-coated vesicles. So far two receptors have been described the well know mannose 6-phosphate receptor and VPs10p domain (VDCRs).

Soluble lysosomal hydrolases, composed of approximately 50 acid hydrolases, including proteases, lipases, nucleases, phosphatases, and sulfatases, are sorted from the TGN towards early or late endosomes via their integration into clathrin-coated vesicles. The sorting mechanism briefly relies on a two-step reaction where the soluble cargoes in the cis-Golgi receive a manose-6-phosphate modification (M6P) allowing its recognition by the M6P receptor which exhibits an 'acidic cluster dileucine (AC-LL) signals' recognized by GGA (Golgi-localizing, γ-adaptin ear domain homology, ARF-binding protein) and AP1 adaptor proteins that leads to recruitment of clathrin from the cytoplasm and the assembly of clathrin-coated vesicles at the TGN. The acidic pH of the endosomal system favors the dissociation of the cargoes from the receptors that would be recycled back to the TGN.

Another M6P-independent transport ensuring sorting of soluble lysosomal proteins have been described in mammalian cells and relies on sortilin or sortilin-related proteins, members of the Vps10p-domain-containing receptors (VDCRs) family (a type I single-pass transmembrane receptors) by direct binding with the cargoes (Hermey 2009) (**Figure 12**).

In addition to the above-mentioned receptors, recent evidence also suggested a role for a carrier protein in transporting Wingless/int (Wnt), a conserved family of secreted proteins that act as morphogens to activate diverse pathways required for major developmental processes and maintain adult tissue homeostasis (Clevers and Nusse 2012). In epithelial cells, Wntless (Wls) together with Wnt3a are sorted from the TGN to the basolateral surface in AP-1- and clathrin-dependent manner (Yamamoto et al. 2013). Therefore, it may be possible that Wls acts as a sorting receptor at the TGN.

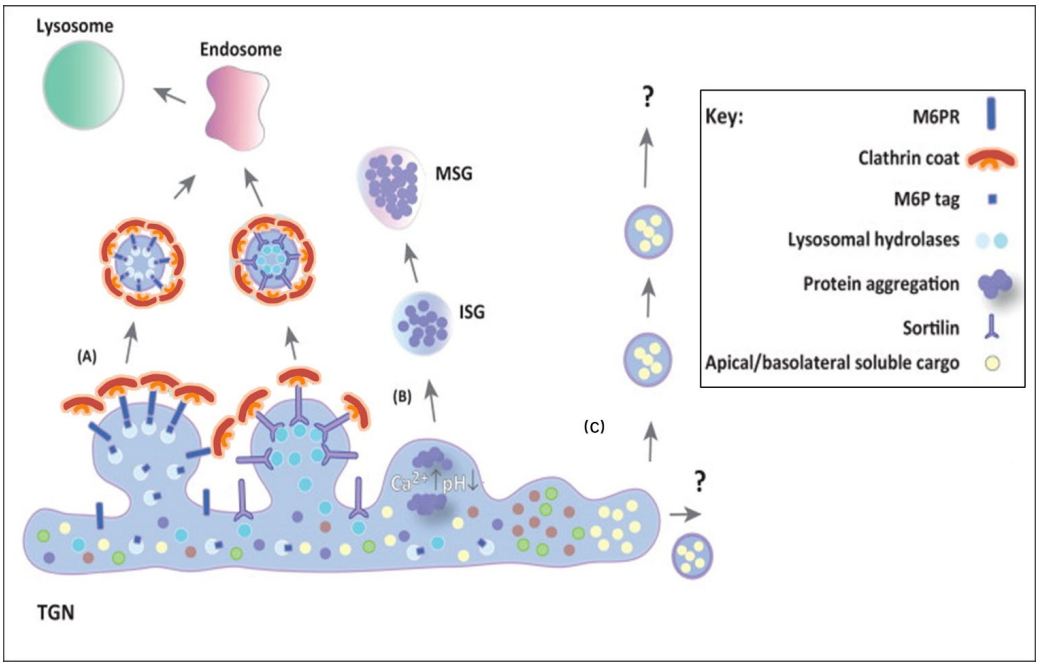

Figure 12. Different mechanisms for sorting of soluble cargo proteins in the TGN lumen.

(A) Soluble proteins are sorted at the TGN by receptor-mediated mechanism 1) either by addition of a M6P (Mannose-6-Phosphate) (blue dot) motif on the soluble cargoes allowing its recognition by the M6P receptor (M6PR) (blue stick) or 2) by direct binding of cargoes to TM sortilin (purple) that via clathrin coated vesicles would be sorted to endosomes and lysosomes (red). (B) Proteins destined for secretory storage granules (SSG) accumulate in TGN subdomains with low pH and high calcium concentrations. These aggregates are then

packed into vesicles which mature into SSGs and are stored inside the cell until an external stimulus releases them. (C) Recently, it was shown that the receptor-independent sorting by CFL1/SPCA1/Cab45 is responsible for the sorting of a subset of soluble cargoes from the TGN for transport to the plasma membrane. Nonetheless, how other soluble secretory proteins destined to the apical and basolateral cell surface are sorted remains poorly understood (Kienzle and von Blume 2014).

4.2.3 Calcium based sorting of soluble cargo

Recently, another molecular machinery involving calcium has been identified as playing a role in the sorting of a subset of soluble cargoes. This mechanism relies on the interplay between actin, calcium ions and resident Golgi calcium binding protein Cab45. This new secretory pathways recently named the Sphingomyelin secretion (SMS) pathway in Hela cells requires specifically the cooperative action of F-actin and Cofilin that interacts with the transmembrane calcium/ manganese pump secretory pathway Ca(2+) -ATPase pump type1 (SPCA1) at the TGN allowing calcium entry that in turn induces Cab45 oligomerization that selectively bind to secretory cargo proteins in a Ca^{2+}-dependent manner to promote their sorting and secretory vesicle formation (von Blume et al. 2009, 2012; Deng et al. 2018; Kienzle and von Blume 2014; von Blume et al. 2011). It was further reported that sphingomyelin in the TGN is essential for SPCA1 pump activity but also for the formation of the secretory vesicles (Deng et al. 2018).

Whether such mechanism could take place in fully polarized epithelial cells is an open question. Therefore, one of the central aims of this doctoral thesis was to understand the molecular mechanisms underlying sorting of constitutively secreted soluble proteins in polarized epithelial cells.

THE AIM OF MY PH.D.

Therefore, the aim of my Ph.D. was to unravel the molecular machinery regulating the apical sorting of GPI-APs in polarized MDCK cells. Then, I wonder whether the Golgi calcium binding Cab45 regulates the apical sorting of soluble cargoes in polarized MDCK cells.

I therefore divided my Ph.D. project in:

(1) Calcium levels in the Golgi complex regulate clustering and apical sorting of GPI-APs in polarized epithelial cells

(2) Cab45 affects apical secretion of the soluble protein PLAP-sec in polarized MDCK cells

EXPERIMENTAL RESULTS

Project 1

(The link to the article in PNAS: https://doi.org/10.1073/pnas.2014709118)

Calcium levels in the Golgi complex regulate clustering and apical sorting of GPI-APs in polarized epithelial cells

Summary of results

Glycosylphosphatidylinositol-anchored proteins (GPI-APs) are a class of membrane proteins which associated to lipid raft by a conserved glycolipid anchor. They are selectively sorted to the apical surface in polarized epithelial cells, where they reside and play diverse vital functions. Cholesterol-dependent clustering of GPI-APs in the Golgi is the key step driving their apical sorting and their further plasma membrane organization and regulate their biological activities. However, the specific molecular factors regulating Golgi clustering of GPI-APs are unknown.

In this study, firstly, we show that the formation of GPI-AP homoclusters (made of single GPI-AP species) in the Golgi relies directly on the level of calcium within cisternae. We further demonstrate that the Golgi transmembrane calcium/manganese pump, SPCA1, regulates calcium levels in the Golgi with a statistical decrease of calcium concentration within the Golgi cisternae upon SPCA1 silencing. Importantly, silencing of SPCA1 impairs Golgi GPI-APs clustering and their apical sorting therefore identifying SPCA1 as regulator of GPI-APs sorting process in polarized MDCK cells.

Subsequently, we found that Cab45, a calcium-binding luminal Golgi resident protein is also involved required for Golgi clustering of GPI-APs and their apical sorting. Specifically, silencing of Cab45 leads to basolateral missorting of exogenous stably expressed apical GPI-APs but also of the endogenous GPI-APs therefore identifying Cab45 as a master regulator of Golgi GPI-APs clustering. In conclusion, our data revealed that beside cholesterol, calcium levels in the Golgi are critical in the mechanism of GPI-AP apical sorting in polarized epithelial cells and we further unravel the molecular machinery involved in the clustering of GPI-APs in the Golgi.

My contribution to this article

I performed all the biochemistry experiments on Cab45 in order to elucidate the role of this Golgi calcium binding protein in the Golgi clustering of GPI-APs and their apical sorting.

Calcium levels in the Golgi complex regulate clustering and apical sorting of GPI-APs in polarized epithelial cells

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Running Title: Sorting and clustering of GPI-APs

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Abstract

Glycosylphosphatidylinositol-anchored proteins (GPI-APs) are lipid-associated luminal secretory cargoes selectively sorted to the apical surface of the epithelia, where they reside and play diverse vital functions. Cholesterol-dependent clustering of GPI-APs in the Golgi is the key step driving their apical sorting and their further plasma membrane organization and activity; however, the specific machinery involved in this Golgi event is still poorly understood.

In this study, we show that the formation of GPI-AP homoclusters (made of single GPI-AP species) in the Golgi relies directly on the levels of calcium within cisternae. We further demonstrate that the TGN calcium/manganese pump, SPCA1, which regulates the calcium concentration within the Golgi, and Cab45, a calcium-binding luminal Golgi resident protein, are essential for the formation of GPI-AP homoclusters in the Golgi and for their subsequent apical sorting. Downregulation of SPCA1 or Cab45 in polarized epithelial cells impairs the oligomerization of GPI-APs in the Golgi complex and leads to their missorting to the basolateral surface. Overall, our data reveal, for the first time, an unexpected role for calcium in the mechanism of GPI-AP apical sorting in polarized epithelial cells and identify the molecular machinery involved in the clustering of GPI-APs in the Golgi.

Significance Statement

Our findings represent a fundamental advance in general understanding of the mechanisms of exocytosis in polarized epithelial cells that are crucial for the establishment and maintenance of epithelial cell polarity.

Moreover, our data also improve the knowledge on the machinery regulating polarized trafficking of GPI-anchored proteins, a class of lipid-associated proteins playing diverse vital functions, unravelling an unexpected role of calcium in their apical sorting.

Introduction

Glycosylphosphatidylinositol (GPI)-anchored proteins (GPI-APs) are localized on the apical surface of most epithelia where they exert their physiological functions, which are regulated by their spatio-temporal compartmentalization.

In polarized epithelial cells the organization of GPI-APs at the apical surface is driven by the mechanism of apical sorting, which relies on the formation of GPI-AP homoclusters in the Golgi apparatus (1, 2). GPI-AP homoclusters (containing a single GPI-AP species) form uniquely in the Golgi apparatus of fully polarized cells (and not in non-polarized cells) in a cholesterol-dependent manner (1, 3, 4). Once formed, GPI-AP homoclusters become insensitive to cholesterol depletion suggesting that protein-protein interactions stabilize them (1, 2). At the apical membrane newly arrived homoclusters coalesce into heteroclusters (containing at least two different GPI-APs species) that are sensitive to cholesterol depletion (1). Of importance, in the absence of homoclustering in the Golgi (e.g., in non-polarized epithelial cells) GPI-APs remain in the form of monomers and dimers and do not cluster at the cell surface (1, 5). Thus, the organization of GPI-APs at the apical plasma membrane of polarized cells strictly depends on clustering mechanisms in the Golgi apparatus allowing their apical sorting. This is different from what was shown in fibroblasts where clustering of GPI-APs occurs from monomer condensation at the plasma membrane, indicating that distinct mechanisms regulate GPI-AP clustering in polarized epithelial cells and fibroblasts (1, 6, 7). Furthermore, in polarized epithelial cells the spatial organization of clusters also appear to regulate the biological activity of the proteins (1), so that GPI-APs are fully functional only when properly sorted to the apical surface, and less active in the case of missorting to the basolateral domain (1, 8, 9). Understanding the mechanism of GPI-AP apical sorting in the Golgi apparatus is therefore crucial to decipher their organization at the plasma membrane and the regulation of their activity. The determinants for protein apical sorting have been

difficult to uncover, compared to the ones for basolateral sorting (10-14). Besides a role of cholesterol, the molecular factors regulating the clustering-based mechanism of GPI-AP sorting in polarized epithelial cells are unknown. Here, we analysed the possible role of the actin cytoskeleton and of calcium levels in the Golgi. The actin cytoskeleton is not only critical for the maintenance of the Golgi structure and its mechanical properties, but also provides the structural support favouring carrier biogenesis (15-18). The Golgi exit of various cargoes is altered in cells treated with drugs either depolymerizing or stabilizing actin filaments (19, 20), as well as the post-Golgi trafficking is affected either by knockdown the expression of some actin-binding proteins, which regulate actin dynamics, or by the overexpression of their mutants (12, 21-23), all together revealing the critical role of actin dynamics for protein trafficking. Only few studies have shown the involvement of actin remodelling proteins in polarized trafficking, mostly in selectively mediating the apical and basolateral trafficking of transmembrane proteins (24-26 and rewieved in 27), thus it remains unclear whether actin filaments play a role in protein sorting in polarized cells.

On the other hand, Golgi apparatus exhibits high calcium levels that have been revealed to be essential for protein processing and sorting of some secreted soluble proteins in non-polarized cells (28-31). Moreover, a functional interplay between actin cytoskeleton and Golgi calcium in modulating protein sorting in non-polarized cells has been shown (22).

In this study, we report that in epithelial cells actin perturbation does not impair GPI-AP clustering capacity in the Golgi and therefore their apical sorting. In contrast, we found that the Golgi organization of GPI-APs is drastically perturbed upon calcium depletion, and that the amount of calcium in the Golgi cisternae is critical for the formation of GPI-AP homoclusters. We further show that the TGN calcium/manganese pump, SPCA1 (Secretory pathway Ca(2+) -ATPase pump type 1), which controls the Golgi calcium concentration (32), and Cab45, a calcium-binding luminal Golgi resident protein previously described to be

involved in the sorting of a subset of soluble cargoes (33, 34), are essential for the formation of GPI-APs homoclusters in the Golgi and for their subsequent apical sorting. Indeed, downregulation of SPCA1 or Cab45 expression impairs the oligomerization of GPI-APs in the Golgi complex and leads to their missorting to the basolateral surface, but does not affect apical or basolateral transmembrane proteins. Overall, our data reveal an unexpected role for calcium in the mechanism of GPI-AP apical sorting in polarized epithelial cells and identify the molecular machinery involved in the clustering of GPI-APs in the Golgi.

Results

Calcium levels regulate homoclustering of GPI-APs in the Golgi apparatus of polarized epithelial cells

In non-polarized cells, the calcium content of the Golgi complex is high and has been shown to regulate essential processes such as protein processing and sorting of secreted soluble proteins (28-31). Of particular interest, some soluble cargoes cluster in a calcium-dependent manner to segregate in secretory vesicles (33-35). Based on this evidence, we analysed the possible role of calcium levels within the Golgi complex in GPI-AP clustering and apical sorting of GPI-APs.

To this aim, we specifically depleted calcium ions from the Golgi by treating polarized MDCK GFP-FR cells with ionomycin (see methods), an ionophore that efficiently promotes a drastic and rapid emptying of Golgi lumenal calcium with respect to others methods used for cytosol or other organelles (e.g., calcium free-medium, calcium chelators) (28, 29). By Number and Brightness (N&B) technique (36, 37) we measured the aggregation state (and number of molecules) of a model apical GPI-AP, GFP-FR, in the Golgi apparatus of polarized MDCK cells (stably expressing this protein; see methods and ref. 1) in control conditions and upon ionomycin treatment. In control condition the brightness of GFP-FR is ~1.20 that

corresponds to protein clusters containing three to four molecules (Figure 1A; see also methods and ref.1), while upon ionomycin treatment the brightness values of GFP-FR significantly decrease to 1.14 ($p<0.0001$) indicating a shift toward monomeric/dimeric forms (Figure 1A). To further assess the assembly of GFP-FR we performed velocity gradient sedimentation experiments on the pool of the proteins in the Golgi apparatus (see methods). This technique allows the proteins to sediment according to their molecular weight, thus revealing if the protein is in its monomeric form or in a high molecular weight (HMW) complex. In control conditions the Golgi pool of GFP-FR mainly sediments in HMW complexes, indicating its capacity to cluster; while upon ionomycin treatment the migration of the Golgi pool of GFP-FR showed a reduction in HMW complexes (Figure 1B). These results support that the amount of calcium in the Golgi lumen regulates the formation of GPI-AP homoclusters in polarized epithelial cells.

If this hypothesis is correct, we postulated that the Golgi apparatus of non-polarized MDCK cells, where GPI-APs do not cluster (1), would contain a lower amount of calcium compared to fully polarized MDCK cells. To test this hypothesis, we measured the calcium concentration ($[Ca^{2+}]$) in the Golgi apparatus of MDCK cells grown in polarized and non-polarized MDCK conditions by using a calcium-sensitive photoprotein (Golgi-aequorin chimera), as previously described (28, 38, 39). We found that the calcium levels in the Golgi apparatus of polarized MDCK cells are higher compared to the Golgi of non-polarized MDCK cells (Figure 1C), thus supporting a role for calcium in the mechanism of GPI-APs clustering in the Golgi apparatus.

The Golgi calcium/manganese ATPase SPCA1 is involved in the calcium dependent regulation of GPI-AP homoclustering

147 The calcium levels in the Golgi apparatus are achieved by the action of two groups of
148 phosphorylation-type calcium-pumps, the well-known SERCAs (sarcoendoplasmic-reticulum
149 Ca-ATPases) and the more recently discovered SPCAs (32). Of interest, in HeLa cells
150 SPCA1 has been shown to regulate the sorting of some secretory soluble cargoes in secretory
151 vesicles at the Golgi level (22, 23, 30, 33) and, differently from SERCA, its activity seems to
152 be dependent on cholesterol and sphingomyelin (40). Thus, we first assessed whether SPCA1
153 regulates the concentration of calcium in the Golgi of polarized epithelial cells.
154 As for HeLa cells (22, 33), we found that endogenous SPCA1 localizes in the Golgi apparatus
155 of both non-polarized (1 day) and fully polarized (3 day) MDCK cells (Figure 2A). In both
156 conditions it co-localized with the trans-Golgi marker TGN46 with a similar Pearson
157 coefficient (0.77+/-0.09 and 0.78+/-0.08 respectively) (Figure 2A). Interestingly, western blot
158 analysis revealed that fully polarized MDCK cells exhibit relative higher amounts of SPCA1
159 compared to non-polarized MDCK cells (Figure 2B), suggesting that the expression levels of
160 SPCA1 may increase with the establishment of polarity. SPCA1 mRNA levels are
161 comparable in fully polarized MDCK cells with respect to non-polarized cells (Figure 2C).
162 In order to investigate whether SPCA1 regulates the concentration of calcium in MDCK cells,
163 we interfered the expression of SPCA1 by stably transfecting MDCK:GFP-FR cells with a
164 specific short hairpin RNA (shRNA). We obtained several clones (SPCA1i) with different
165 degrees of silencing ranging between 20-70% compared to scrambled interfered cells
166 (CTRLi) as assessed by western blotting and immunofluorescence (Figure S1). We used the
167 clones with the highest degree of silencing for further studies. In these cells we found a
168 strong reduction in the concentration of calcium in the Golgi (Figure 3A), indicating that
169 SPCA1 pump regulates calcium uptake into the Golgi apparatus of MDCK cells, similar to
170 HeLa cells (22, 31). According to this hypothesis, like in wild-type MDCK cells (Figure 1C)
171 in control interfered cells calcium levels increase upon polarization (Figure S1C), whereas in

SPCA1 silenced cells the concentration of calcium in the Golgi is comparable in non-polarized vs polarized conditions (Figure S1C).

Of importance, we found that the brightness of GFP-FR in the Golgi was significantly reduced in SPCA1i cells compared to scramble-interfered cells (from 1.26 to 1.1, $p<0.0001$; Figure 3B). Overall these results show that SPCA1 regulates the concentration of calcium in the Golgi of polarized MDCK cells and its downregulation affects GPI-AP homoclustering.

Because clustering of GPI-APs in the Golgi is crucial for their apical sorting (2, 4) we postulated that SPCA1 depletion would also alter their sorting and trafficking to the apical surface. To test this hypothesis, we monitored the transport kinetics of GFP-FR from the Golgi apparatus to the cell surface using cells grown to confluence on coverslips (Figure 3C). We used time-lapse confocal experiments based on temperature block assays (at 19.5°C) and subsequent warm up at 37°C (Figure 3C), as previously described (41). After temperature block (time 0), the protein is accumulated in the Golgi and the Pearson's coefficient of colocalization between GFP-FR and giantin/furin, two Golgi markers, is high in both control and SPCA1-silenced cells (Figure 3C). As expected, in control cells 1 hour after release from the Golgi block, this colocalization decreases (Figure 3C, Pearson coefficient of colocalization 0.64+/-0.07 at time 0 and 0.11+/-0.1 after 1 hour; $p<0.0001$) consistent with the exit of GFP-FR from the Golgi apparatus towards the surface. On the contrary, in the majority of SPCA1-silenced cells (~70%) the colocalization between GFP-FR and giantin/furin remains high even 1 hour after release from the Golgi block (Figure 3C, Pearson coefficient of colocalization 0.75+/-0.05 at time 0 and 0.55+/-0.09 after 1 hour; $p<0.05$), indicating that a high amount of GFP-FR remains in the Golgi apparatus. In addition, it appeared that GFP-FR was also missorted to the basolateral surface (Figure 3C) suggesting that the impairment of GFP-FR homoclustering observed in SPCA1i cells (Figure 3B) affects both the Golgi exit and the apical sorting. In order to further investigate this hypothesis, we compared the distribution

of GFP-FR at steady state in control and SPCA1-silenced cells grown on filters in fully polarized conditions. As shown in Figure 3D and in Supplementary Figure S2A, while in control cells GFP-FR is present almost exclusively on the apical surface (80-85%), in the SPCA1i cells it is found in large amounts (about 50-60%) at the basolateral surface.

Next, to test whether SPCA1 had a specific role in the apical sorting of GPI-APs we analyzed the plasma membrane distribution of an endogenous apical transmembrane protein, GP114, in CTRLi and SPCA1i cells. Our data show no change in the polarized localization of this apical transmembrane protein indicating that the loss of SPCA1 specifically affects the sorting of GPI-APs (Figure 3D). As an additional control we analysed both the integrity of the monolayer and the sorting of an endogenous basolateral protein, E-cadherin. The distribution of the junctional protein ZO-1 and E-cadherin was comparable between control and silenced cells (Figures S2B and C), showing that SPCA1 knockdown does not alter the assembly of junctional complexes and therefore the integrity of epithelial cell monolayers, and does not have a role in basolateral sorting of transmembrane proteins.

All together these data reveal that SPCA1 plays a specific role in regulating the calcium-dependent homoclustering of GPI-APs in the Golgi, and their subsequent sorting and trafficking towards the apical surface.

Recently, a functional interplay between actin and SPCA1 via the actin-filament severing protein ADF/cofilin has been shown to promote the sorting of a subset of secretory proteins in HeLa cells (22, 23, 34). A similar mechanism could take place in polarized epithelial cells. To investigate this issue, we first asked whether perturbation of the actin cytoskeleton would affect GPI-APs protein sorting. To this aim, we measured the aggregation state of GFP-FR in the Golgi apparatus of polarized MDCK cells in control conditions and upon perturbation of the actin cytoskeleton by using latrunculin A (6 μM) as previously described (3, 42, 43) (Figure S3A). We found that upon latrunculin treatment the brightness values of GFP-FR did

not vary compared to control cells (1.24 and 1.26, respectively; Figure S3A). Consistently, latrunculin A did not affect the migration of the Golgi pool of GFP-FR on velocity gradients, thus indicating that actin perturbation does not modulate its oligomeric status (Figure S2B). Furthermore, in agreement with the role of clustering for GPI-AP apical sorting (2, 4), upon latrunculin addition GPI-APs were correctly sorted to the apical surface (Figure S2C).

Overall, these results suggest that actin cytoskeleton is not involved in Golgi GPI-AP clustering and apical sorting in polarized MDCK cells.

The Golgi calcium-binding protein Cab45 is involved in the regulation of GPI-AP homoclustering

SPCA1-dependent calcium levels in the Golgi apparatus have been previously shown to regulate the segregation into secretory vesicles and export from the Golgi of a subset of secretory proteins (Cartilage Oligomeric Matrix Protein (COMP) and lysozyme) in HeLa cells (22, 33). This was shown to be dependent on Cab45, a Golgi lumenal protein, which oligomerizes upon calcium binding (44) and selectively interacts with these soluble secretory cargoes allowing their export (33, 34, 45, 46). These data prompted us to investigate whether Cab45 could be involved in the regulation of apical sorting of GPI-APs in polarized cells. We first analysed the expression and localization of Cab45 in both polarized and non-polarized MDCK cells and we found that, similar to HeLa cells, Cab45 is enriched in the TGN and colocalizes with TGN46 marker in both conditions (Figure 4A; Pearson coefficient: 0.9+/-0,06 and 0,94+/-0,02 in non-polarized and polarized cells, respectively). qRT-PCR and western blot analyses showed that mRNA and protein levels of Cab45 are higher in non-polarized MDCK cells compared to polarized conditions (Figure 4B, C). Interestingly, by purification on velocity gradients we observed that Cab45 is mostly monomeric in non-polarized MDCK cells, while it forms HMW complex in polarized MDCK cells (Figure 4D),

supporting a correlation between Cab45 clustering and the higher levels of calcium in the Golgi apparatus in polarized vs non-polarized conditions (Figure 1C). To understand the function of Cab45 in protein sorting in polarized MDCK cells we generated stable knockdown Cab45 MDCK:GFP-FR cells (Cab45i). After infection with lentiviral particles containing a specific short hairpin RNA (shRNA) sequence targeted against Cab45 we selected MDCK GFP-FR clones exhibiting a decrease in Cab45 expression (Figure S4A, B).

Next, we analyzed the aggregation state of GFP-FR and found that the brightness of GFP-FR is significantly reduced in the Golgi of Cab45i cells in comparison to CTRLi (Figure 5A), indicating a reduction of GFP-FR homoclustering in the knockdown cells. By performing time-lapse microscopy, we monitored the trafficking of GFP-FR towards the surface in control and Cab45-silenced cells (Figure 5B). Strikingly, while GFP-FR is similarly enriched in the Golgi apparatus in both CTRLi and Cab45i cells, after temperature block (time 0), we monitored a strong delay in GFP-FR Golgi exit in Cab45i cells, as shown by high Pearson's coefficient of colocalization with giantin/furin after 1 hour release of the Golgi block at 37°C (Figure 5B, Pearson coefficient of colocalization 0.65+/-0.09 and 0.2+/-0.092 in Cab45i and CTRLi cells, respectively). In addition, it appeared that the majority of GFP-FR was missorted to the basolateral membrane in Cab45i cells (Figure 5B) indicating that impairment of GFP-FR homoclustering observed in Cab45i cells (Figure 5A) affects both the Golgi exit and the apical sorting. These data were sustained by the analysis of the protein distribution at steady-state in cells grown on filters in fully polarized conditions, where we observed more than 50% of GFP-FR at the basolateral surface in Cab45i cells compared to the apical enrichment in control cells (Figure 5C, S2A). Overall, these data show that in the absence of Cab45 GPI-APs do not cluster in the Golgi and they are missorted to the basolateral surface. Importantly, we observed that the polarized distribution of apical and basolateral transmembrane proteins, GP114 and E-cadherin respectively, was unaffected in Cab45

knockdown cells (Figure 5C and S4C), further supporting the specificity of the role of Cab45 in the apical sorting of GPI-APs. The effects of knockdown of Cab45 expression mimic the ones of calcium lowering, indicating that Cab45 might be the calcium dependent modulator of GPI-AP clustering and apical sorting.

Besides cholesterol, calcium plays a key role in apical sorting of GPI-APs

The above data have revealed the crucial role of calcium in homoclustering and apical sorting of the model GPI-AP GFP-FR. To further corroborate these findings, we analysed whether the suppression of SPCA1 or Cab45 impacts the sorting of a native GPI-AP, PLAP (Placental Alkaline Phosphatase), and of endogenous proteins. To this aim, we generated knockdown MDCK:PLAP cells for SPCA1 (Figure S5) or Cab45 by using the same methods aforementioned. In agreement with data obtained for GFP-FR, we observed that PLAP (about 30-35%) is basolaterally missorted in SPCA1-silenced cells, unlike 3-5% of PLAP delivered to the basolateral side in control-interfered cells (Figure 6A). Consistently, SPCA1 knockdown affects the PLAP homoclustering as shown by the reduction of HMW complexes on velocity gradients and a shift towards monomeric and dimeric forms (Figure 6B). Moreover, comparable results were obtained in Cab45-silenced cells where about 55-60% of PLAP is localized on the basolateral surface (Figure 6C), further highlighting the essential role of Cab45 as a regulator of apical GPI-APs sorting.

Taking advantage of the use of a fluorescent-conjugated version of the bacterial toxin aerolysin, which binds with high affinity GPI-APs (47), we analysed the surface distribution of endogenous GPI-APs upon Cab45 knockdown (Figure 6D). As expected in CTRLi cells about 70% (+/-9%) of GPI-APs are localized on the apical surface (Figure 6D), while in Cab45-silenced cells they are largely missorted to the basolateral surface (82% +/-3%),

strengthening the role of a calcium-dependent mechanism in homoclustering and apical sorting of GPI-APs.

Importantly, we could show that the polarized distribution of a basolateral GPI-AP, GFP-PrP, was unaffected in Cab45 knockdown cells (Figure S6), indicating that this calcium dependent mechanism is specific for GPI-AP apical sorting, and it does not work for basolateral pathway.

Next, to gain more insight into this mechanism, we analysed whether Cab45 suppression affects the sorting of the chimeric GPI-AP in which the GFP protein is exclusively fused to the GPI-attachment signal of PrP (3, 4). We have previously shown that this protein is basolaterally sorted and the exogenous addition of cholesterol is sufficient to determine its oligomerization and redirect it to the apical surface (3, 4). We generated MDCK:GFP-PrP (GPI attachment signal) cells stably knockdown for Cab45 and we analysed the impact of cholesterol addition.

As expected, in CTRLi cells upon cholesterol addition a larger amount of GFP-PrP (GPI attachment signal) is sorted to the apical surface (44% vs 23%, treated vs untreated cells) (Figure 7). On the contrary, the exogenous cholesterol addition is not sufficient to re-direct GFP-PrP (GPI attachment signal) to the apical surface (23% vs 25% treated vs untreated cells) in Cab45i cells (Figure 7), further supporting the critical role of Cab45 as a calcium-dependent modulator in GPI-AP apical sorting.

Overall, these data indicate that both cholesterol and calcium are key regulators of Golgi GPI-AP clustering and apical sorting and are both crucial for these processes.

Discussion

Proper protein sorting and trafficking to the cell surface is essential for the establishment and maintenance of epithelial properties and functions of epithelial cells.

GPI-APs are selectively localized at the apical surface in the majority of epithelia (reviewed in 8, 27, 48)) and are sorted at the TGN (41, 49), the major protein sorting station (50). It has been demonstrated that protein oligomerization is the key step to determine the apical sorting of GPI-APs in epithelial cells of different origins (2, 51). In particular, clusters of single GPI-AP species (named homoclusters) form in the Golgi apparatus of fully polarized cells when proteins traverse the medial Golgi (2, 51). The formation of GPI-AP homoclusters in the Golgi is dependent on the cholesterol concentration, while afterwards the formed homoclusters become insensitive to cholesterol depletion (1, 2, 4). It has been shown that the mechanisms responsible for homoclustering and apical sorting of GPI-APs regulate their subsequent organization in heteroclusters at the apical plasma membrane and their functional activity (1). This is important as this mechanism would ensure that only correctly sorted proteins to the apical membrane are organized in functional clusters, while the missorted ones remain monomeric/dimeric and inactive (1, 8, 48). This implies that in epithelial cells the functional cluster organization of GPI-APs at the apical plasma membrane is strictly linked with the acquisition of epithelial polarity (1). Hence, in non-polarized conditions GPI-APs are not clustered at the cell surface due to a lack of clustering in the Golgi (1).

This is very different from what occurs in fibroblasts where GPI-APs are organized in clusters in response to surface cues (6, 52-57). The mechanism that would assure a rapid change in the Golgi of polarized cells compared to non-polarized conditions remains unclear.

Lipidomic analyses have shown that epithelial cells undergo drastic changes in lipid compositions during cell polarization, among which is an increase in cholesterol (58). Previously we have shown that the levels of cholesterol in the Golgi of polarized and non-

346 polarized cells are different (1); however, while high cholesterol concentration is necessary
347 for the clustering of GPI-APs this is not sufficient for their apical sorting (4).
348 Here we discovered that another difference between the Golgi of polarized and non-polarized
349 MDCK cells is the calcium concentration, which increases substantially during the acquisition
350 of the fully polarized phenotype (Figure 1C). This was never reported before; nonetheless the
351 calcium content of the Golgi complex is known to be high (28, 29, 59) and has been shown to
352 regulate protein processing and sorting of secreted soluble proteins in non-polarized cells (30,
353 31). High calcium concentrations in the TGN promote the selective aggregation of secretory
354 granule cargoes such as granins, which is a key step for the sorting of regulated proteins (60-
355 62). Therefore, we assessed whether calcium had a role in the mechanism of GPI-AP
356 clustering occurring in the Golgi of polarized MDCK cells prior to their apical sorting. We
357 show that ionomycin, an agent perturbing/lowering the calcium levels, impaired the
358 homoclustering of GPI-APs in the Golgi, thus indicating that calcium levels are critical for
359 this process.
360 Two groups of phosphorylation-type calcium-pumps, the well-known SERCAs and the more
361 recently discovered SPCAs, regulate Golgi calcium levels (32). Interestingly, SPCA1 was
362 found mainly in detergent-resistant fractions (where GPI-APs abound) and its activity is
363 inhibited upon cholesterol depletion, while SERCA function was unaffected (40). Moreover,
364 recent findings have shown that SPCA1, by mediating calcium influx in the Golgi, plays a
365 key role in the trafficking of secretory cargoes and virus maturation (23, 30, 33, 63). Here we
366 show that the concentration of calcium in the Golgi of MDCK cells is dependent on SPCA1.
367 Most importantly, its protein expression increases during polarization, supporting that calcium
368 in the Golgi lumen is a crucial determinant in the establishment of epithelial polarized
369 phenotype. Remarkably, SPCA1 knockdown results in the loss of GPI-AP oligomerization
370 and missorting to the basolateral surface highlighting a role of SPCA1 in the apical sorting of

371 GPI-APs. This appears to be specific for GPI-APs as SPCA1 knockdown does not alter
372 sorting of apical transmembrane proteins, further supporting that the mechanisms regulating
373 GPI-AP and transmembrane protein sorting to the apical surface are different.
374 It is conceivable that Golgi calcium homeostasis is influenced by other intracellular calcium
375 stores (e.g., ER, cytosol, endosomes) or, vice-versa, the SPCA1 knockdown may impact the
376 calcium concentration of other organelles. While initial focus centered on membrane contact
377 sites between the ER and mitochondria (crucial for the transfer of calcium and lipids), in the
378 last years it is becoming clear that all organelles can contact each other, including the Golgi
379 apparatus (64). Efficient calcium transfer was ascribed to the ER-plasma membrane contact
380 sites and to ER-Golgi contacts (64, 65). To investigate whether the integration of calcium
381 signals from diverse compartments may synergically regulate their homeostasis and polarized
382 trafficking will be important in the future.
383 How SPCA1 would be regulated in the Golgi of polarized epithelial cells is an important
384 question. We showed here that actin cytoskeleton does not regulate Golgi GPI-AP clustering
385 and apical sorting, suggesting that the SPCA1-mediated sorting of GPI-APs is independent of
386 actin in epithelial cells. These data indicate that the mechanism regulating the activity of the
387 SPCA1 in polarized cells and in HeLa cells is different. Interestingly, it was reported that
388 SPCA1 is localized in cholesterol-enriched microdomains in colon adenocarcinoma and
389 kidney cells (40) and that SPCA1 pump's activity seems to be regulated by the levels of
390 cholesterol (40). Cholesterol levels in the Golgi cisternae of polarized MDCK cells are higher
391 compared to non-polarized MDCK cells (1, 58) and we have previously shown that high
392 cholesterol levels in the Golgi are required, but not sufficient for GPI-AP oligomerization (4).
393 Indeed, this process needs favourable cholesterol-enriched membrane environments but
394 depends also on protein-protein interactions of the GPI-AP ectodomains (2, 4, 27). Therefore,
395 in light of our results, one possibility is that cholesterol would indirectly favour GPI-AP

396 homoclustering by regulating the activity of the SPCA1, thereby controlling Golgi calcium
397 levels.
398 Moreover, a recent study showed that in HeLa cells SPCA1 activity is regulated by the levels
399 of sphingomyelin in Golgi membranes (35). Therefore, another open question is whether
400 sphingomyelin would also contribute to regulate SPCA1 activity in polarized MDCK cells.
401 To date, the role of sphingolipids in GPI-AP sorting is still not clear. In thyroid polarized
402 epithelial cells the treatment with the ceramide synthase inhibitor fumonisin B1 leads to the
403 basolateral missorting of GPI-APs (66), but also impairs the apical transport of
404 transmembrane proteins supporting a role of sphingolipids in promoting apical vs basolateral
405 segregation rather than specifically affecting GPI-APs (66). Similar conclusions arose from
406 studies showing that in MDCK cells, the knockdown of FAPP2 (four-phosphate-adaptor
407 protein 2), a protein which is required for the synthesis of glycosphingolipids, resulted in
408 delayed delivery and/or intracellular accumulation of apical raft-associated proteins (either
409 transmembrane and GPI-AP), whereas the basolateral transport was unaffected (67). On the
410 other hand, a recent study showing a functional link between GPI remodelling and
411 glycosphingolipid biosynthesis (68) points to a novel critical role for sphingolipid pathways in
412 the segregation and formation of apical GPI-AP vesicles. It will be interesting to test this
413 hypothesis in the future.
414 Besides interplay between lipids and calcium, calcium per se would exert its effect on GPI-
415 AP clustering through binding of specific calcium binding proteins. Several calcium-binding
416 proteins, such as calmodulin, have been shown to mediate fusion between yeast vacuoles, the
417 later steps of fusion vesicle trafficking and endosome fusion. At the level of the Golgi two
418 luminal calcium-binding proteins, Cab45 and p54/NEFA, have been identified. In non-
419 polarized cells, in response to SPCA1-mediated calcium influx, the Golgi-resident binding
420 protein Cab45 oligomerizes and in turns promotes oligomerization of soluble cargoes, and by

421 sequestering them within subdomains favours their export from the TGN in sphingomyelin-
422 enriched vesicles (30, 33-35, 45, 46).
423 Here we demonstrate that Cab45 localizes in the TGN of MDCK cells and forms oligomers
424 only in fully polarized MDCK cells (and not in non-polarized conditions), which is in good
425 correlation with the higher levels of calcium in the Golgi of these cells. We further show that
426 silencing of Cab45 induces reduction of homoclusters of GFP-FR and leads to its basolateral
427 missorting. Importantly, we highlighted the requirement of Cab45 for the apical sorting of the
428 native GPI-AP PLAP and of endogenous GPI-APs, further strengthening its role in GPI-AP
429 sorting. Also in this case, as shown for SCPA1, the knockdown of Cab45 affects specifically
430 the polarized trafficking of apical GPI-APs but not of basolateral ones as well as apical and
431 basolateral transmembrane proteins indicating that Cab45 is critical for Golgi clustering of
432 GPI-APs and their apical sorting. Moreover, the fact that mRNA of both SPCA1 and Cab45
433 are higher in cells expressing apical GPI-APs (Figure S7) strengthens the key role of these
434 two factors in GPI-AP apical sorting. On the other hand, in these cells we detected higher
435 protein levels only for SPCA1 and not for Cab45, which may imply different mechanisms
436 regulating the activity of these proteins.
437 This calcium-dependent mechanism is crucial for GPI-AP sorting because when it is inhibited,
438 the increased Golgi cholesterol levels, which are sufficient to re-direct apically a basolateral
439 GPI-AP (3, 4), are unable to promote their apical sorting. Thus, both cholesterol and calcium
440 are key determinants and concur to apical sorting of GPI-APs.
441 Overall, this study revealed for the first time in polarized epithelial cells an unexpected role
442 for the calcium levels in mediating Golgi clustering and sorting of GPI-APs, and unravelled
443 key players of the molecular machinery regulating these processes.
444 It is worth noting that both SPCA1 and Cab45 undergo changes during polarization (increased
445 protein expression and oligomerization, respectively), clearly indicating that their activity is

strictly related to the acquisition of a polarized cell phenotype. Hence, the control of these two players may fall in the global epithelial polarity program (69, 70). Post-translational rather than transcriptional mechanisms regulate SPCA1 expression. For example, SPCA1 may undergo a lower and/or slower turnover of protein levels. Its increased stability might correlate with its partitioning into lipid microdomains (40), whose main lipid components increase during cell polarization (58). Further studies will be necessary to test this hypothesis. On the other side, why Cab45 expression decreases during polarization remains an intriguing open question. One possibility is that Cab45 might be implicated in other processes important for the establishment of polarized cell phenotypes.

Moreover, another key question is through which mechanism Cab45 can sort GPI-APs. Pulldown experiments of HeLa cell lysates combined with mass-spectrometry analysis suggest that Cab45 interacts with secretory proteins (34), and this also could be the case for GPI-APs. Whether and how Cab45 may recognize protein ectodomains and/or lipid anchors, whether Cab45 interacts with monomers favouring their clustering or binds GPI-AP oligomers by stabilizing them remain the most intriguing questions. The fact that Cab45 is mainly localized in the TGN of MDCK cells when the oligomers are already formed, as previously shown in pulse-chase experiments (2), supports the role of Cab45 as a stabilizer of GPI-AP clusters. So far, no evidence exists showing that different GPI-APs share some structural similarities except that their C-terminal portion is characterized by four regions (including the GPI-attachment signal, amino acids upstream of the omega site), all determining the efficiency of the GPI trans-amidation reaction (71). Moreover, post-translational modifications and/or lipid remodelling of the GPI anchor may influence this interaction. A deep comparative analysis of protein ectodomains and lipid anchors of different GPI-APs will be the next challenge as well as that to unravel the common structural elements between GPI-APs and secreted proteins.

We propose a model of Golgi GPI-APs clustering in polarized MDCK cells in which the cholesterol environment could i) allow the establishment of the lipid environment favourable for GPI-AP oligomerization and ii) regulate the activity of the pump, which in turn is important for the oligomerization of Cab45 that stabilizes GPI-AP oligomers in a cholesterol-independent manner and facilitates their segregation in apically sorted vesicles (Figure 8).

Likely, this mechanism may operate in epithelial cells derived from different tissues and/or organs since the mRNA expression of both SPCA1 and Cab45 is quite comparable in organs composed of epithelial tissues [such as colon, kidney, liver, lung, thyroid etc. (44, 72, 73)]; higher mRNA and protein expression have been observed in rat brain and testis as compared to other tissues (74). Interestingly, higher mRNA levels of SPCA1 were observed in foetal organs with respect to the adult counterpart (73). This is in agreement with our data and suggests that a higher level of the SPCA1 pump is critical in the early phases of epithelial differentiation.

Among epithelia, hepatocytes are peculiar because they sort the majority part of apical proteins, including GPI-APs, through transcytosis. Whether and at which level of the transcytotic route the calcium-mechanism plays a role in the polarized trafficking of GPI-APs to liver canaliculi will be very interesting to study in the future.

Materials and Methods

Cell cultures, transfections and antibodies

MDCK cells were grown in DMEM (Sigma-Aldrich) containing 5% FBS. MDCK cells stably expressing the GPI-APs: GFP-FR, PLAP, GFP-PrP full-length or GFP-PrP carrying exclusively the GPI attachment signal of PrP were generated previously (2-4).

Cells were stably knockdown using either short hairpin RNAs or lentiviral vector (for details see Supplementary appendix).

Antibodies used were listed in Supplementary appendix.

Velocity gradients

Velocity gradients allowing purifying proteins according to their molecular weight, independently of their association with membrane domains were performed as previously described (75, 76), for details see Supplementary Appendix.

Calcium measurements

We measured the concentration of calcium in the Golgi apparatus by using the chimeric photoprotein Golgi-aequorin (GoAEQ) following a previously described protocol (28, 39), for details see Supplementary appendix.

Immunofluorescence

All details regarding the immunofluorescence assays, image acquisition and analyses are in Supplementary appendix.

N&B experiments

The Number & molecular Brightness method, a technique based on moment-analysis for the measurements of the average number of molecules and brightness in each pixel in fluorescence microscopy images (37), provides the state of aggregation of molecules in living cells with high spatial and temporal resolution. N&B experiments were carried out as previously described (1) and all details are in Supplementary appendix.

Statistical analysis

In N&B experiments we used a two-tailed student test for statistical analysis as well as to quantify all immunofluorescence mean intensities, in RT-qPCR one-way anova using prism was employed.

Further information (such as perturbation of actin cytoskeleton or cellular calcium content, temperature block, deglycosylation assay, RT-qPCR, labelling of endogenous GPI-APs) in Supplementary appendix.

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Author contributions

C.Z., S.L., S.P. conceived the project and designed experiments. S.P. planned and performed N&B. S.P, S.L designed biochemical and imaging experiments performed by S.P, S.L and

D.L. S.L designed and performed qRT-PCR. S.P. designed the experiments of calcium measurements, which were carried out by herself and M.N. in the P.P.'s lab, and P.P. helped with data analysis. S.L, S.P, and C.Z wrote the manuscript. All authors discussed the results and manuscript text.

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Figure legends

Figure 1: The content of calcium within the Golgi lumen, which is higher in fully polarized than non-polarized MDCK cells, is crucial for GFP-FR homoclustering

(A) N&B analysis of GFP-FR in the Golgi of polarized MDCK cells in control conditions or upon ionomycin treatment. Briefly, MDCK cells, grown on filters, were treated with trypsin 25 μg/ml for 25 min) exclusively at the apical side in order to remove the pool of GFP-FR already present at the plasma membrane and then incubated for 40 min at 4°C in Krebs-Ringer modified buffer (CTR) or in the same buffer supplemented with ionomycin (5 μM) and 600 μM EGTA (+ ionomycin) and imaged at the Golgi level. On the left, quantification of the brightness of GFP-FR in the Golgi compartment from three independent experiments either in control conditions (CTR, red bar) or upon calcium chelation (+ionomycin, blue bar). On the right, graphical representation of the percentage of pixels falling into the different

771 classes of B values (from monomer to hexamer) on the basis of the calibration curve (1).
772 Values are expressed as the mean of three independent experiments, n>25 cells. (B) MDCK
773 cells, grown on filters and treated as in (A), were lysed and run on a velocity gradient.
774 Fractions were collected from the top (Fraction 1) to bottom (Fraction 9), TCA-precipitated,
775 run on a SDS-PAGE gel and revealed by western blotting with a specific anti-GFP antibody.
776 Molecular weight markers are indicated on top of the panels. The molecular weight of the
777 monomeric form of GFP-FR is indicated together with the band at 43 kDa, which represents a
778 partially denatured dimer of GFP (2). On the right panel, the distribution of GFP-FR in the
779 fractions of the gradient is expressed as a percentage of the total protein. Mean values of two
780 independent experiments are shown.
781 (C) Golgi [Ca^{2+}] quantification in fully polarized and non-polarized MDCK cells. MDCK
782 cells transiently expressing Golgi-aequorin mutant were grown on coverslips for 1 day (non-
783 polarized) or for 3 days (polarized). Before carrying out the experimental procedure for
784 aequorin reconstitution with coelenterazine (see Methods), cells were treated 5 min at 37°C in
785 Ca-free medium containing ionomycin (5 μM) and EGTA (2 mM) in order to completely
786 empty the calcium from the Golgi apparatus. The coverslip with cells was placed in the
787 thermostatic chamber of the luminometer at 37°C and perfused with KRB supplemented with
788 0.1 mM EGTA. Where indicated, the EGTA was replaced with 1 mM $CaCl_2$. The [Ca^{2+}] in
789 the Golgi apparatus of polarized vs non-polarized MDCK cells is shown as a mean of three
790 independent experiments. Representative curves are shown.
791 Error bars, ± SD; NS, not significant; ** $p<0.01$; *** $p<0.001$, Student's *t*-test.
792
793 **Figure 2: SPCA1 is localized at the TGN of fully polarized and non-polarized MDCK**
794 **cells and its expression levels increase during polarization**

795 (A) MDCK cells after 1 and 3 days were stained with SPCA1 and TGN46 antibodies and
796 revealed by using alexa-488 and alexa-546, respectively. Experiments were performed three
797 different times and the Pearson's coefficient for colocalization analysis was measured; n>70
798 cells. Scale bars, 10 and 5 μm (1° day and 3° day, respectively). (B) MDCK cells grown for 1
799 and 3 days were tested for the expression of SPCA1 (101 kDa) by Western blotting. elF4A
800 was used as a loading control and the relative optical density of SPCA1 was normalized to
801 elF4A levels. Quantification was made from 8 different experiments and is expressed as a
802 mean. (C) SPCA1 mRNA levels of MDCK cells grown for 1 or 3 days were analysed by RT-
803 qPCR and normalized to HPRT and Ubch5 mRNA levels; experiments were performed 3
804 independent times.
805 Error bars, ± SD; NS, not significant; * $p<0.05$, Student's *t*-test.
806
807 **Figure 3. The loss of SPCA1 affects Golgi homoclustering and sorting of GPI-APs**
808 (A) Golgi [Ca^2] quantification in MDCK:GFP-FR cells control-interfered (CTRLi) or
809 SPCA1-interfered (SPCA1i) cells were measured following the same procedure described in
810 Figure 1C. The data represent the mean of four independent experiments performed in two
811 knockdown clones. **(**B**)** N&B analysis of GFP-FR in the Golgi of CTRLi and SPCA1i cells.
812 Representative B and I maps of SPCA1i cells are shown; scale bars, 0.9 μm. Quantification of
813 the brightness of GFP-FR in the Golgi compartment from three independent experiments
814 either in CTRLi (red bar) or SPCA1i, (blue bar) cells. **(**C**)** CTRLi and SPCA1i cells grown for
815 3 days on a coverslip were subjected to a temperature block (19.5°C) to accumulate proteins
816 in the TGN. Then cells were warmed at 37°C for the indicated times, fixed and treated for
817 confocal microscopy. Representative images taken at the top and at the middle of the cells are
818 shown. Pearson's coefficient between GFP-FR and giantin/furin is shown as mean of three
819 different experiments, n>60 cells. Scale bars, 4 μm. **(**D**)** CTRLi or SPCA1i cells grown for 4

820 days on a filter were imaged in live conditions or stained with anti-GP114 antibody. Mean
821 fluorescence intensities at the apical and basolateral surface were measured and expressed as
822 percentages of the total fluorescence. Scale bars, 12 and 6 μm in GFP-FR and GP114 panels,
823 respectively.
824 Error bars, ± SD; NS, not significant; ** p<0.01; *** *p*<0.001, Student's *t*-test.
825
826 **Figure 4. Cab45 localizes in the TGN and form oligomers in polarized MDCK cells**
827 (A) MDCK cells after 1 and 3 days were stained with Cab45 and TGN46 antibodies and
828 revealed by using alexa-488 and alexa-546, respectively. Experiments were performed three
829 different times and the Pearson's coefficient for colocalization analysis is shown; n> 65 cells.
830 Scale bars, 10 and 5 μm (1° day and 3° day, respectively). (B) Cab45 mRNA levels of MDCK
831 cells grown for 1 and 3 days were analysed by RT-qPCR and normalized to HPRT and Ubch5
832 mRNA levels. Experiments were performed 3 independent times. (C) Cells were also tested
833 for the expression of Cab45 (45 kDa) by western blotting. Tubulin was used as a loading
834 control and the relative optical density of Cab45 was normalized to tubulin levels. The mean
835 of 4 different experiments is shown. **(**D**)** Cells were lysed and run on velocity gradients;
836 samples were analyzed as described in Figure 1B. Molecular weight markers are indicated on
837 top of the panels. The molecular weight of the monomeric form of Cab45 is indicated.
838 Error bars, ± SD; NS, not significant; * p<0.05, Student's *t*-test.
839
840 **Figure 5: Cab45 is essential for apical sorting of GPI-APs**
841 (A) N&B analysis of GFP-FR in the Golgi of polarized scrambled and Cab45-silenced
842 MDCK cells. Quantification of the brightness of GFP-FR in the Golgi compartment from
843 three independent experiments either in CTRLi (red bar) or Cab45i (blue bar) clones. **(**B**)**
844 CTRLi and Cab45i cells grown for 3 days on a coverslip were subjected to a temperature

block assay as described in Figure 3C. Representative images taken at the top and at the middle of the cells are shown. Pearson's coefficient between GFP-FR and giantin /furin is shown as mean of three different experiments. Scale bars, 4 μm. **(**C**)** MDCK:GFP-FR CTRLi or Cab45i cells grown for 4 days on a filter were imaged in live conditions or stained with anti-GP114 antibody. Mean fluorescence intensities at the apical and basolateral surface were measured and expressed as percentages of the total fluorescence. Scale bars, 6 μm.

Error bars, ± SD. NS, not significant; * p<0.05; ** p<0.01; *** *p*<0.001, Student's *t*-test.

Figure 6: Calcium is critical for clustering and apical sorting of GPI-APs

MDCK:PLAP CTRLi or SPCA1i cells, grown for 4 days on filters, were subjected to different assays. (A) Cells were stained with anti-PLAP antibody and revealed with Alexa-546 secondary antibody. Mean fluorescence intensities at the apical and basolateral surface were measured and expressed as percentages of the total fluorescence. Scale bar, 6 μm. (B) Cells were lysed and run on velocity gradients. Fractions were collected from the top (Fraction 1) to bottom (Fraction 15), TCA-precipitated, run on a SDS-PAGE gel and revealed by western blotting with anti-PLAP antibody. Molecular weight markers are indicated on top of the panels. The molecular weight of the monomeric form of PLAP is indicated. (C) Cells were processed as in A. Scale bar, 10 μm. (D) MDCK CTRLi or Cab45i cells, grown on filter for 4 days, were incubated with ASSP (aerolysin mutant) conjugated to alexa-488 before fixation. Scale bar, 10 μm. Mean fluorescence intensities at the apical and basolateral surface were expressed as percentages of the total fluorescence.

Error bars, ± SD. ** p<0.01; *** *p*<0.001, Student's *t*-test.

Figure 7: Cholesterol is not sufficient to re-direct the basolateral GFP-PrP to the apical surface.

MDCK:GFP-PrP (GPI-attachment signal) CTRLi and Cab45i cells, grown for 4 days on filter, were incubated or not for one hour at 37°C with water soluble cholesterol (3, 4) before fixation. Mean fluorescence intensities at the apical and basolateral surface were expressed as percentages of the total fluorescence.

Error bars, ± SD. NS, not significant; *** $p<0.001$, Student's *t*-test.

Figure 8: Model of GPI-AP clustering and sorting in MDCK cells.

The scheme depicts the role of calcium in the Golgi clustering of GPI-APs (for simplicity only one GPI-AP is shown). A GPI-AP oligomerizes (forming a homocluster) after its association to lipid microdomains in the medial Golgi. The Golgi homoclustering regulates apical sorting of GPI-APs. Calcium uptake in the TGN, governed by SPCA1, is essential for GPI-AP clustering. In control cells, SPCA1 allows the uptake of calcium within the Golgi that would lead to oligomerization of Cab45, which in turn stabilizes GPI-AP clustering and apical sorting. Upon SPCA1 knockdown, less calcium is uptaken by the Golgi leading to the impairment of GPI-AP clustering that results in their missorting. Similarly, upon Cab45 knockdown, Golgi GPI-AP clustering is impaired leading to their basolateral missorting.

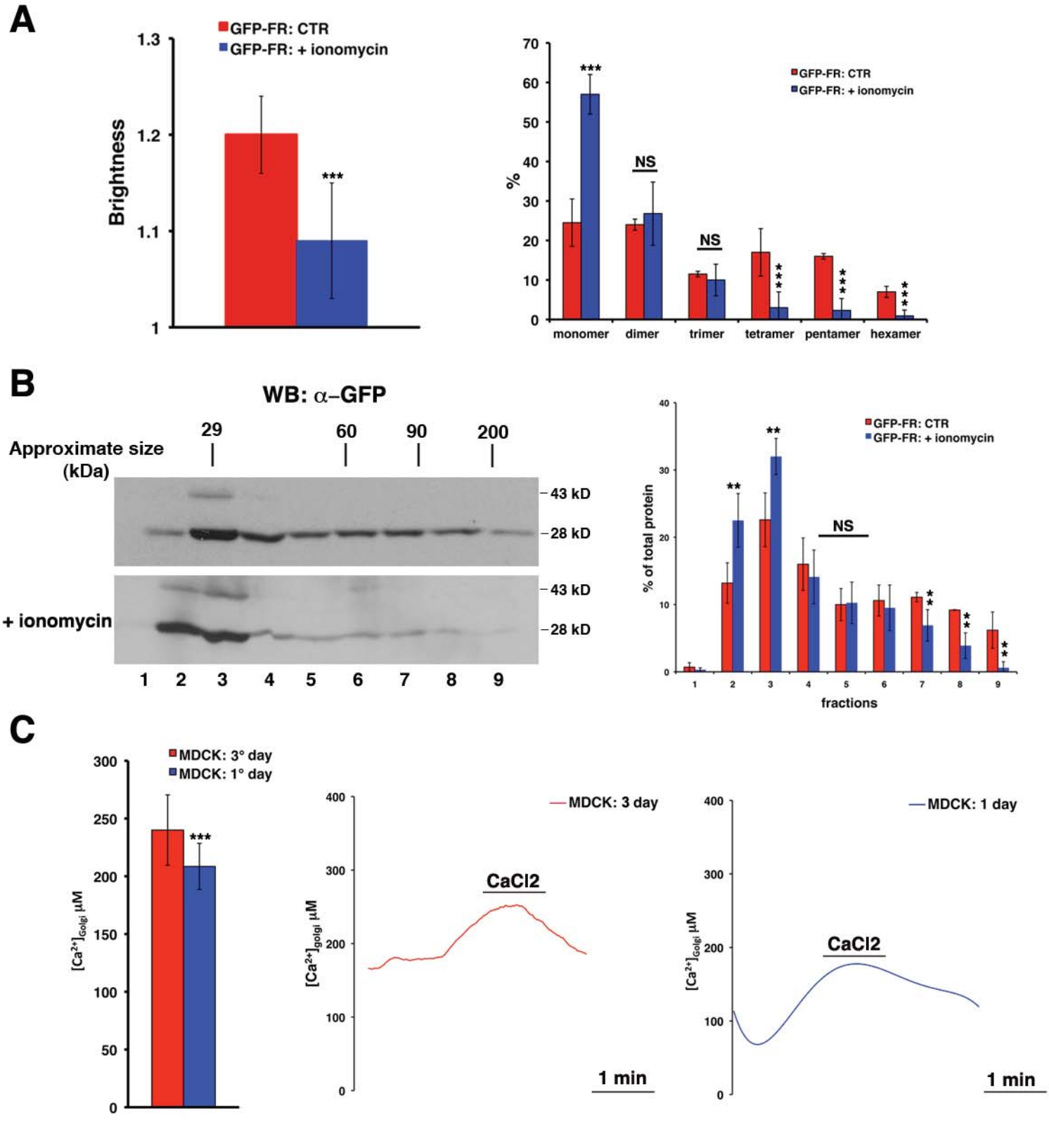

A
GFP-FR: CTR
GFP-FR: + ionomycin
Brightness
1.3
1.2
1.1
1
***
70
60
50
40
30
20
10
0
%
NS
NS
***
monomer
dimer
trimer
tetramer
pentamer
hexamer
B
WB: α–GFP
Approximate size (kDa)
29
60
90
200
43 kD
28 kD
+ ionomycin
1 2 3 4 5 6 7 8 9
% of total protein
fractions
**
NS
C
MDCK: 3° day
MDCK: 1° day
[Ca2+]Golgi µM
300
250
200
150
100
50
0
MDCK: 3 day
MDCK: 1 day
CaCl2
400
1 min

Figure 1

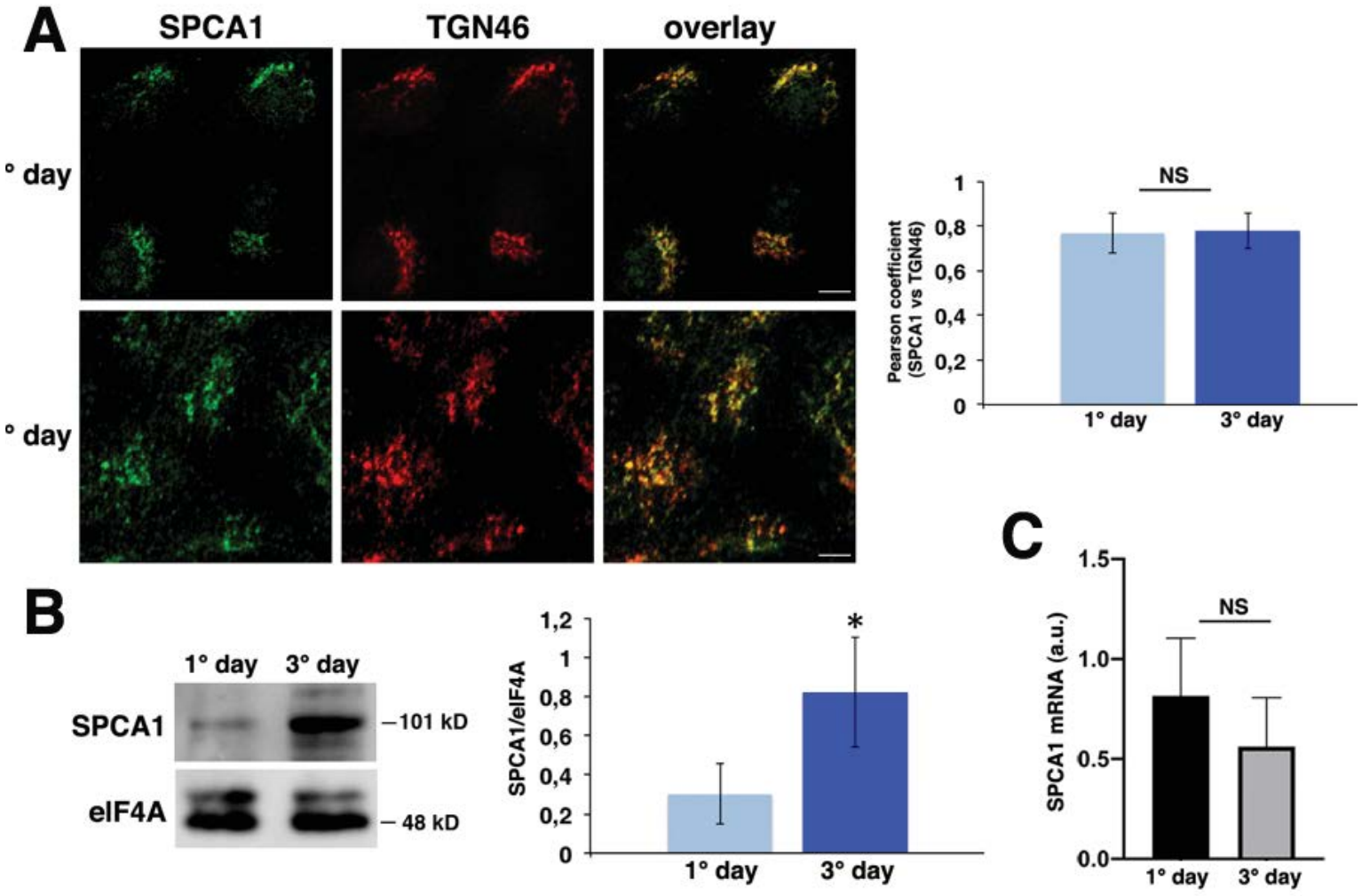

A
SPCA1
TGN46
overlay
° day
° day
Pearson coefficient (SPCA1 vs TGN46)
1
0,8
0,6
0,4
0,2
0
NS
1° day
3° day
B
1° day
3° day
SPCA1
eIF4A
101 kD
48 kD
SPCA1/eIF4A
1,2
1
0,8
0,6
0,4
0,2
0
*
1° day
3° day
C
SPCA1 mRNA (a.u.)
1.5
1.0
0.5
0.0
NS
1° day
3° day

Figure 2

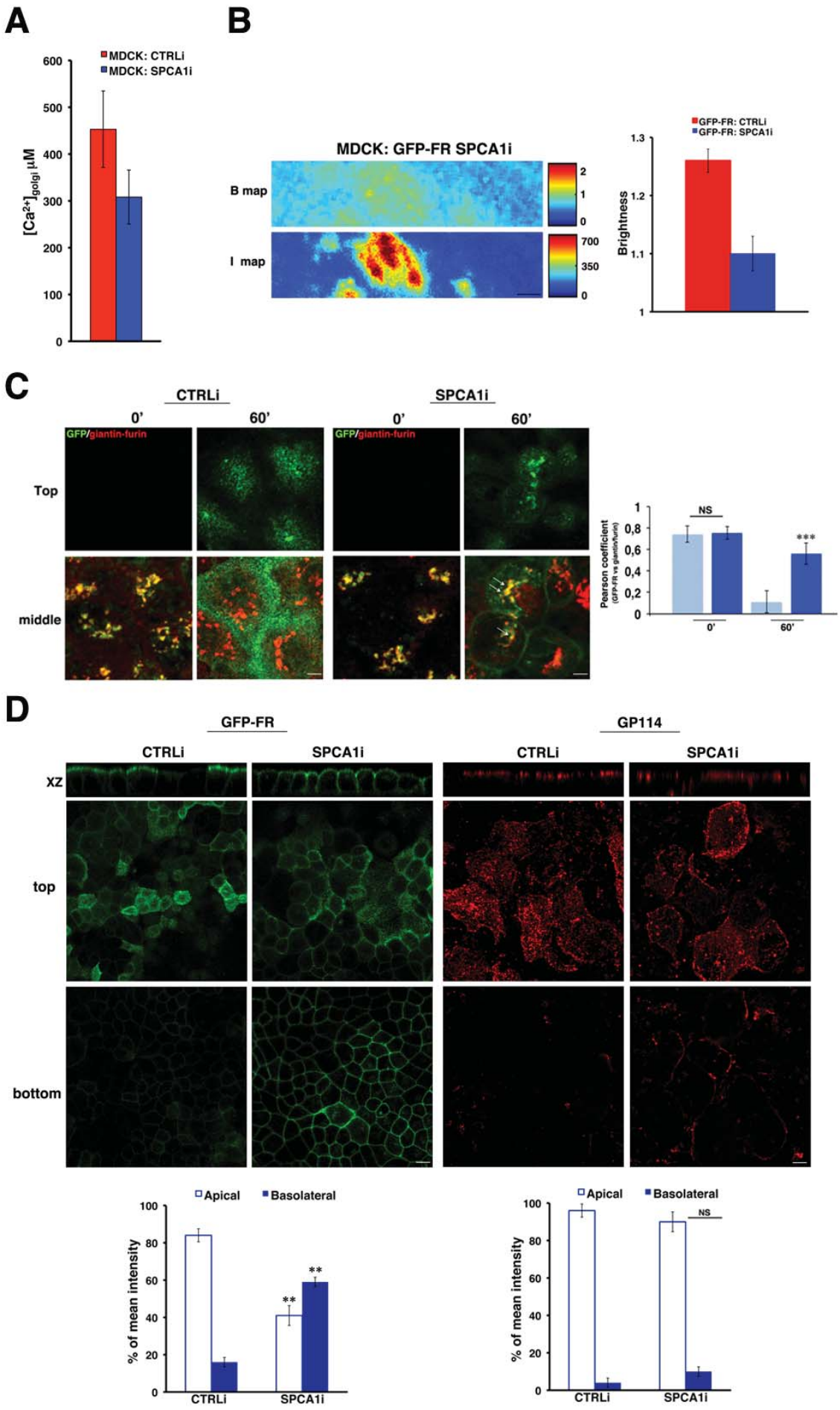
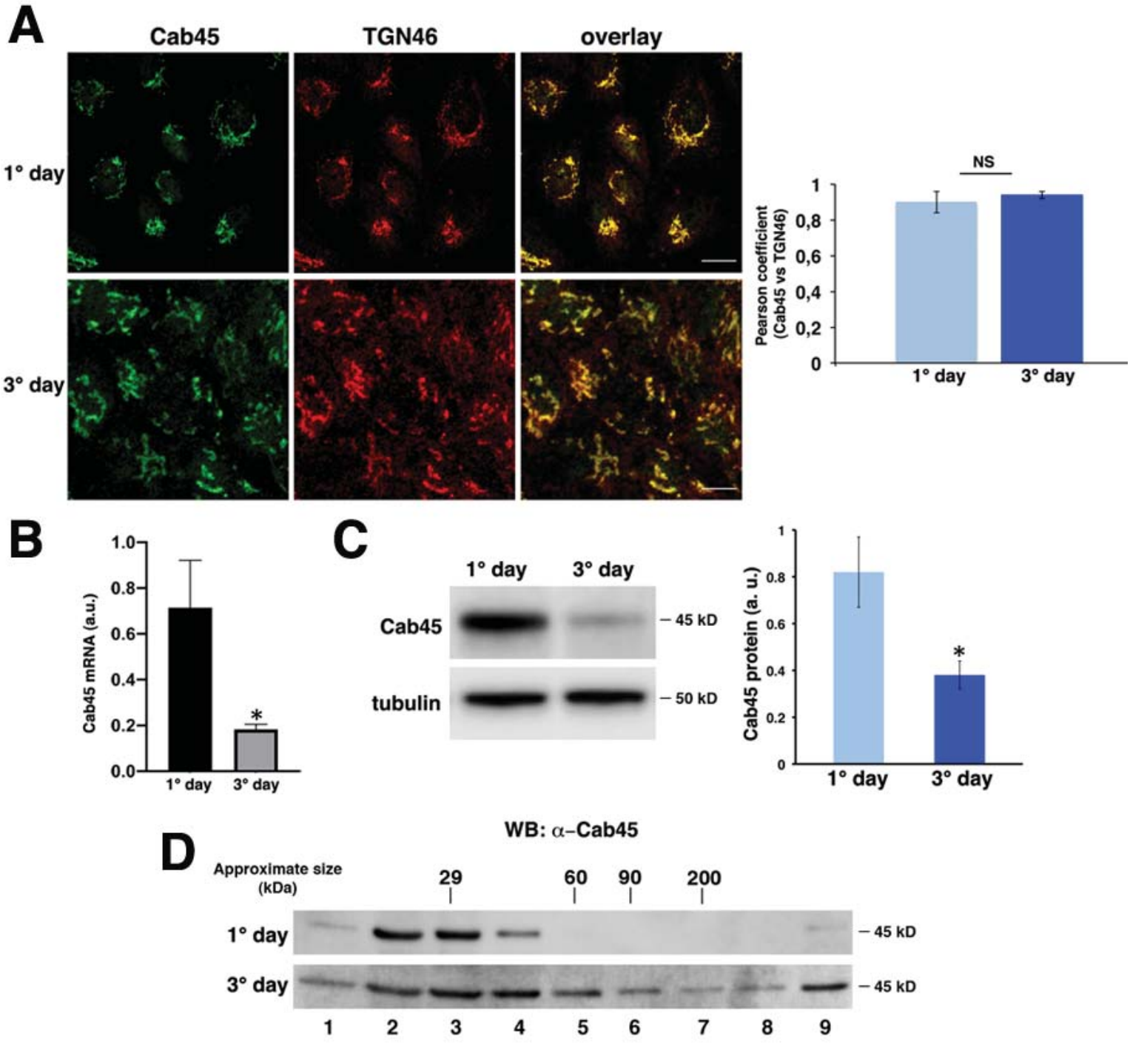

A
MDCK: CTRLi
MDCK: SPCA1i
[Ca2+]golgi µM
600
500
400
300
200
100
0
B
MDCK: GFP-FR SPCA1i
B map
I map
2
1
0
700
350
0
GFP-FR: CTRLi
GFP-FR: SPCA1i
Brightness
1.3
1.2
1.1
1
C
CTRLi
SPCA1i
0'
60'
0'
60'
GFP/giantin-furin
GFP/giantin-furin
Top
middle
Pearson coefficient
(GFP-FR vs giantin/furin)
1
0,8
0,6
0,4
0,2
0
NS
***
0'
60'
D
GFP-FR
GP114
CTRLi
SPCA1i
CTRLi
SPCA1i
XZ
top
bottom
Apical
Basolateral
% of mean intensity
100
80
60
40
20
0
**
**
CTRLi
SPCA1i
Apical
Basolateral
NS
% of mean intensity
100
80
60
40
20
0
CTRLi
SPCA1i

Figure 3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Cab45
TGN46
overlay
1° day
3° day
NS
Pearson coefficient (Cab45 vs TGN46)
1
0,8
0,6
0,4
0,2
0
1° day
3° day
B
Cab45 mRNA (a.u.)
1.0
0.8
0.6
0.4
0.2
0.0
*
1° day
3° day
C
1° day
3° day
Cab45
tubulin
– 45 kD
– 50 kD
Cab45 protein (a. u.)
1
0.8
0.6
0.4
0.2
0
*
1° day
3° day
D
WB: α–Cab45
Approximate size (kDa)
29
60
90
200
1° day
3° day
– 45 kD
– 45 kD
1
2
3
4
5
6
7
8
9

Figure 4

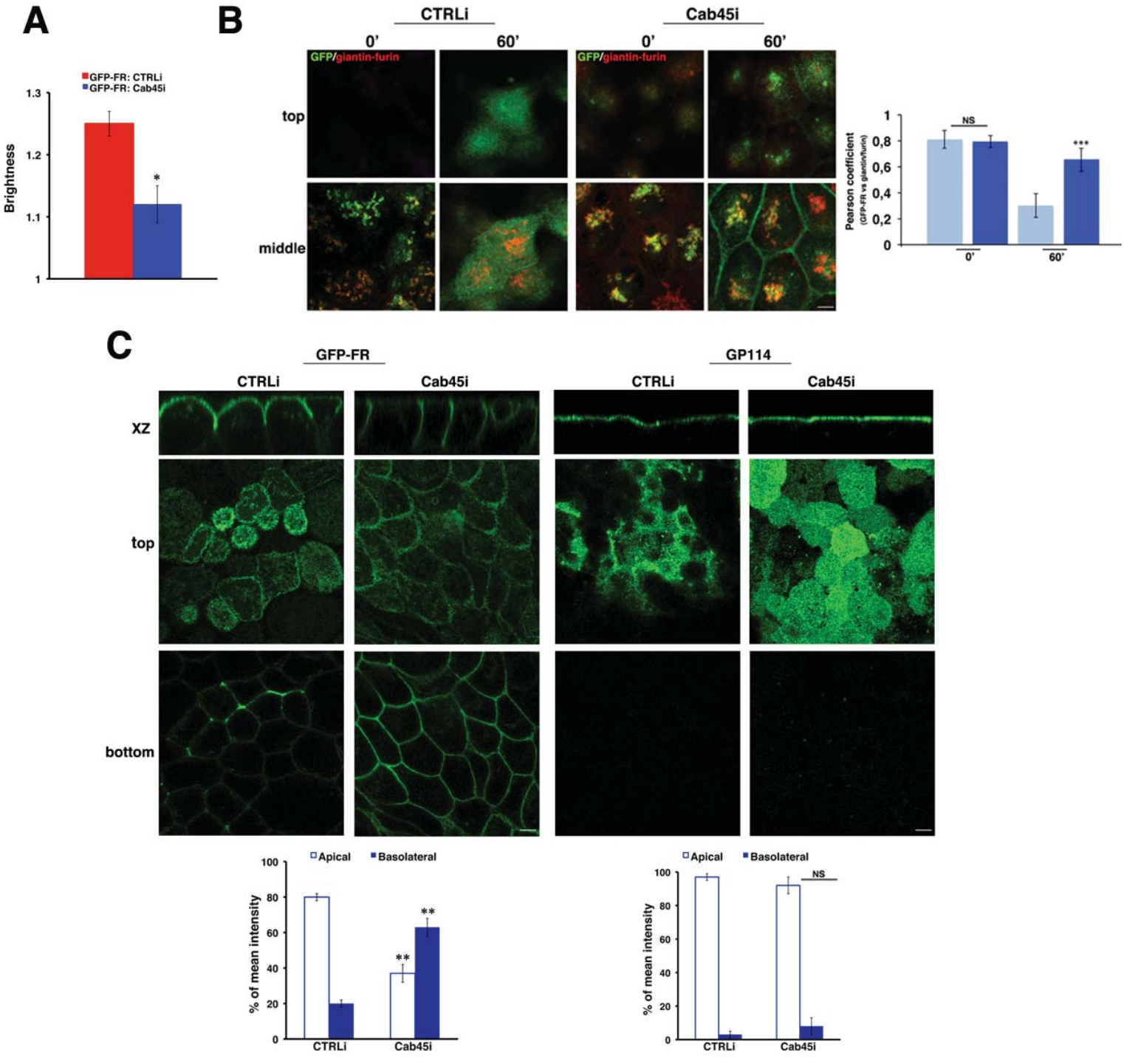

A
GFP-FR: CTRLi
GFP-FR: Cab45i
Brightness
1.3
1.2
1.1
1
*
B
CTRLi
Cab45i
0'
60'
0'
60'
GFP/giantin-furin
top
middle
Pearson coefficient
(GFP-FR vs giantin/furin)
NS
***
1
0,8
0,6
0,4
0,2
0
0'
60'
C
GFP-FR
GP114
CTRLi
Cab45i
CTRLi
Cab45i
XZ
top
bottom
Apical
Basolateral
% of mean intensity
100
80
60
40
20
0
**
**
CTRLi
Cab45i
Apical
Basolateral
NS
% of mean intensity
100
80
60
40
20
0
CTRLi
Cab45i

Figure 5

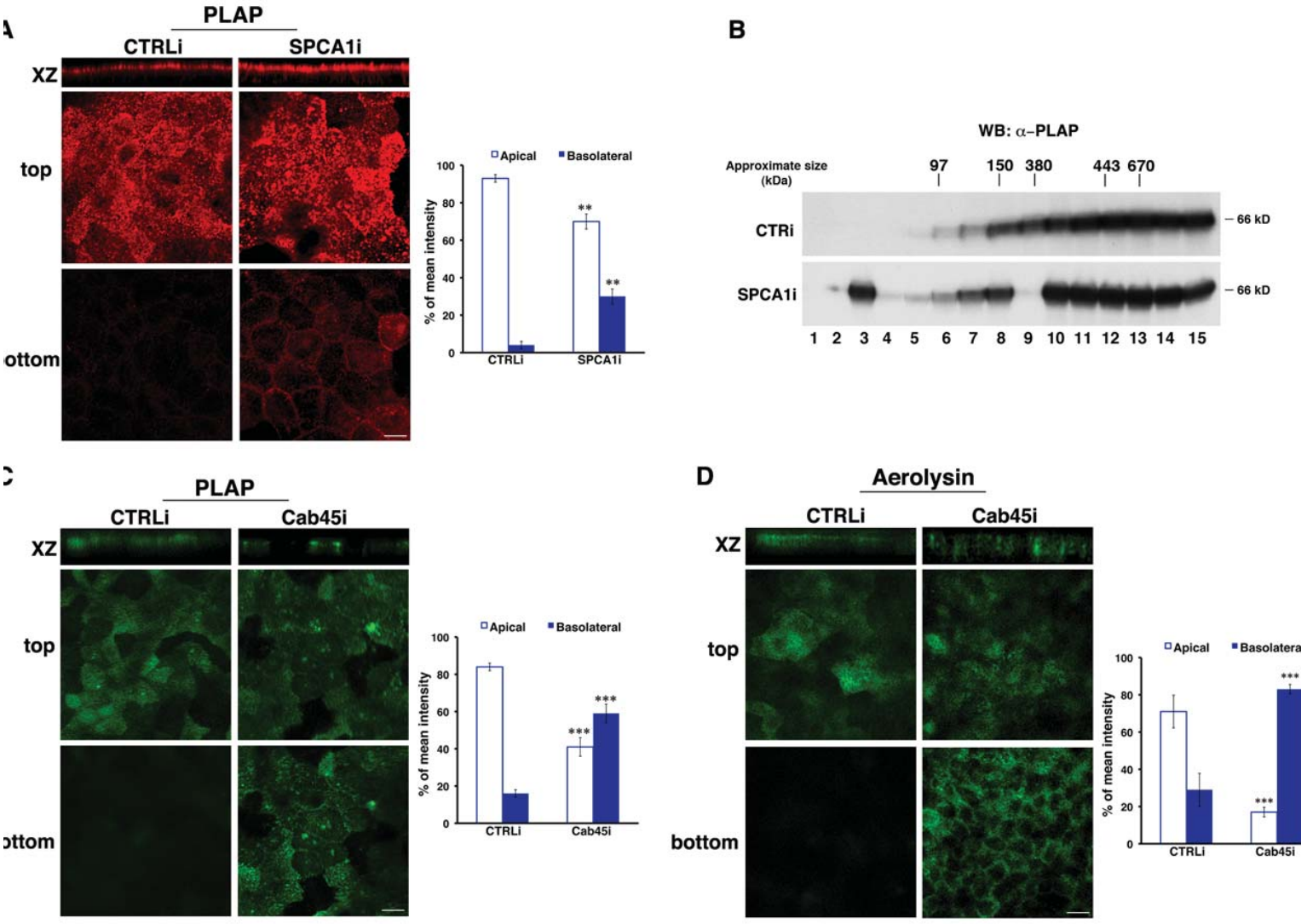

A
PLAP
CTRLi
SPCA1i
XZ
top
ottom
Apical
Basolateral
% of mean intensity
CTRLi
SPCA1i
B
WB: α–PLAP
Approximate size (kDa)
97
150
380
443
670
CTRi
SPCA1i
66 kD
66 kD
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
C
PLAP
CTRLi
Cab45i
XZ
top
ttom
Apical
Basolateral
% of mean intensity
CTRLi
Cab45i
D
Aerolysin
CTRLi
Cab45i
XZ
top
bottom
Apical
Basolatera
% of mean intensity
CTRLi
Cab45i

Figure 6

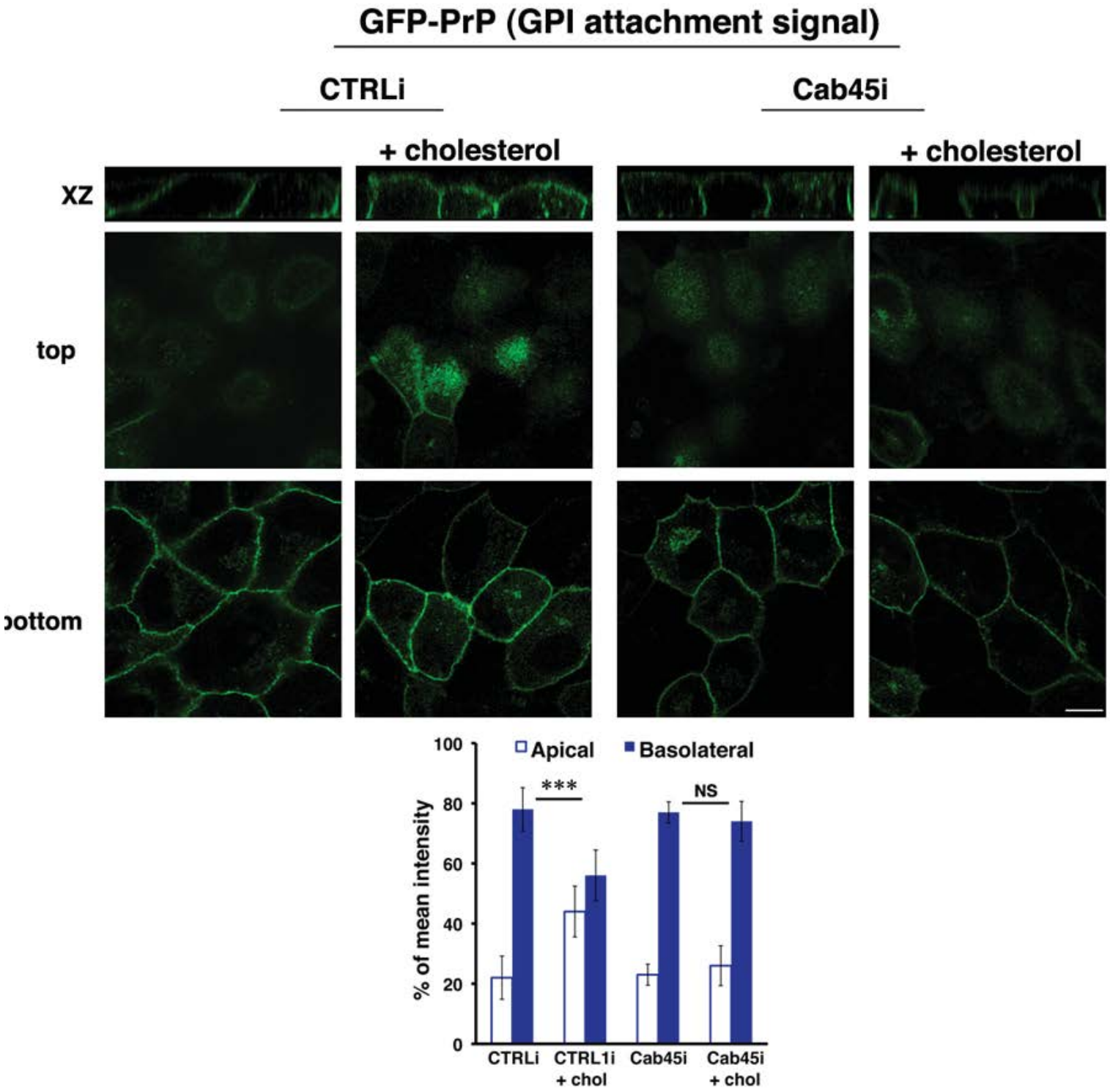

GFP-PrP (GPI attachment signal)
CTRLi
Cab45i
+ cholesterol
+ cholesterol
XZ
top
bottom
Apical
Basolateral
***
NS
% of mean intensity
100
80
60
40
20
0
CTRLi
CTRL1i + chol
Cab45i
Cab45i + chol

Figure 7

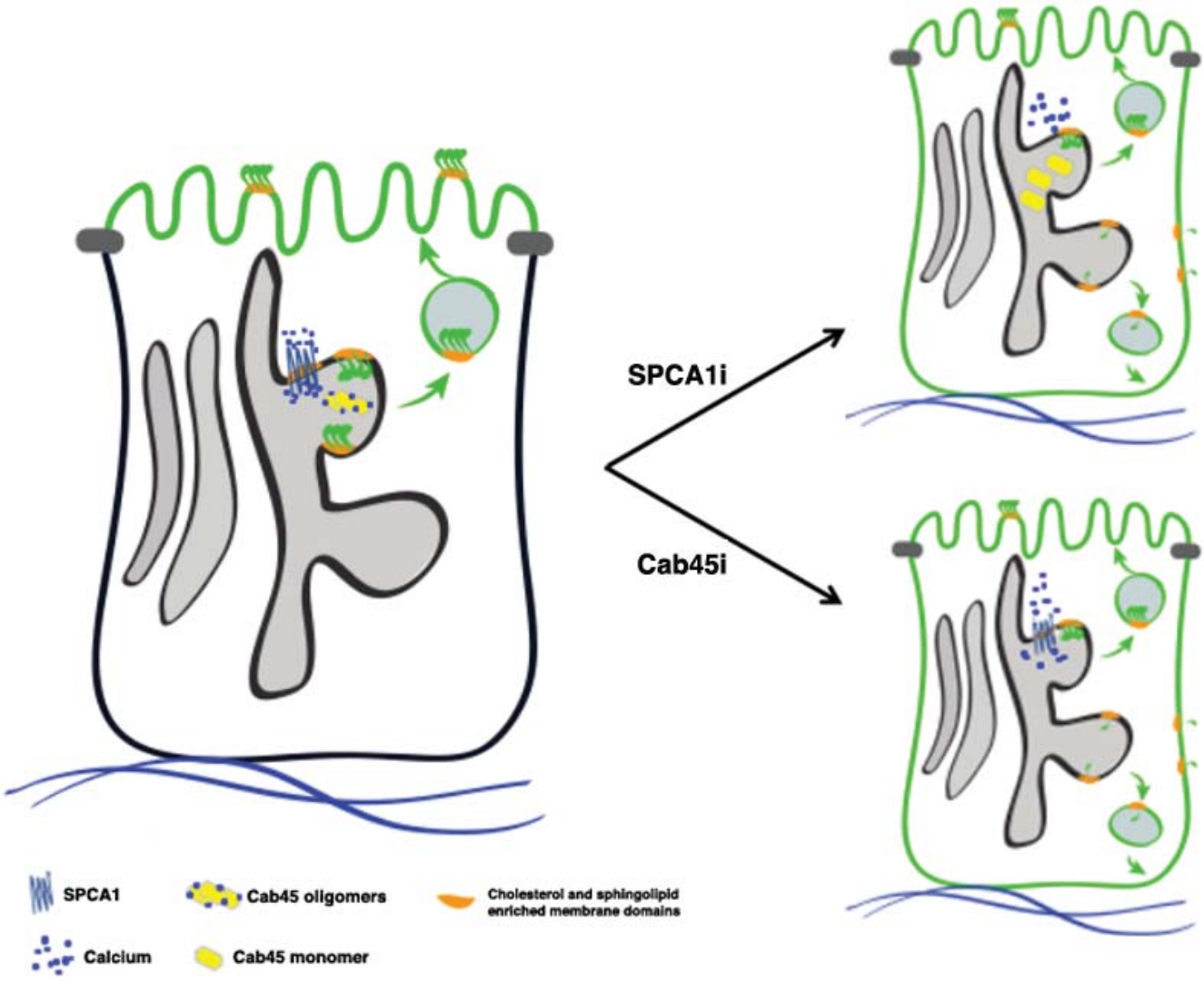

SPCA1i
Cab45i
SPCA1
Cab45 oligomers
Cholesterol and sphingolipid enriched membrane domains
Calcium
Cab45 monomer

Figure 8

Supplementary Appendix

Supplementary Figure legends

Figure S1: **Generation of MDCK GFP-FR cell line stably silenced for SPCA1**

(A**)** The levels of SPCA1 expression in MDCK GFP-FR stably interfered with scrambled (CTRLi) or specific short hairpin RNA (SPCA1i) were tested by Western blotting. Tubulin was used as loading control. Note that the transfection of shRNAs did not affect the expression of GFP-FR. Densitometric analyses of three different experiments are shown. Results show the percentage of SPCA1 expression in SPCA1i clones compared with scrambled interfered cells (set equal to 100%). (B) CTRLi and SPCA1i MDCK cells were stained with SPCA1 and revealed wit Alexa-546 antibodies. Scale bar, 10 μm. (C) Golgi [Ca^2] quantification in MDCK:GFP-FR cells control-interfered (CTRLi) or SPCA1-interfered (SPCA1i) cells, in fully polarized and non-polarized conditions, were measured following the same procedure described in Figure 1C. The data represent the mean of four (for polarized conditions) and three (for unpolarized condition) independent experiments performed in two knockdown clones.

Figure S2: SPCA1 knockdown does not alter apical GFP-FR distribution, epithelial polarity or apical and basolateral localization of endogenous proteins.

(A) MDCK GFP-FR CTRLi, SPCA1i or Cab45i were grown for 4 days on filter were imaged in live conditions by using confocal microscopy. Mean fluorescence intensities on the apical and basolateral surface were measured and expressed as percentages of the total fluorescence. Scale bar, 6 μm. Error bars, ± SD; ** $p<0.01$; Student's *t*-test. (B, C) MDCK CTRLi and SPCA1i cells were grown on filters for 4 days and then processed for immunofluorescence using specific antibody against E-cadherin (B, left) and ZO-1 (C). Scale bars, 6 μm.

Figure S3: Alteration of actin dynamics does not affect GPI-AP Golgi homocluster organization and apical sorting in MDCK cells

(A) After 4 days in culture, polarized MDCK cells expressing GFP-FR were treated or not with latrunculin and imaged *in vivo* for N&B at the level of the Golgi complex. Representative B and I maps are shown on the left. Scale bars, 0.9 μm. On the right upper panel, quantification of the brightness of GFP-FR from three independent experiments is plotted. On the right lower panel, graphical representation of the percentage of pixels falling into the different classes of B values (from monomer to hexamer) on the basis of the calibration curve (1). Values are expressed as the mean of three independent experiments, n>25 cells. Error bars, ± SD. (B) In order to analyze exclusively the GFP-FR Golgi pool, MDCK cells were grown on filters and were treated with trypsin (25 μg/ml for 25 min) exclusively at the apical side in order to remove the pool of GFP-FR already present at the plasma membrane and purified on a velocity gradient. Cells were lysed and run on a velocity gradient as described in methods. Fractions were collected from the top (Fraction 1) to the bottom (Fraction 9), TCA-precipitated, run on a SDS-PAGE gel and revealed by western blotting with a specific anti-GFP antibody. Molecular weight markers are indicated on the top of the panels. The molecular weight of the monomeric form of GFP-FR is indicated together with the band at 43 kDa, which represents a partially denatured dimer of GFP. On the right panel, the distribution of GFP-FR in the fractions of the gradient is expressed as a percentage of the total protein. Mean values of two independent experiments are shown. (C) MDCK GFP-FR cells, grown on filters, were treated with trypsin as in (B), then incubated or not with latrunculin for 30 min and imaged in live conditions by using confocal microscopy. Mean fluorescence intensities on the apical and basolateral surface were measured and expressed as percentages of the total fluorescence. Scale bar, 6 μm.

Error bars, ± SD. NS, not significant.

Figure S4: Cab45 knockdown does not alter apical and basolateral localization of endogenous proteins

(A) The levels of Cab45 expression in MDCK GFP-FR stably interfered with scrambled (CTRLi) or specific short hairpin RNA (Cab45i) was tested by Western blotting. Tubulin was used as loading control. Densitometric analyses of three different experiments are shown. Results show the percentage of Cab45 expression in Cab45i cells compared with scrambled interfered cells (set equal to 100%). **(**B**)** CTRLi and Cab45i MDCK cells were stained with Cab45 and revealed with Alexa-633 antibodies. Scale bar, 6 μm. (C) CTRLi and Cab45i MDCK cells, grown on filters for 4 days, were subjected to immunofluorescence assay using E-cadherin antibody. Scale bars, 6 μm.

Figure S5. Generation of MDCK PLAP cell line stably silenced for SPCA1

(A**)** The levels of SPCA1 expression in MDCK PLAP stably interfered with scrambled (CTRLi) or specific short hairpin RNA (SPCA1i) were tested by Western blotting. The PLAP expression levels are also shown. Densitometric analyses of two different experiments are shown. Results show the percentage of SPCA1 expression in SPCA1i clones compared with scrambled interfered cells (set equal to 100%).

Figure S6: Cab45 knockdown does not alter basolateral localization of GFP-PrP

MDCK GFP-PrP CTRLi or Cab45i were grown for 4 days and then fixed and imaged by using confocal microscopy. Mean fluorescence intensities on the apical and basolateral surface were measured and expressed as percentages of the total fluorescence. Scale bar, 6 μm. Error bars, ± SD; NS, not significant; Student's *t*-test.

Figure S7: The expression of apical GPI-APs correlates with an increase of mRNA of SPCA1 and Cab45

(A) SPCA1 and Cab45 mRNA levels in MDCK wild-type (wt) and stably expressing GPI-APs (PLAP, GFP-FR and GFP-PrP), grown for 3 days, were analysed by RT-qPCR and normalized to HPRT and Ubch5 mRNA levels; experiments were performed 3 independent times. (B) SPCA1 and Cab45 protein levels in MDCK wild-type and stably expressing the aforementioned proteins, grown for 3 days, were tested by Western blotting. Tubulin and calnexin-1 were used as loading control. Densitometric analyses of two different experiments are shown. Results show the amount of SPCA1 and Cab45 expression in the different MDCK cells expressing GPI-APs compared with wild-type cells (set equal to 1). Error bars, ± SD; NS, not significant; * $p<0.05$, Student's *t*-test.

Materials and Methods

Gene silencing and antibodies

RNA interference was obtained by transfecting specific short hairpin RNAs (from Open Biosystems) bearing this targeting sequence: sh-1 ACCATTGTGCGTGAAGGAAA; sh-2 GAGGCCTTAATTGCTCTTGCAAT. As a negative control, we used an shRNA against GFP, GGCACAAGCTGGAGTACAACTA. Transfection was performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Stably transfected cells were obtained after selection with puromycin (1.5 μg/ml, Sigma).

Cab45 knockdown was performed by using a lentiviral vector pRFP-CB-sh sequence TGTGAATACTGACCGGAAGATAAGCGCCA (Origene); as a negative control a non-effective 29-mer scrambled shRNA cassette in the same p-RFP-CB-sh Lenti Vector (ref. TR30033) was used. MDCK cells were infected for 24h with lentiviral particles and then stable clones were collected after selection with blasticidin. Screening of positive clones was carried out by analyzing RFP fluorescence.

We used the following antibodies: polyclonal anti-GFP (Clontech), polyclonal anti-PLAP (from Rockland), polyclonal anti-giantin (from Ozyme), polyclonal anti-furin convertase (from Thermofisher), monoclonal anti-ATP2C1 (from Biorad) and polyclonal elF4a (from Cell Signaling); monoclonal anti-TGN46 (from ABD Serotec); polyclonal Cab45 from J.V. Blume laboratory.

Perturbation of actin cytoskeleton

To perturb the actin cytoskeleton we incubated cells in culture medium at 37°C with 6 μM latrunculin A (Molecular Probes) for 5 minutes as previously described (3).

Perturbation of cellular calcium content

To perturb the cellular calcium we used the calcium ionophore ionomycin (39, 40). Cells were incubated for 40 min in modified Krebs-Ringer buffer (135 mM NaCl, 5 mM KCl, 1 mM K_2PO_4, 1 mM $MgSO_4$, 5.5 mM glucose, 20 mM HEPES, pH 7.4) supplemented with ionomycin (5 μM) and 600 μM EGTA.

Deglycosylation assay

Deglycosylation of protein extracts was carried out by using Protein Deglycosylation Mix II kit (New England biolabs) according to the manufacturer's protocol.

Velocity gradients

Purification of proteins on velocity gradients is a biochemical method that allows purifying proteins according to their molecular weight, independently of their association with membrane domains as previously described (4, 79, 80). Briefly, cells were lysed in 20 mM Tris, pH 7.4, 100 mM NaCl, 1% TX-100 (with or without 0.4% SDS). Lysates were scraped, sheared through a 26-g needle and layered on top of a discontinuous sucrose gradient (30-5%) or a glycerol gradient

(20-40%) in the same buffer containing 0.1% TX-100. After centrifugation at 45,000 rpm for 16 h in a Beckman SW50 ultracentrifuge, fractions of 500 µl (for 5-30% gradient) and 300 µl (for 20-30% gradient) were harvested from the top of the gradient.

Temperature block

To achieve an almost complete protein block in the TGN, we used a previously published protocol (2, 47). Filter-grown cells were incubated at 19.5°C for 2 h in areal medium (F12 Coon's modified medium without NaHCO3 and with 0.2% BSA and 20 mM Hepes, pH 7.4). In the last hour at 19.5°C, they were treated with 150 µg/ml cycloheximide.

RT-qPCR

Total RNA extraction from the different MDCK cell lines was performed using the RNeasy Mini Kit from Qiagen. Reverse transcription was done using the Biorad iScript gDNA Clear cDNA Synthesis Kit. Oligonucleotides were designed using Prime PCR Look Up Tool (Bio-Rad) and purchased from Eurofins Genomics. Quantitative PCR was performed using the Bio-Rad iTaqTM universal SYBR® Green supermix and analysed using a CFX96TM real-time PCR detection system under the CFX Manager software (Bio-Rad). Gene expression was normalized to hypoxanthine-guanine phosphoribosyltransferase (HPRT) and Ubiquitin-conjugating Enzyme H5b (UBCH5B). Each experiment was performed in triplicates and the results are the mean of 3 independent experiments. Oligonucleotides used in qPCR are presented below:

ATP2C1 (gene name for SPCA1) forward 5'-GAGGCGGGTTGTGTATGCAATG-3', reverse 5'-GATATTCAGCTTTTCTGACATAGTCC-3';

SDF4 (gene name for Cab45) forward 5'-CCATGATCCAGTGCTGCATC-3'; reverse 5'-AGGAGCAGGCGGAAGCTGAT-3';

HPRT forward 5'-TAATTGGTGGAGATGATCTCTCAAC-3'; reverse 5' TGCCTGACCAAGGAAAGC 3';

UBCH5 forward 5'-TGAAGAGAATCCACAAGGAATTGA-3'; reverse 5'-CAACAGGACCTGCTGAACACTG-3'.

Calcium measurements

We measured the concentration of calcium in the Golgi apparatus by using as a probe the chimeric photoprotein Golgi-aequorin (GoAEQ) following a previously described protocol (39, 45). Cells were electroporated with GoAEQ and seeded onto glass coverslips to confluence and allowed to grow for 1 or 3 days. To produce the functional calcium-sensitive luminescent protein, aequorin was reconstituted for 40 min at 4°C in modified Krebs–Ringer buffer (KRB; 135 mM NaCl, 5 mM KCl, 1 mM K_2PO_4, 1 mM $MgSO_4$, 5.5 mM glucose, 20 mM HEPES, pH 7.4) supplemented with 5 μM mutated coelenterazine, the Calcium-ionophore ionomycin (5 μM) and 600 μM EGTA (the latter ones reduce the calcium content of the Golgi, which is necessary for measurements). After this incubation, cells were washed 3 times with KRB supplemented with 2% bovine serum albumin and 1mM EGTA (to remove calcium mobilized from intracellular compartments), followed by 3 washes with KRB buffer containing 100 μM EGTA and then transferred into perfusion chamber of a custom built aequorinometer (45). Interestingly, while this experimental procedure was suitable for non-polarized MDCK cells, the reconstitution with coelenterazine was highly inefficient in polarized MDCK cells, indicating that the Golgi apparatus of these cells is endowed with a high [Ca^{2+}]. Thus, to better empty the calcium from the Golgi apparatus, cells were treated 5 minutes at 37°C in calcium-free medium containing ionomycin and 2 mM EGTA before the reconstitution.

The cells were perfused with KRB containing 100 μM EGTA at 37°C. After a 30 sec baseline recording, the perfusion solution was switched to KRB supplemented with 1 mM calcium and recording continued until the light signal reached a steady-state level. At the end of each experiment, for quantification of the intra-organellar calcium levels, the cells were perfused with a hypotonic calcium-rich solution (10 mM $CaCl_2$ in H_2O) supplemented with 0.1 % TX-100 to

discharge the remaining aequorin pool. The aequorin luminescence data were calibrated off-line into [Ca^{2+}] values using an algorithm as previously described (45).

Immunofluorescence

MDCK cells grown on coverslips for 1 or 3 days were washed with phosphate-buffered saline containing $CaCl_2$ and $MgCl_2$, fixed with 4% paraformaldehyde or methanol (for SPCA1 staining) depending on the antibodies used, quenched with 50 mM NH_4Cl, and stained with specific antibodies (SPCA1 1/500 or Cab45 1/500 and TGN46 1/500 or Giantin 1/400 and furin 1/100, PLAP 1/300) in permeabilized (0.2% TX-100) conditions. Primary antibodies were detected with Alexa 488 or 546 (ThermoFisher Scientific). The images were acquired using a laser scanning confocal microscope (LSM 510 or LSM 700; Carl Zeiss MicroImaging, Inc.) equipped with a Plan Apo 63× oil immersion (NA 1.4) objective lens. The quantification of mean fluorescence intensities in random selected regions of interest was performed using the LSM 510 software (Carl Zeiss MicroImaging, Inc.) as previously described in (47). Co-localization analyses were carried out using Image J. Specifically, the Pearson's coefficient was measured using Costes' method with the Jacob plugin in Image J. The analyses were carried out on a Z-stack of images (16 bit) by considering individual cells manually defined as a region of interest.

Labelling of endogenous GPI-APs

The bacterial toxin aerolysin binds GPI-APs with high affinity. Specifically, the aerolysin mutant, ASSP, carrying a double mutation (residues 202 and 445 are changed to cysteines) that remains inactive unless it becomes reduced, was used to assess the localization of endogenous GPI-APs. CTRLi or Cab45i cells, grown on filters, were incubated with ASSP-alexa 488 (20 mM) for 1h at 4°C as previously reported (51) before fixation and confocal microscopy analysis.

N&B experiments

The Number & molecular Brightness method, a technique based on moment-analysis for the measurements of the average number of molecules and brightness in each pixel in fluorescence microscopy images (37), provides the state of aggregation of molecules in living cells with high spatial and temporal resolution. N&B experiments were carried out as previously described (1).

Microscopy and image analysis

50 frame time-series were acquired with a Zeiss LSM 510 META equipped with a plan apo 63x oil-immersion (NA 1.4) objective lens by using the following settings: 488 nm Argon laser, 0.05 mW of output power, 505-550 nm emission, gain less or equal to 850, offset 0.1, digital gain 1. Scanning parameters were: 512x512 frame window, 25.61 μs/pixel dwell time, no average, zoom 6x, ROI (x, y) 256x64, pinhole corresponding to 1 mm optical slice. Images were collected with resolutions of 70 nm/pixel. All measurements were performed in cells displaying comparable levels of fluorescence intensity. Data from each cell were analyzed by SimFCS software (Globals Software, East Villa Grove, IL 61956, USA) following a described procedure (37). A correction was applied for taking into account the analog detection of fluorescence by the photomultiplier tubes of the confocal microscope in order to express the molecular brightness (e) in terms of photons/s/molecule (36). Briefly, the correction parameters S (the conversion factor between one photon detected and the number of digital levels produced by the electronics), offset and sigma0 were determined for each experiment by plotting the measured average intensity (<I>) vs the average variance (<Var>) of 50 frame time-series acquired using the same settings as above except that 4 different values of the laser transmission percentages and filters and beam splitters configured to get reflection images, in order to detect the defined amount of light originating directly from the laser. The obtained plots were linearly interpolated and the equation of a straight line ($R \geq 0.99$) was used to extract the parameters S and offset based on the following equation: $\langle Var \rangle = S * \langle I \rangle + q$ (parameter related to readout noise). The parameter sigma0 was

estimated from time-series acquired with laser off, as the half maximum width of the histogram peak of the dark-counts. Its value was constantly lower than 0.1, and consequently was assumed to be zero in all the calculations.

In an analog system, the brightness was calculated pixel by pixel from the following equation $B= V(x,y)/(S*Ix,y)$ and the relationship with molecular brightness is described by the following equation: $B/S= e+1$. Here we indicate with the term brightness the ratio B/S. Hence, the measured brightness (B/S) is > 1 from the pixels with mobile components, while B/S= 1 from the pixels with immobile features.

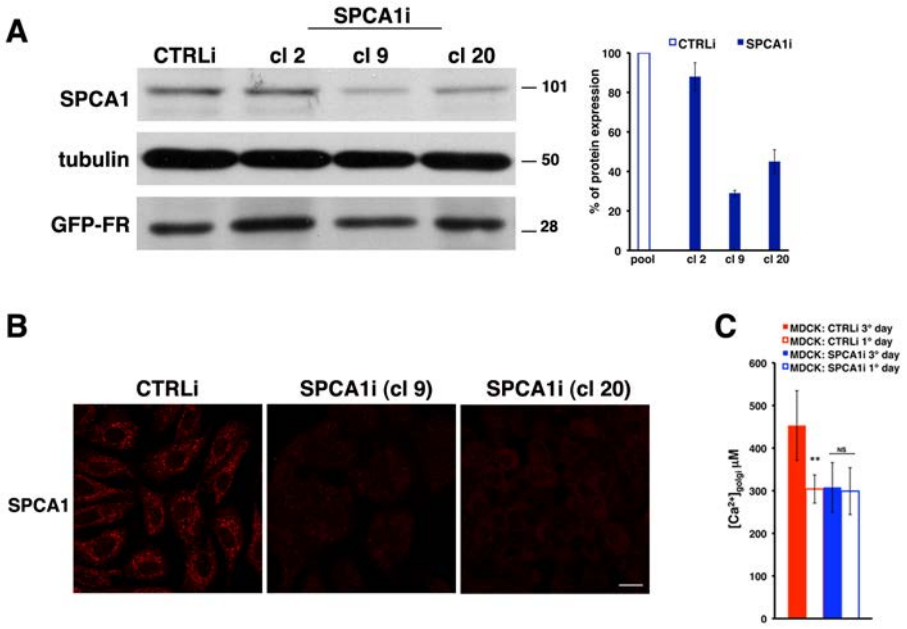
Photobleaching correction (photobleaching rate measured from the experimental data) has been included in the algorithms used to analyze N&B data (37). Specifically, we used a high-pass filter to the intensity as a function of time of each pixel, which we experimentally verified to be able to remove slowly varying signals. After removal of the trend, we added a constant equal to the average intensity at that pixel. Therefore, the variance of the ‘‘immobile’’ part is unaffected by bleaching after correction and we can recover the variance of the mobile part (37).

In all experiments a detrend function (the same used for bleaching correction) was applied to image stacks before determining the B in order to avoid that slow changes of the intensity due to the cell movement or protrusion/retraction events could interfere with our measurements (36, 37). Finally, all acquisitions where we monitored aberrant movements (e.g., microvilli movement or fluctuations of the apical membrane) were discarded.

Data Analysis

As previously showed (1), by using the K-means function in Matlab (The Mathworks Inc. Natick, MA) we partitioned, with an interval of 0.5, the observed brightness values upon different experimental conditions into N exclusive groups with statistical reliability. In particular, for each experiment (number of cells > 15) we obtained the percentage of pixels in each group (calculated as an average of single cell values from an experiment). The range of B values were ascribed to

monomer, dimer, trimer etc. on the basis of extrapolation of the standard curve obtained by plotting the experimental B values for monomeric, dimeric, trimeric GFP (mGFP, mGFP-mGFP, mGFP-mGFP-mGFP) vs number of units per aggregate (for detail see Supplementary Fig. 4 in ref. 1).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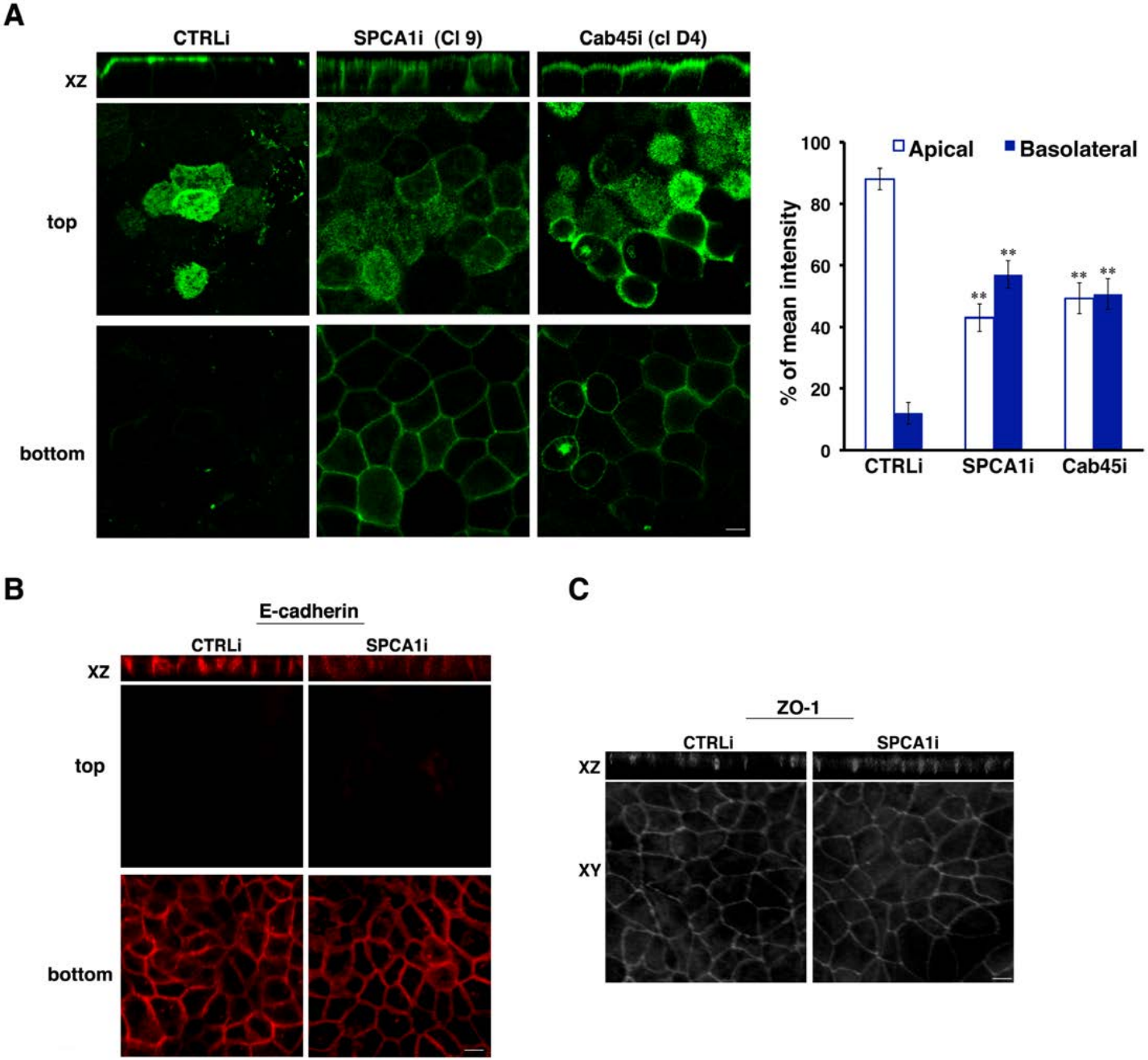
SPCA1i
CTRLi
cl 2
cl 9
cl 20
SPCA1
tubulin
GFP-FR
101
50
28
CTRLi
SPCA1i
% of protein expression
100
80
60
40
20
0
pool
cl 2
cl 9
cl 20
B
CTRLi
SPCA1i (cl 9)
SPCA1i (cl 20)
SPCA1
C
MDCK: CTRLi 3° day
MDCK: CTRLi 1° day
MDCK: SPCA1i 3° day
MDCK: SPCA1i 1° day
600
500
400
300
200
100
0
**
NS

Figure S1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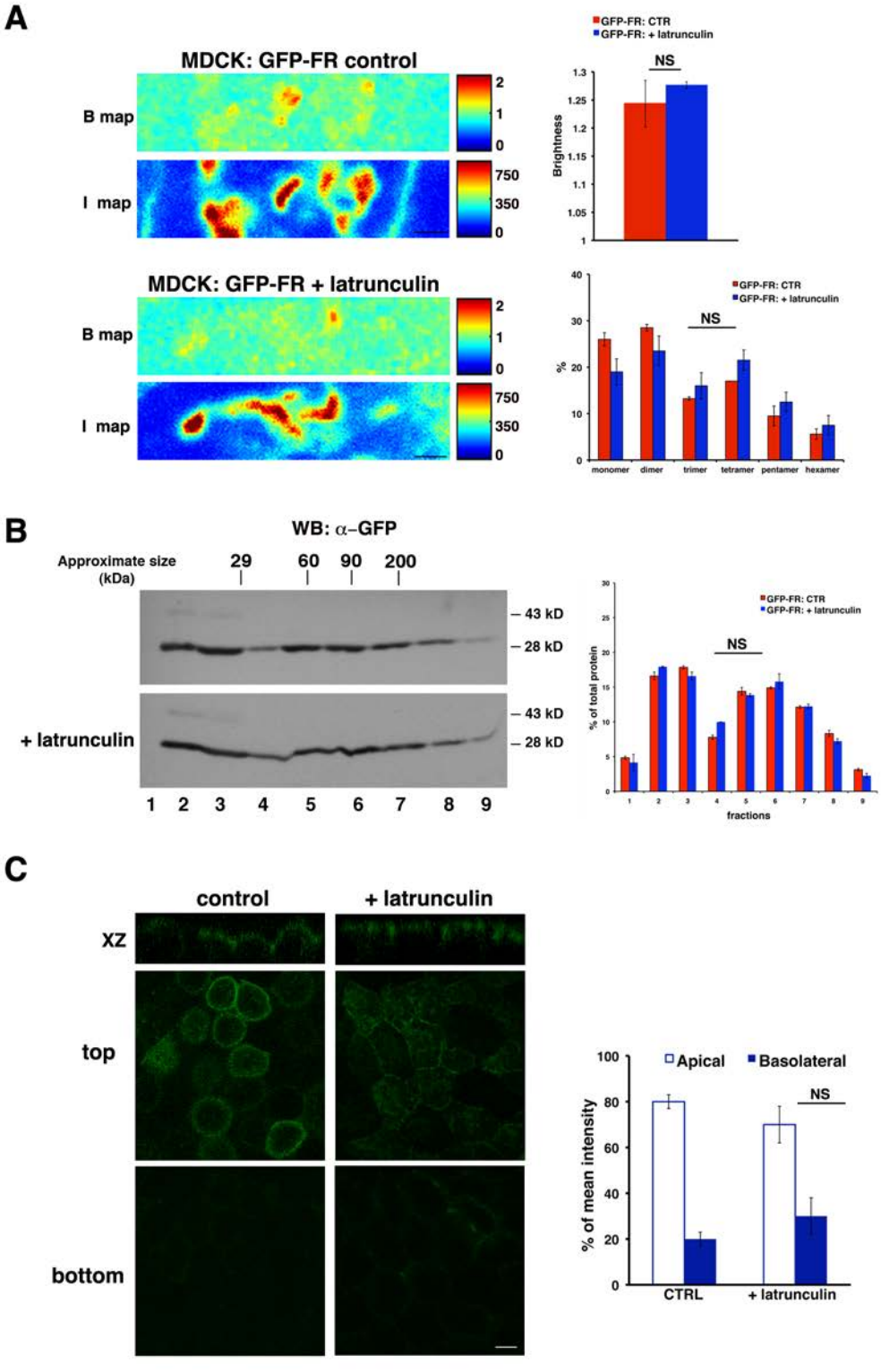
CTRLi
SPCA1i (Cl 9)
Cab45i (cl D4)
XZ
top
bottom
Apical
Basolateral
% of mean intensity
100
80
60
40
20
0
CTRLi
SPCA1i
Cab45i
**
B
E-cadherin
CTRLi
SPCA1i
XZ
top
bottom
C
ZO-1
CTRLi
SPCA1i
XZ
XY

Figure S2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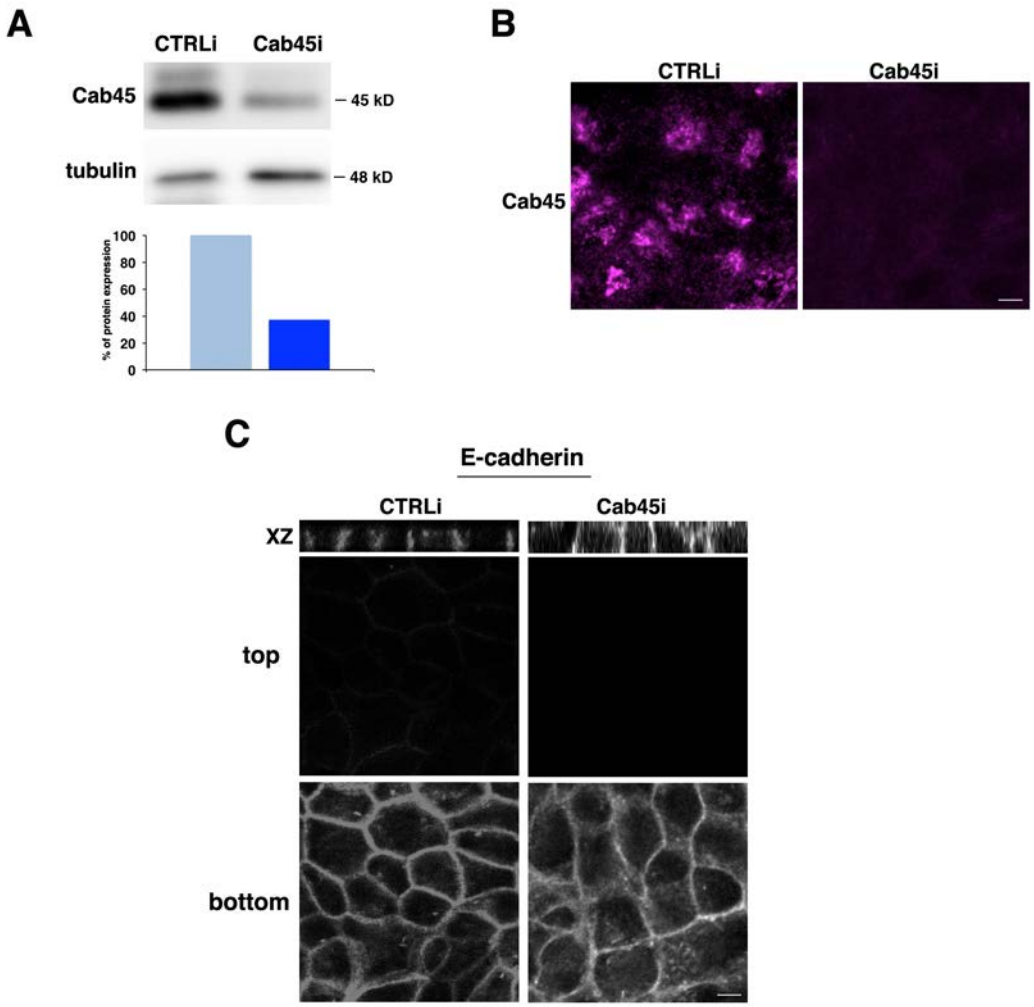
MDCK: GFP-FR control
B map
I map
2
1
0
750
350
0
GFP-FR: CTR
GFP-FR: + latrunculin
NS
Brightness
1.3
1.25
1.2
1.15
1.1
1.05
1
MDCK: GFP-FR + latrunculin
B map
I map
2
1
0
750
350
0
GFP-FR: CTR
GFP-FR: + latrunculin
NS
%
40
30
20
10
0
monomer
dimer
trimer
tetramer
pentamer
hexamer
B
WB: α–GFP
Approximate size (kDa)
29
60
90
200
– 43 kD
– 28 kD
+ latrunculin
– 43 kD
– 28 kD
1 2 3 4 5 6 7 8 9
GFP-FR: CTR
GFP-FR: + latrunculin
NS
% of total protein
fractions
C
control
+ latrunculin
XZ
top
bottom
Apical
Basolateral
NS
% of mean intensity
100
80
60
40
20
0
CTRL
+ latrunculin

Figure S3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CTRLi
Cab45i
Cab45
– 45 kD
tubulin
– 48 kD
% of protein expression
100
80
60
40
20
0
B
CTRLi
Cab45i
Cab45
C
E-cadherin
CTRLi
Cab45i
XZ
top
bottom

Figure S4

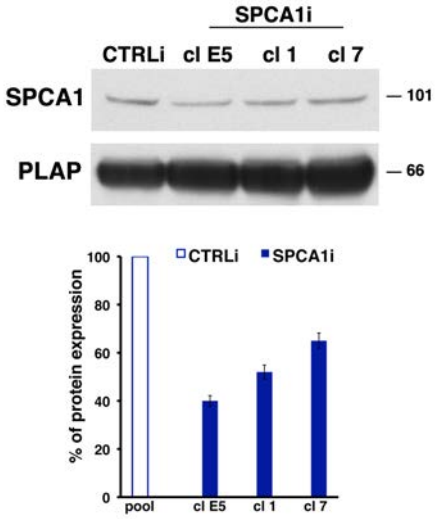
SPCA1i
CTRLi
cl E5
cl 1
cl 7
SPCA1
101
PLAP
66
CTRLi
SPCA1i
% of protein expression
100
80
60
40
20
0
pool
cl E5
cl 1
cl 7

Figure S5

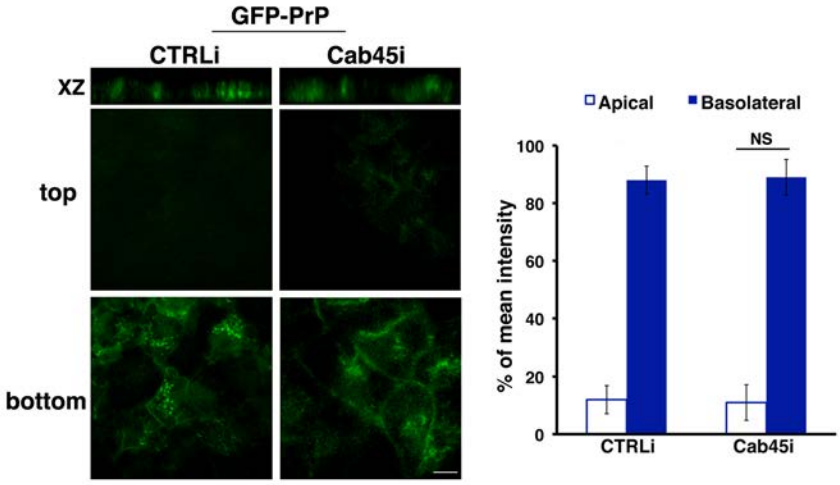
GFP-PrP
CTRLi
Cab45i
XZ
top
bottom
Apical
Basolateral
NS
100
80
60
40
20
0
% of mean intensity
CTRLi
Cab45i

Figure S6

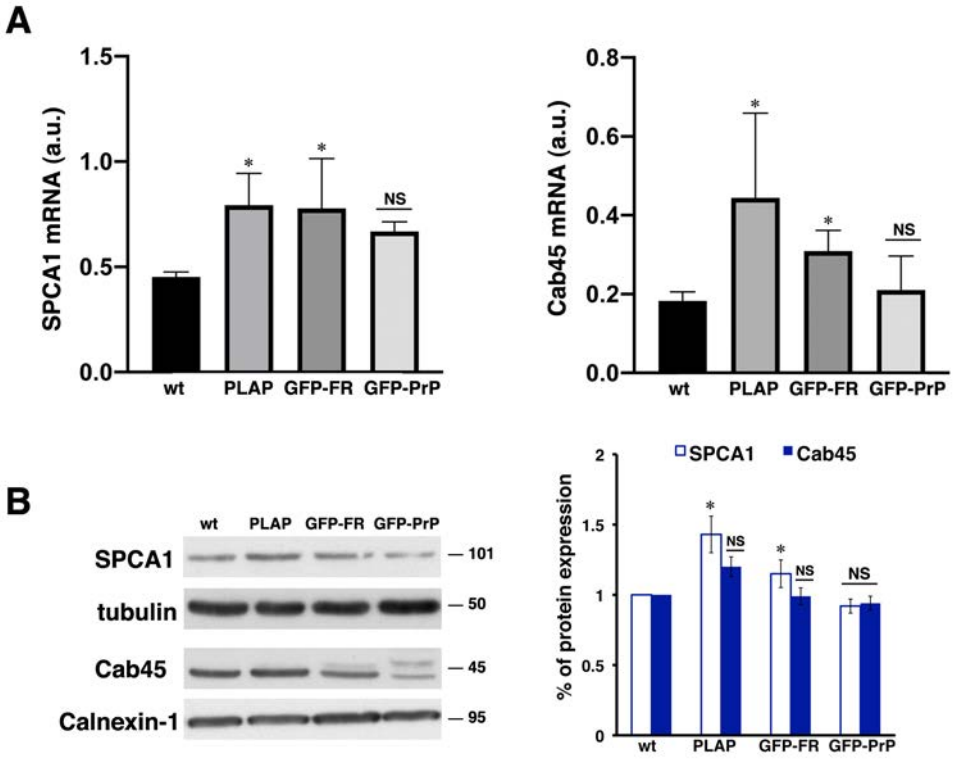
A
SPCA1 mRNA (a.u.)
1.5
1.0
0.5
0.0
wt
PLAP
GFP-FR
GFP-PrP
NS
Cab45 mRNA (a.u.)
0.8
0.6
0.4
0.2
0.0
B
SPCA1
tubulin
Cab45
Calnexin-1
101
50
45
95
SPCA1
Cab45
% of protein expression
2
1.5
1
0.5
0

Figure S7

Project 2: (Manuscript in preparation LIU et al.) Cab45 affects apical secretion of the soluble protein PLAP-sec in polarized MDCK cells

Abstract

Epithelial polarity relies on the proper sorting of proteins and lipids that mostly occurs at the level of the trans-Golgi network (TGN), the main sorting hub in cell. Protein sorting is tightly regulated in polarized epithelial cells and we recently uncovered an unexpected role of calcium ions in the apical sorting of a class of raft-associated protein GPI-APs (Glycosylphosphatidylinositol-anchored proteins). We reported that the calcium /manganese pump SPCA1 which regulates calcium uptake in the TGN, and of a TGN luminal calcium binding protein Cab45 are master regulators of the apical GPI-APs sorting by stabilizing Golgi GPI-AP clusters that govern GPI-AP apical sorting. Importantly, Cab45 is critical for the apical sorting of GPI-APs specifically since it does not alter the sorting of basolateral GPI-APs and of both apical and basolateral transmembrane proteins therefore revealing the existence of several secretory pathways in polarized epithelial cells.

We further revealed that Cab45 is essential for the apical secretion of apical soluble cargoes PLAP-sec, a non-raft associated proteins. Recent work in Hela cells have shown that Cab45 regulates the sorting of a subset of soluble cargoes by direct binding with its clients. Importantly, in Hela cells silencing of Cab45 leads to Golgi accumulation of soluble cargoes while in polarized MDCK cells silenced for Cab45, PLAP-sec becomes mis-secreted to the basolateral surface. It is intriguing that the same TGN luminal protein Cab45 regulates the apical sorting of both raft GPI-APs and non-raft associated soluble protein PLAP-sec in polarized MDCK cells.

We are currently deciphering the mechanism by which Cab45 regulates apical secretion of soluble cargoes in polarized MDCK cells.

Materials and Methods

Cell cultures, transfections and antibodies

MDCK cells were cultured in DMEM (Sigma-Aldrich, D6429) containing 5% FCS.

The cDNA encoding PLAP-sec is a gift of Andre Le Bivic and have been described in Berger et al 1989. MDCK stably expressing Wnt11 cell lines is a kind gift from A. Kikuchi laboratory.

MDCK cells stably expressing the PLAP-sec were generated by the transfection of PLAP-sec cDNA by using lipofectamine 2000. After 48 hours transfection, a serial dilution of the cells in 96 wells plates (0.5-1 cell/ well) with G418 (800ug/ml) (GIBICO/FISHER, 10131-019) for selection was performed. After amplification, cells and cellular medium were collected to monitor the expression level of PLAP-sec.

In order to silence Cab45, MDCK PLAP-sec cells were infected using lentiviral vector pRFP-CB-sh sequence TGTGAATACTGACCGGAAGATAAGCGCCA (Origene) or with non-effective 29-mer scrambled shRNA cassette in the same p-RFP-CB-sh Lenti Vector (ref. TR30033) as negative control. MDCK cells were infected with lentiviral particles for 24h and then stable clones were selected by using blasticidin (10ug/ml). Screening of positive clones was carried out by analyzing RFP fluorescence.

The antibodies used for biochemistry: polyclonal anti-PLAP 1:500 (from Rockland), polyclonal anti-Wnt11 1:3000 antibody (Abcam, ab31962); polyclonal Cab45 1:1000 from J.V. Blume laboratory. The secondary antibodies considered were rabbit (GE Healthcare UK, NA934V) and mouse (Cytiva, NXA931). Regarding immunostaining, polyclonal KDEL 1:200 (from ABR Affinity/Ozyme, SPA-827), polyclonal GM130 1:100 (from DB transduction, 610823), the same PLAP 1:500 and Cab45 1:500

antibody. The secondary antibodies Alexa Fluor 488 rabbit (A11034), Alexa Fluor 633 rabbit (A21070), Alexa Fluor 546 rabbit (A11035), Alexa Fluor 546 mouse (A11030), and Alexa Fluor 633 mouse (A21050), Alexa Fluor 488 mouse (A11029), were purchased from Thermo Fisher Scientific. Monoclonal anti-PLAP 1:1000 (Sigma-Aldrich, A2951) was used in Immunoprecipitation experiment.

Immunofluorescence

MDCK cells grown on coverslips for 1 or 4 days were washed with phosphate-buffered saline containing $CaCl_2$ and $MgCl_2$, fixed with 4% paraformaldehyde for 20 minutes, quenched with 50 mM NH_4Cl for 10 minutes. After a specific saturation and permeabilization of the cells for 30 minutes in PBS CaCl2/ MgCl2 0.2% gelatin, 0.075% saponin, cells were incubated for 30 minutes with specific antibodies in permeabilized conditions. Primary antibodies were detected with Alexa 488 or 546 (Thermo Fisher Scientific). The images were acquired using a laser scanning confocal microscope (LSM 700; Carl Zeiss MicroImaging, Inc.) equipped with a Plan Apo 63×oil immersion (NA1.4) objective lens.

Medium Collection

MDCK cells PLAP-sec or Wnt11 were seeded on filters (2 million) for 4 days (Corning 3450) or on 10cm dishes (1 million) for 1 day or 4 days. Cells were washed 2 times with serum-free medium and then incubate in serum-free medium (Sigma-Aldrich, D1145) for 4h at 37°C. More precisely, in case of fully polarized cells grown on filters 0,7ml in both apical and basolateral chambers was used while 3ml was added for cells grown on dish. Then, the medium from each condition was collected and concentrated by 10 KD VIVASPIN6 (DUTSCHER, 28-9322-96) at 4°C 4000 rpm for all the proteins above 10 KD. The corresponding cell lysates were collected in parallel.

Western Blot

Cell lysates or concentrated cellular medium were reduced in laemmli containing 5% beta-mercaptoethanol and boiled at 100°C for 5min before being loaded onto 1) 4 - 12% Bis-Tris gels (BioRad, 3450124) or 2) 8% acrylamide/bisacrylamide gel. The gel was run using MOPS buffer (BioRad, 161-0788) or TGS buffer (BioRad, 1610732) at 90V constant and then transfer onto 0.45mm polyvinylidene difluoride membranes (DUTSCHER, 10600023) with transfer buffer (BioRad, 1610734) at 90V for 90 min. When appropriate gels were cut for Coomassie blue staining before transfer or membranes were stained with Ponceau S staining after transfer. The membranes were blocked with 5% non-fat milk in TBST (Tris-buffered saline with 0.1% Tween 20) for 1 hour at room temperature and incubated overnight at 4°C with specific primary antibodies. After 3 washes of 10 minutes each membrane were incubated with secondary antibody for 3 hours before being wash and reveal using ECL prime reagent (DUTSCHER, RPN2236).

Velocity gradients

Velocity gradients, a biochemical method that allows to purify proteins according to their molecular weight, independently of their association with membrane domains, were performed as previously published (Scheiffele et al., 1998, Tivodar S, et al., 2006). Briefly, cells were lysed in 20 mM Tris, pH 7.4, 100 mM NaCl, 0. 2% TX-100, with 0.4% SDS. Lysates were scraped, sheared through a 26-g needle or the concentrated medium were layered on top of a discontinuous glycerol gradient (20-40%) in the same buffer containing 0.2% TX-100. After centrifugation at 45,000 rpm for 16 h in a Beckman SW50 ultracentrifuge, fractions of 500 µl were harvested from the top of the gradient and trichloroacetic acid-precipitated. The proteins were revealed by western blot using specific antibodies.

Native PAGE

Cellular medium was collected and protein inhibitor was added immediately after concentration of the medium using vivaspin and then supplemented with 5% G-250 native sample buffer additive (Thermo Fisher Scientific, BN2004) and native sample buffer (Thermo Fisher Scientific, BN2003) and then load on a Native PAGE Novex Bis-Tris Gel 3–12% (Thermo Fisher Scientific, BN1001) and run at 200V. The SDS page protein ladder (Thermo Fisher Scientific, 26619) was loaded to label protein migration and Native PAGE maker (Thermo Fisher Scientific, Invitrogen, LC0725) was loaded and stain using Coomassie blue. Proteins were transferred onto a polyvinylidene fluoride membrane for 90 min at 90V, then the membrane was immersed in 6% acetic acid for 5 min, air-dried and washed with 100% methanol. Finally, membranes were blocked in 4% Bovine Serum Albumin (Sigma-Aldrich, A9647) in TBS overnight at 4°C and incubated with the primary antibody PLAP antibody overnight at 4°C.

Immunoprecipitation (IP)

PLAP-sec expressed in MDCK cell lysates were precleared with Sepharose protein A beads (Cytiva, 17-0780-01) for 1 hour at 4°C before being incubated with PLAP antibody (Sigma-Aldrich, A2951) 1:1000 overnight at 4°C. Then Sepharose protein A beads were incubated with cell lysate for 2h at 4°C before centrifugation at 500 rpm for 2min. Then beads are collected and washed gently to remove the unbound proteins and boiled at 100 °C for 5min in 2X laemmli buffer containing 5% beta-mercaptoethanol to elute and denature the bound proteins. The MDCK WT cells were considered in parallel as negative control. The input and elution were loaded on SDS PAGE and revealed with antibodies PLAP and Cab45 antibodies respectively.

Temperature block

Temperature block is used to accumulate protein in the Golgi in order then upon release at 37°C to synchronise the protein sorting from the Golgi and their trafficking in MDCK cells. To this aim, we used a protocol which was published previously (Paladino S et al 2004). Cells were seeded on the coverslips and incubated at 19.5°C for 2 h in areal medium (F12 Coon's modified medium without NaHCO3 and with 0.2% BSA and 20 mM Hepes, pH 7.4). In the last hour of incubation, the cells were incubated in areal medium supplemented with 150 μg/ml cycloheximide.

Tunicamycin treatment

MDCK PLAP-sec cells were seeded on filters for 4 days with or without tunicamycin (Sigma-Aldrich, T7765) 10μg/ml added in the last 20 hours. Cellular medium and cell lysate were collected as described above for further biochemistry detection.
Regarding immunofluorescence experiments MDCK PLAP-sec cells were seeded on coverslip for 4 days and incubated for the last 20 hours with or without tunicamycin 10μg/ml before fixation and immunofluorescence protocol.

Statistical analysis

Image analyses were performed by using image J and then t-test was used to address the statistical significance.

Results

Exocytosis of soluble cargoes from the TGN occurs though different mechanisms such as protein aggregation, receptor-mediated or via the sphingomyelin secretion (SMS) pathway recently described in HeLa cells that involves an interplay between actin, calcium and sphingomyelin. More precisely, cofilin recruits actin cytoskeleton that binds and activates the calcium/manganese pump SPCA1 allowing calcium ion uptake in the TGN that in turn leads to oligomerization of the Golgi luminal calcium binding Cab45 that through direct bindings with its client favour the exocytosis of a subset of soluble cargoes such as Cartilage Oligomeric Matrix Protein (COMP) or lysozyme C (LyzC) (Blank and von Blume 2017; von Blume et al. 2012; Crevenna et al. 2016; Pakdel and von Blume 2018; Scherer et al. 1996; von Blume et al. 2011). We recently revealed an unexpected role of calcium ion, SPCA1 and Cab45 in the apical sorting of GPI-APs in polarized MDCK cells (Lebreton et al. under revision in PNAS). These data prompted us to investigate whether Cab45 could be involved in the regulation of apical sorting of soluble protein in polarized epithelial MDCK cells.

PLAP-sec is apically secreted in polarized MDCK cells

In order to decipher whether Cab45 regulates the apical secretion of soluble cargoes in polarized MDCK cells, we investigated the sorting of the secretory form of Placental Alkaline Phosphatase (PLAP), PLAP-sec. While the glycosylphosphatidylinositol-anchored PLAP protein contains 513 amino acids (**Figure 1A**) PLAP-sec devoid of its membrane-bound property contains 489 amino acids excluding the phosphatidylinositol-glycan PI-G tail. By transfection of PLAP-sec cDNA, we generated several MDCK PLAP-sec stable cell lines where we could monitor by western blot secreted PLAP-sec in the cellular medium but also intracellularly (**Figure S1A** and **Figure 1B**). By performing immunostaining of PLAP-sec in MDCK cells, we monitor the intracellular localization of PLAP-sec that is colocalizing with KDEL, an endoplasmic marker with a pearson coefficient of

0,36+/-0,08 (**Figure 1C**). Next, we monitored the level of PLAP-sec secreted in MDCK PLAP-sec cells plated for 1 day or 4 days by collecting and subjecting equivalent volume of media and cell lysates to western blotting and found that secreted PLAP-sec levels normalize to tubulin are similar independently of the polarization state of MDCK cells (**Figure 1D**). Then, we analysed the polarity of secretion of PLAP-sec in MDCK cells plated for 4 days in filters and found that PLAP-sec protein is largely secreted (73%+/-5) in the apical cellular medium as previously reported in FRT cells, another epithelial cell line (Berger et al. 1989) (**Figure 1E**).

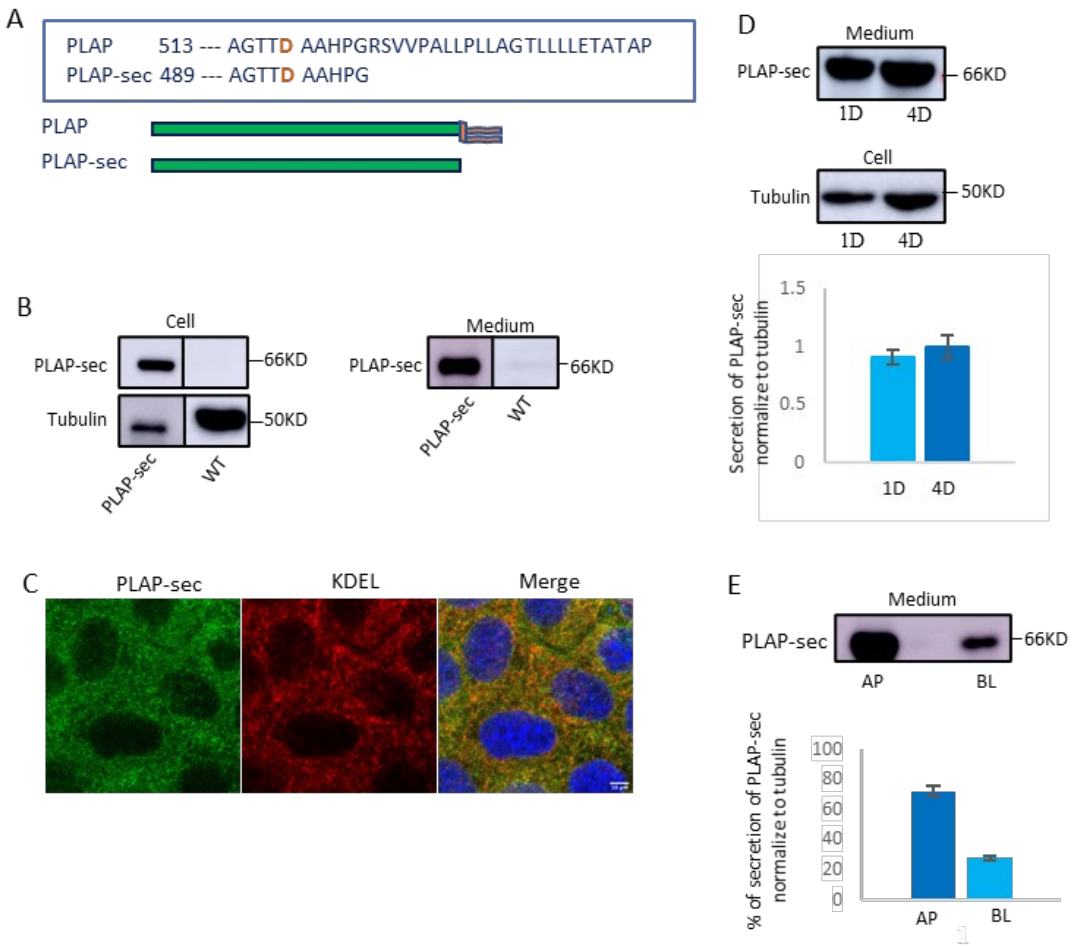

Figure 1. PLAP-sec is apically secreted in polarized MDCK cells.

(A) Carboxyl-terminal amino acid sequences of Placental Alkaline Phosphatase made of 513 amino acids and below its truncated form devoid of the GPI-anchor PLAP-sec containing 489 amino acids. The amino acid sequences are presented in one-letter code (Modified by (Berger et al. 1989)). Asp-484 (ω-site), appears to serve as the amino acid determinants for cleavage and GPI attachment (Kodukula et al. 1993). Below scheme of PLAP and the PLAP-sec (Modified from (Lipardi et al. 2000)) (B) Cell lysates (left) and cellular medium (right) of MDCK WT cells transfected with PLAP-sec cDNA or not were considered to monitor by western blot PLAP-sec expression. Tubulin is used as loading control. (C) Immunostaining of PLAP-sec in MDCK PLAP-sec cells to monitor the intracellular localization of PLAP-sec. KDEL is used as endoplasmic reticulum marker. (D). MDCK PLAP-sec cells seeded on 10cm dish for 1 day (non-polarized) or 4 days (polarized) were used to monitor the level of PLAP-sec secretion in the cellular medium. The secretion of PLAP-sec was normalized to tubulin level in cell lysate. Experiments were performed 3 independent times and the quantification is shown in the histogram below. (E) MDCK PLAP-sec cells were seeded on filters for 4days. The medium was collected from apical and basolateral chambers and PLAP-sec secretion was monitored. The histogram below is the mean of three independent experiments (± SD).

Apical secretion of PLAP-sec relies on Cab45

To evaluate whether Cab45 has a role in the apical secretion of PLAP-sec we generated stable knockdown Cab45 MDCK PLAP-sec cells. After infection with lentiviral particles containing specific short hairpin RNA (shRNA) sequence targeted against Cab45 (Cab45i) or scrambled shRNA (CTRLi) we selected MDCK PLAP-sec Cab45i clones exhibiting decrease of Cab45 expression levels compared to the control condition (Lebreton et al. under revision in PNAS) (**Figure 2A**). PLAP-sec localization and immunostaining intensity are similar in MDCK PLAP-sec CTRLi

and Cab45i (**Figure 2B**). We first compared PLAP-sec secretion in MDCK PLAP-sec CTRLi and Cab45i cells plated on coverslip for 1 and 4 days by collecting and subjecting equivalent volume of media and cell lysates to western blotting as previously (**Figure 1D**). In MDCK PLAP-sec CTRLi cells as previously reported in MDCK PLAP-sec cells (**Figure 1D**), levels of secreted PLAP-sec normalize to tubulin is similar in both 1 and 4 days grown cells (**Figure 2C**). Furthermore, MDCK PLAP-sec Cab45i exhibit similar levels of secreted PLAP-sec in both 1 day and 4 days conditions compared to MDCK PLAP-sec CTRLi (**Figure 2C**) therefore suggesting that silencing of Cab45 does not alter the levels of secreted PLAP-sec. Next, we wonder whether the polarity of the secretion would be modified upon Cab45 silencing. According to this aim, we plated MDCK PLAP-sec CTRLi or Cab45i on filters and evaluate the levels of secreted PLAP-sec in the apical and basolateral chambers. After cultured for 4 days, PLAP-sec was monitored by western blotting with PLAP antibody after enriched from cellular medium of apical or basolateral cellular medium by immunoprecipitation. While in control cells, we could monitor mostly PLAP-sec in the apical cellular medium in case of MDCK PLAP-sec Cab45i cells we monitor PLAP-sec secreted exclusively in the basolateral medium (**Figure 1D**) therefore revealing the critical role of Cab45 in the apical secretion of PLAP-sec in polarized MDCK cells.

In order to assess whether Cab45 is regulating the apical secretion of other soluble cargoes we monitor the secretion of soluble protein Wnt11 whose apical secretion in polarized MDCK cells requires Galectin3 binding (Yamamoto et al 2013). As previously we generated MDCK Wnt11 CTRLi and Cab45i cells (**Figure S1C**) and monitor Wnt11 secretion in apical and basolateral medium of cells plated for 4 days on filters. As previously reported, in MDCK Wnt11 CTRLi cells, Wnt11 is largely secreted in the apical medium and its apical secretion is unaffected by Cab45 silencing (**Figure S1D**). This latter data highlights the specificity of Cab45 as

regulator of the apical secretion of PLAP-sec and suggest, that differently from Wnt11, the apical secretion of PLAP-sec is not relying on Galecin-3 binding.

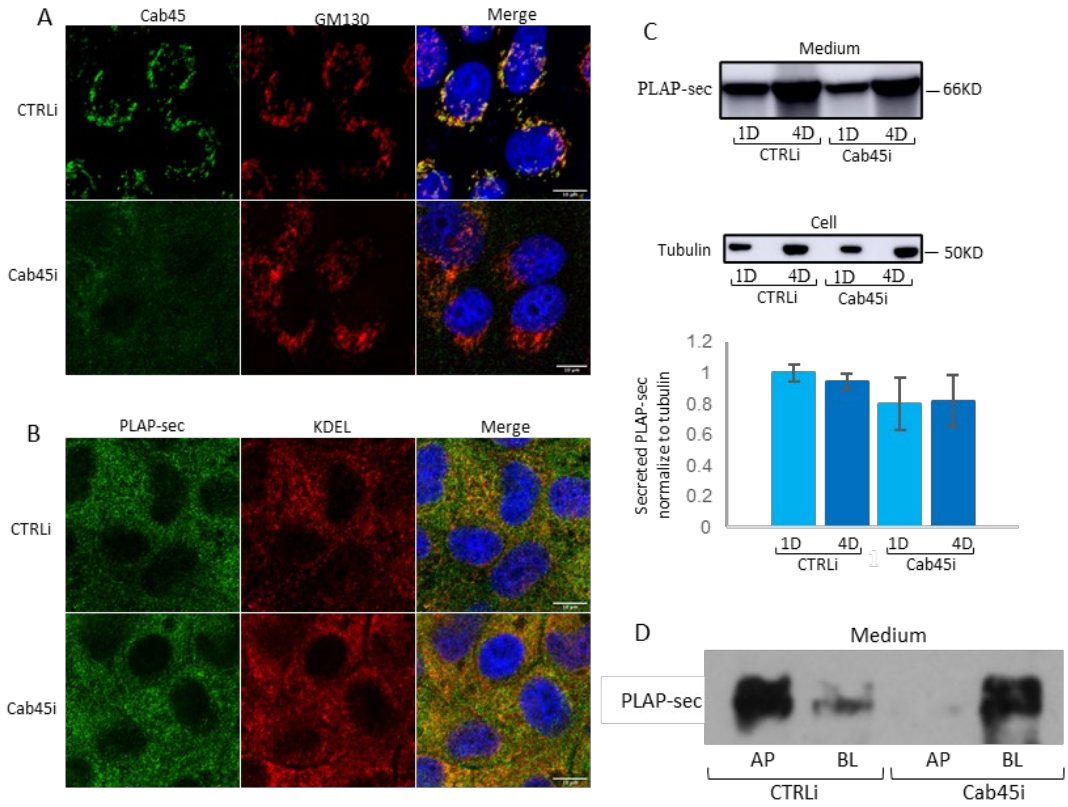

Figure 2. Cab45 is essential for the apical secretion of PLAP-sec.

(A) (B) MDCK PLAP-sec, infected with lentiviral scrambled shRNA particles (CTRLi) or shCab45 particles (Cab45i), were plated on coverslip for 4 days for immunostaining of Cab45 and GM130 a cis-medial Golgi marker (A) or PLAP-sec and KDEL, ER marker (B). (C) Cellular medium of MDCK PLAP-sec CTRLi and Cab45i plated for 1 day or 4 days were collected to monitor PLAP-sec levels normalize to tubulin level. Experiment was performed three independent times and quantification is shown in the histogram below. (D) Cellular medium of MDCK PLAP-sec CTRLi and Cab45i plates for 4 days on filters were collected from apical and basolateral chamber respectively to monitor PLAP-sec levels after

immunoprecipitation. The experiment was performed twice. Standard molecular weights are indicated. Scale bars, 10um. Error bars, ± SD.

Secreted PLAP-sec clustering state is not affected by Cab45 silencing

In order to decipher how Cab45 is governing the apical secretion of PLAP-sec, we analysed the clustering state of secreted PLAP-sec. We grew fully confluent MDCK PLAP-sec CTRLi and Cab45i cells for 4 days on dishes and loaded the cellular medium on NATIVE-PAGE to address the clustering state of secreted PLAP-sec and found that secreted PLAP-sec in both control and Cab45 silenced cells migrates around 240kd indicating that PLAP-sec is mostly organized as trimers (**Figure 3A**). To further assess the assembly of secreted PLAP-sec we performed velocity gradient sedimentation experiments on the cellular medium of MDCK PLAP-sec CTRLi and Cab45i. This technique allows the proteins to sediment according to their molecular weight, thus revealing if the protein is in its monomeric form or in a high molecular weight (HMW) complex. In MDCK PLAP-sec CTRLi cells, PLAP-sec is mostly migrating from fraction 3 to 6 indicating that PLAP-sec is organized as monomer, dimer and trimer. Importantly, PLAP-sec clustering state is not affected by the silencing of Cab45 since the migration pattern of PLAP-sec on velocity gradient is similar when considering the cellular medium of MDCK PLAP-sec Cab45i as shown by the quantification curves (**Figure 3B**).

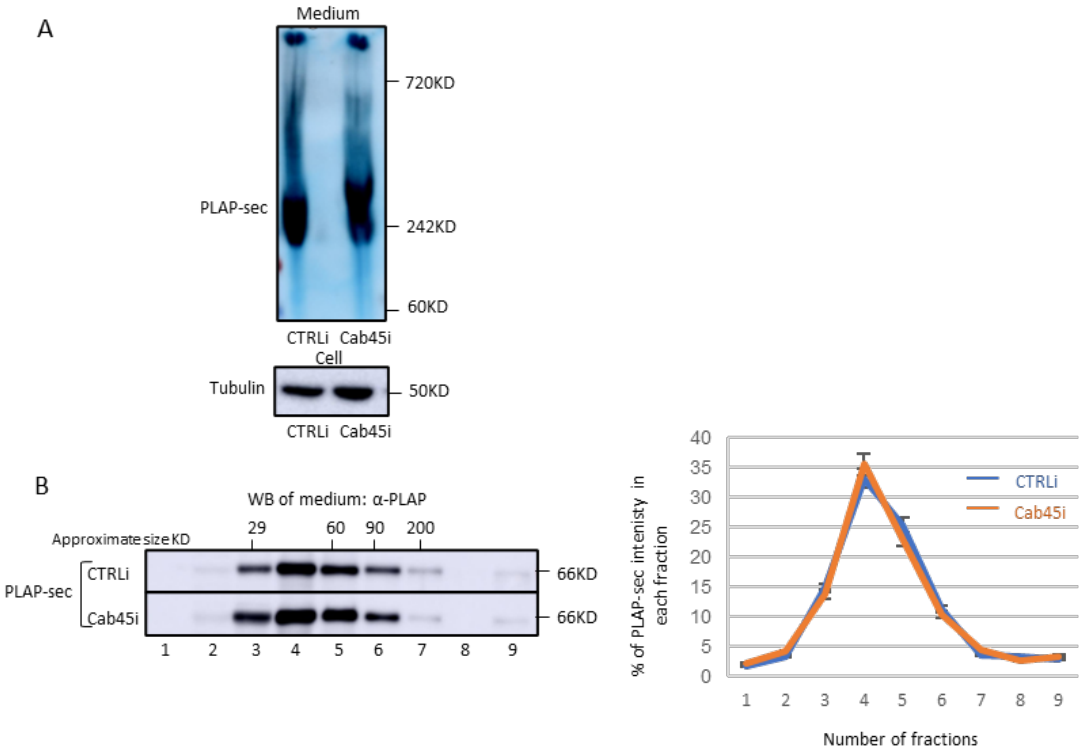

Figure 3. The loss of Cab45 does not affect the clustering state of secreted PLAP-sec.

(A)(B) Cellular medium of MDCK PLAP-sec CTRLi and Cab45i cells plated for 4 days were analyzed by (A) Native PAGE or (B) by velocity gradient where fractions collected from top (fraction 1) to bottom (fraction 9) were loaded on SDS-PAGE gel to monitor clustering state of secreted PLAP-sec. The representative molecular weight markers are indicated. Native-PAGE experiments were performed 3 times and velocity gradient 5 times with the quantification shown on the right.

The intracellular PLAP-sec form Cab45-independent clusters

Here we showed that PLAP-sec is mostly apically secreted in polarized MDCK cells and that the apical secretion relies on Cab45. We recently reported that Cab45 in the TGN of polarized MDCK cells is essential for the apical sorting of GPI-APs known to form high molecular weight complexes or cluster in the Golgi prior to their apical sorting. Therefore, we propose that Cab45 would stabilize GPI-APs cluster to favor

their apical sorting (Lebreton et al. under revision in PNAS). Furthermore, in professional secretory cells, calcium-dependent soluble protein aggregation has been shown to be essential for their sorting from the TGN, we therefore wonder whether PLAP-sec could cluster intracellularly and whether Cab45 could play a role in this clustering mechanism. In order to address this point, we performed velocity gradient sedimentation experiments by considering cell lysates of MDCK PLAP-sec CTRLi or Cab45i grown for 4 days on dishes. In control cells, 20% of PLAP-sec intensity signal is found in the higher fraction of the gradient indicating that PLAP-sec form high molecular weight complexes (**Figure 4A**). Interestingly, in MDCK PLAP-sec Cab45i cells, the pattern of PLAP-sec sedimentation of velocity gradient is similar to the control condition with 18% of PLAP-sec signal intensity found in the higher fractions (**Figure 4A**) indicating that intracellular PLAP-sec organization does not rely on Cab45. Then, we wonder whether Cab45 could directly interact with PLAP-sec as reported in HeLa cells for its client COMP and LysC (von Blume et al. 2012). Accordingly, we grew MDCK WT or MDCK PLAP-sec cells for 4 days on dish in fully confluent conditions and immunoprecipitated PLAP-sec by considering the cell lysates. Then the eluted PLAP immunoprecipitated is run and reveal either with an anti-PLAP or anti -Cab45 antibody. Although, we could monitor an enrichment of PLAP-sec in the eluted immunoprecipitation (IP) compared to the sample collected before the IP (input) (**Figure 4B** upper gel), we could not detect interaction between PLAP-sec and endogenous Cab45 therefore suggesting that i) Cab45 does not interact with PLAP-sec or ii) alternatively their interaction is transient.

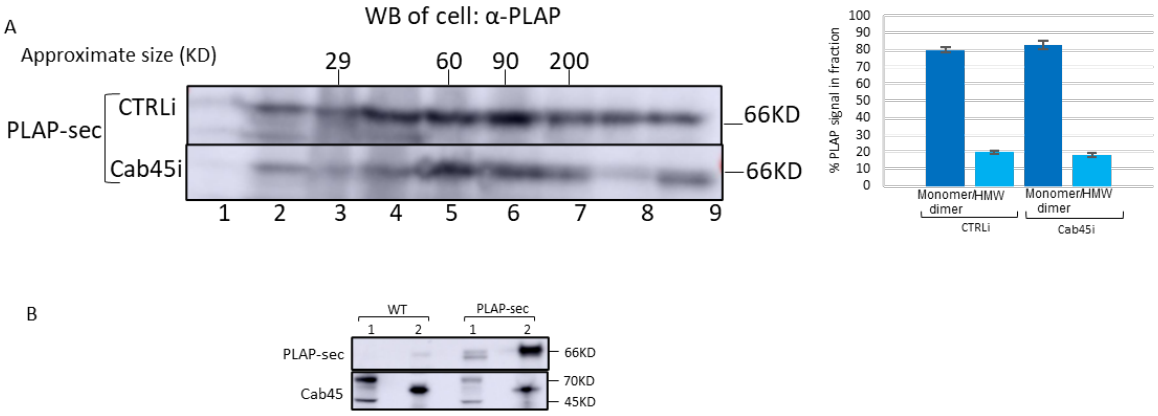

Figure 4. The loss of Cab45 does not affect the intracellular clustering of PLAP-sec.

(A) Cell lysates of MDCK PLAP-sec CTRLi Cab45i cells seeded for 4 days were collected and ran on velocity gradient as described in method and ***Figure 3****. Fractions were collected from top (fraction 1) to bottom (fraction 9), then trichloroacetic acid (TCA) precipitated and analyzed by SDS-PAGE with PLAP antibody. The experiment was repeated 4 times and the quantification is shown on the right). (B) Cell lysates of MDCK PLAP-sec or MDCK WT cells seeded for 4 days were immunoprecipitated with PLAP antibody together with the sepharose protein A beads and revealed with either anti-PLAP (top) or anti-Cab45 (bottom) antibodies. An aliquot of cell lysates (1: Input; 2: pulldown) were loaded. Note that no interaction could be monitored between PLAP-sec and Cab45.*

N-glycosylation of PLAP-sec is essential for its exocytosis

Importantly, as reported earlier carbohydrates act as apical sorting signal for secretory proteins in polarized MDCK cells (Kitagawa et al. 1994; Schelffele, Perinen, and Simons 1995; Urban et al. 1987). Furthermore, the apical secretion of Wnt11 has been reported to rely on the N-glycosylation also, therefore we wonder whether the N-glycosylation of PLAP-sec is regulating the apical secretion of the soluble cargoes in

fully polarized MDCK cells. Would it be possible that N-glycosylation of PLAP-sec is involved in the Cab45-dependent apical secretion? In order to investigate this possibility, we plated MDCK PLAP-sec cells for 4 days on filter and treated or not the cells with tunicamycin (10μg/ml) for the last 20 hours of the culture to block N-glycosylation (Catino et al. 2008). Apical and basolateral cellular medium were collected, concentrated and loaded on SDS-PAGE to monitor the polarity of secretion of PLAP-sec. While in non-treated cells as previously found (**Figure 1E**), PLAP-sec is mostly secreted in the apical medium, upon tunicamycin treatment although by using S-Ponceau we monitored the presence of proteins both in apical and basolateral cellular medium (**Figure S2A**), we barely reveal PLAP-sec in the apical medium **(Figure5A).** Importantly, as we collected and loaded equivalent volume of media and cell lysates to monitor PLAP-sec levels, we found that the drastic decrease of secreted PLAP-sec upon tunicamycin treatment of MDCK PLAP-sec cells is correlating with a statistical increase of the intracellular pool of PLAP-sec that without N-glycans is exhibiting a shift in its migration (55kD) on SDS-PAGE (**Figure 5B**). The immunostaining of intracellular PLAP-sec reveals an increase of fluorescence intensity in MDCK PLAP-sec cells treated with tunicamycin compared to control conditions **(Figure 5C)** further indicating that PLAP-sec exocytosis is blocked upon impairment of N-glycosylation by tunicamycin treatment.

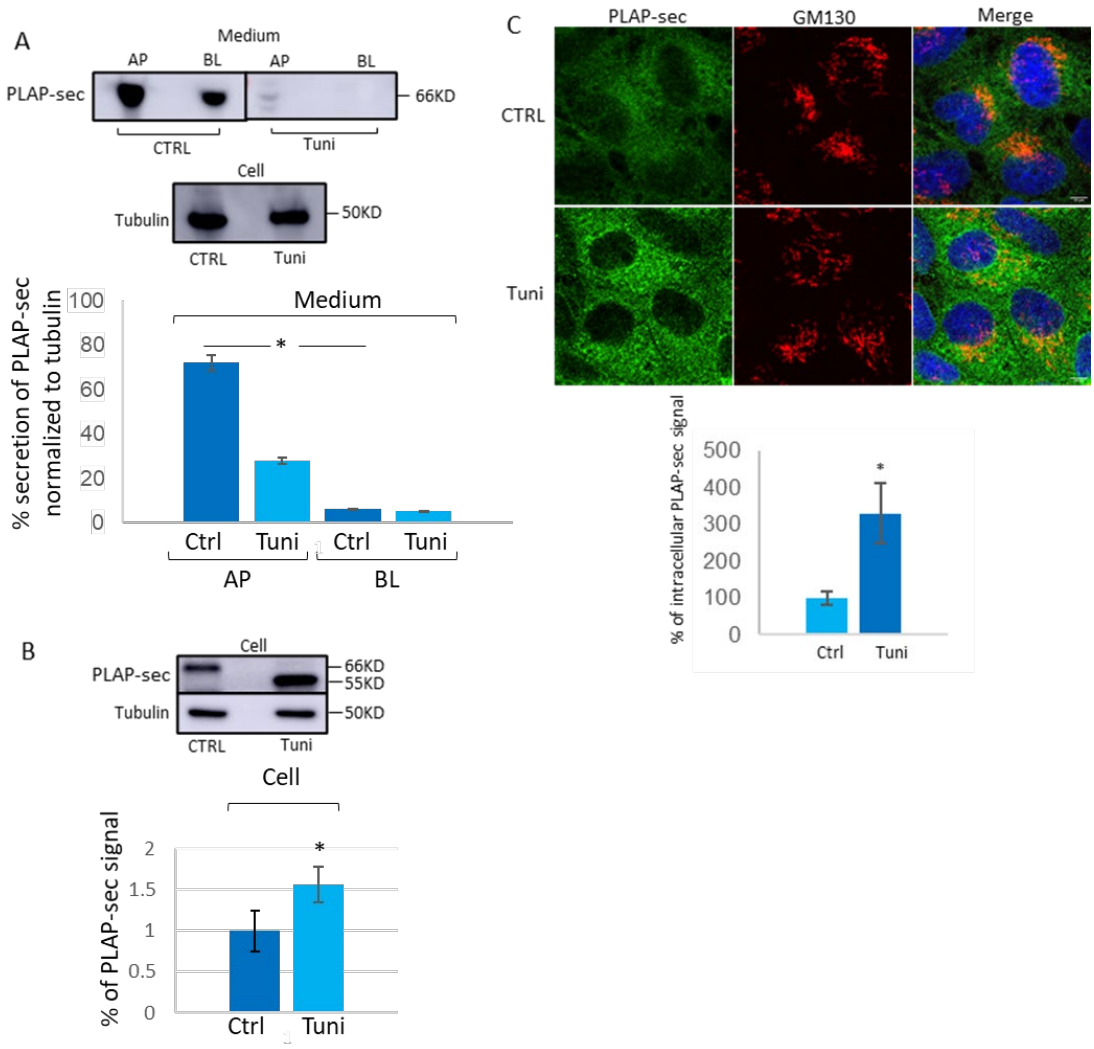

Figure 5. N-glycosylation of PLAP-sec is crucial for its exocytosis.

(A) Apical and basolateral cellular medium of MDCK PLAP-sec cells plated on filters for 4 days and treated or not (CTRL)with tunicamycin (Tuni) were collected and analyzed to monitor PLAP-sec expression. Tubulin is used to normalize PLAP-sec secretion. The same volume of corresponding cell lysates was collected as loading control and western blotting with tubulin antibody. Experiments were repeat three independent times and the quantification are shown below the image. (B) Cell lysates of MDCK PLAP-sec cells seeded for 4 days on filers and treated or not with tunicamycin as in (A) were loaded to monitor intracellular PLAP-sec in both conditions and normalized to tubulin level. Experiments were repeat three independent

*times and the quantification are shown below. (C) MDCK PLAP-sec seeded on coverslip for 4days were treated or not (CTRL) with tunicamycin (Tuni) were considered for PLAP-sec immunostaining and the fluorescence intensity of PLAP-sec in both conditions was evaluated by using image J software in 60 cells. Scale bars, 10 um. Error bars, ± SD; * p<0.05, Student's t-test.*

Overall, exocytosis of PLAP-sec occurs only if PLAP-sec is N-glycosylated and the apical secretion of this soluble cargoes relies on Cab45.

Interestingly in HeLa cells, it was reported that silencing of SPCA1 or Cab45 leads to a retention of Cab45 clients in the TGN that results from a delay of exocytosis while in MDCK cells upon Cab45 silencing 1) we did not monitor accumulation of PLAP-sec in the Golgi (**Figure 2A**) and 2) after 4 hours of secretion the levels of PLAP-sec monitored in the cellular medium are similar to the ones observed in control conditions.

In the next section, I will further discuss the remaining open questions that I have to address and their further implications.

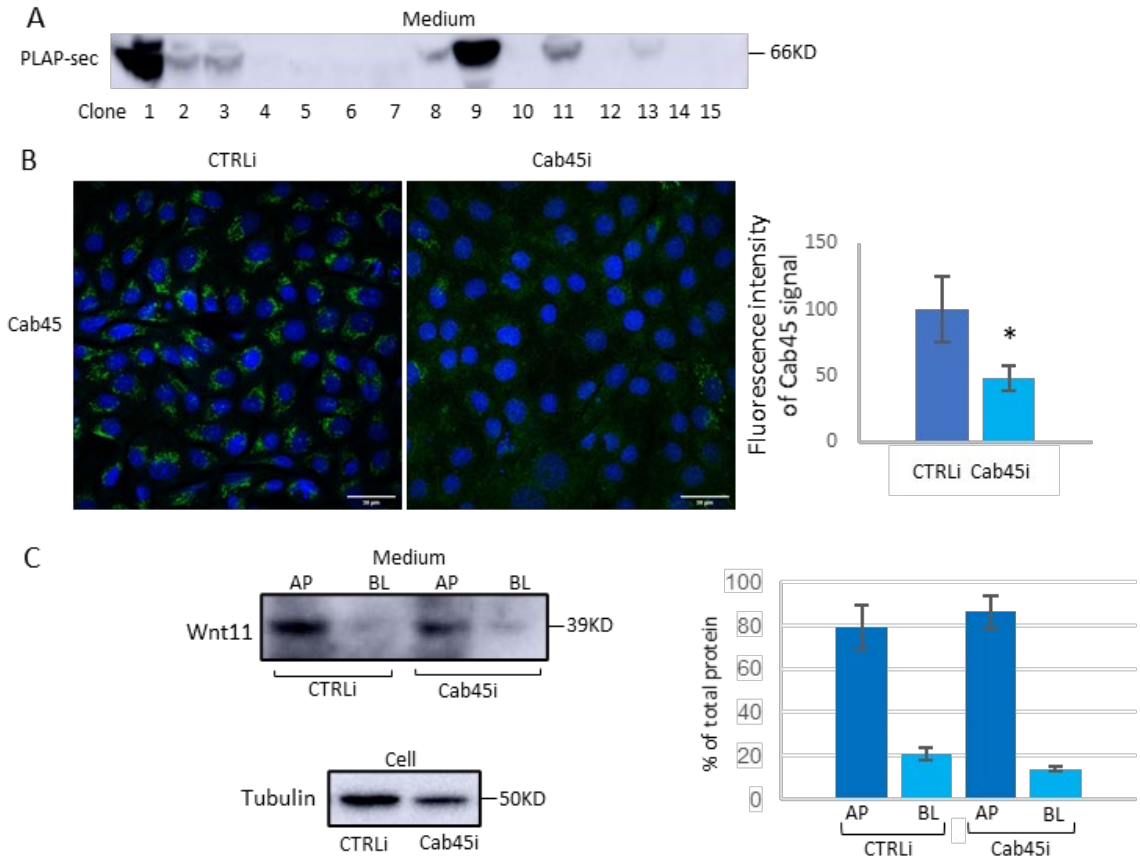

Figure S1. Cab45 is required for the apical secretion of PLAP-sec but not of Wnt11.

*(A) Cellular medium of different MDCK PLAP-sec clones grown on coverslip were loaded and analyzed by western blot to monitor PLAP-sec expression. (B) MDCK Wnt11 cells infected with scrambled shRNA particles (CTRLi) or shCab45 particles (Cab45i) were grown on coverslip and immunostained to monitor Cab45. Fluorescence intensity in both samples was quantified as shown on the histogram on the right; (C) Cellular medium of MDCK Wnt11 CTRLi and Cab45i cells grown on filter for 4 days were collected from apical and basolateral chambers respectively and analyzed by western blot to monitor Wnt11 levels normalize to intracellular levels of tubulin. Experiments were repeated 3 times and the quantification shown on the right. Scale bars, 30 μm. Error bars, ± SD; * $p<0.05$, Student t-test.*

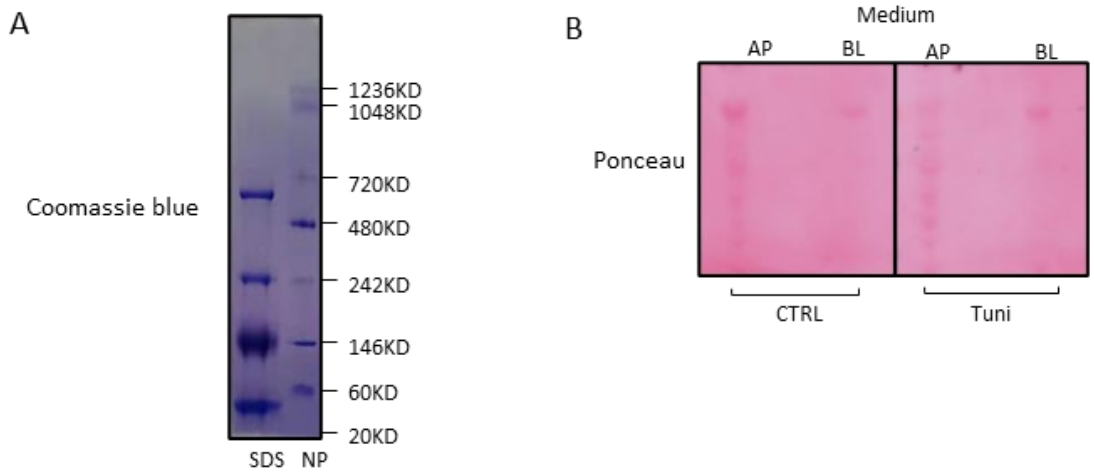

Figure S2. Tunicamycin does not block general protein exocytosis.

(A) The SDS page protein ladder was loaded to visualize protein migration and Native PAGE maker was loaded and stain by Coomassie blue to evaluate the molecular weight. SDS, SDS page protein ladder; NP, Native PAGE maker. (B) Cellular medium of MDCK PLAP-sec CTRL and tunicamycin treated cells were loaded on SDS-PAGE membrane and incubated with S- ponceau. Note that in case of tunicamycin treated cells proteins can be monitored both from apical and basolateral chambers.

DISCUSSION

The establishment and maintenance of epithelial properties and functions rely on the proper protein sorting and trafficking to the cell surface. GPI-APs are lipid-anchored proteins that are sorted at the TGN (Hua et al. 2006; Paladino et al. 2006) and are selectively localized at the apical surface in the majority of epithelia (Lebreton, et al. 2018, 2019; Paladino et al. 2015).

We showed that in order to be apically sorted in different epithelial cell lines, GPI-APs need to clusters in the Golgi apparatus (Paladino et al. 2004, 2007b). We further showed that Golgi clustering of GPI-APs that drives their apical sorting also regulates their apical plasma membrane organization and biological functions (Lebreton et al. 2018; Paladino et al. 2014, 2015). We previously reported that the levels of cholesterol in the Golgi apparatus is critical but not sufficient to drive oligomerization of GPI-APs (Paladino et al. 2004, 2008, 2014). Beside the role of cholesterol, we here show that the level of calcium in the Golgi apparatus is also essential for GPI-APs clustering and their apical sorting. We further revealed that Golgi level of Calcium is regulated in MDCK cells by the calcium manganese pump SPCA1 (Secretory Pathway Ca^{2+} -ATPase) allowing uptake of calcium in the Golgi that in turn leads to Cab45 oligomerization. Silencing of either SPCA1 or Cab45 results in impairment of GPI-APs oligomerization in the Golgi and their mis-sorting to the basolateral surface. It is also interesting that higher expression levels of mRNA SPCA1 and Cab45 have been monitored in polarized MDCK cells stably expressing apical GPI-APs reinforcing their role in this cellular process. Importantly, SPCA1 or Cab45 silencing does not alter the apical and basolateral sorting of transmembrane proteins (gp114 and E-cadherin respectively) reinforcing the existence of several anterograde routes in polarized MDCK cells. We further found that Cab45 is not regulating the basolateral sorting of GPI-APs but only of apical ones (exogenous or endogenous).

The apical GPI-APs start to oligomerize concomitantly to their raft-association in the Golgi (Paladino et al. 2004) and SPCA1 and Cab45 localizes in the TGN therefore suggesting that Cab45 would not regulate cluster formation of GPI-APs but stabilize the cluster of GPI-APs prior to their apical exocytosis. However, many open questions remain. Whether Cab45 interacts with apical GPI-APs as reported in case of Cab45 clients in Hela cells? To address this question, we performed coimmunoprecipitation experiment by using GFP-Trap. Briefly we incubate the lysate of MDCK cells stably expressing GFP-FR with GFP agarose beads to pull down GFP-FR and reveal Cab45 to monitor whether Cab45 binds to GFP-FR. We could not reveal any direct interaction between these two proteins, GFP-FR and Cab45 (**Figure I A)**. This latter data could result from the limiting amount of endogenous Cab45 that could interact with overexpressed GFP-FR or could be due to the specificity of the chimeric protein made just of the GFP ectodomain fused to the GPI signal attachment of folate receptor. Therefore, we next repeated the same experiment by considering overexpressed GFP-Cab45 in MDCK cells stably expressing apical GPI-AP FRα, folate receptor α full length. By performing GFP-Trap, we still could not monitor a direct interaction between GFP-Cab45 and FRα (**Figure I B)**. These data can be interpreted in different manners: 1. that Cab45 does not interact with apically sorted GPI-APs in the Golgi; 2. that this interaction is transient or 3. that interaction occurs but indirectly through another partner that could be a transmembrane protein for instance. Although Cab45 is luminal protein it has been reported to directly interact with SPCA1 a transmembrane protein in Hela cells (von Blume et al. 2012). To this aim, it will be interesting to further investigate the spatio-temporal relationship between Cab45 and GPI-APs by using FRET/FLIM to see if Cab45 and GFP-FR are in the same close environment. Alternatively, mass spectrometry in polarized MDCK cells stably expressing GFP-Folate receptor alpha full length or expressing GFP-Cab45 could be done upon GFP-Trap (Zi-Liang et al. 2014).

In HeLa cells, it has been shown that Cab45 forms oligomers in the TGN in a calcium dependent manner and regulates the exocytosis from the TGN of a subset of soluble protein as LysC, HRP and COMP. Cab45 can directly binds to its clients in order to favor their exocytosis. Therefore, we wonder whether Cab45 could regulate the apical sorting of soluble cargoes in MDCK cells. We first investigated whether Cab45 expression affects secretory cargoes PLAP-sec in polarized MDCK cells. Our results show that silencing of Cab45 drives the basolateral mis-secretion of PLAP-sec that in control conditions is apically secreted. Moreover, we did not detect any interaction between Cab45 and intracellular PLAP-sec (**Figure 4**B). Importantly, in HeLa cells silencing of Cab45 leads to a retention of its clients in the Golgi after 2 or 4 hours monitoring the protein in the medium and the cell by western blot and the microscopy (von Blume et al. 2012; Crevenna et al. 2016) while we never monitor any Golgi accumulation of PLAP-sec in Cab45 silenced cells. Importantly in polarized MDCK cells stably expressing apical soluble cargoes Wnt11, we found that silencing of Cab45 did not alter Wnt11 apical secretion that has been previously reported to be relying on both N-glycosylation and Galectin-3 (Yamamoto et al. 2013). This would indicate that several exocytosis pathways exist to target the apical secretion of soluble cargoes in polarized MDCK cells. The key question remains how Cab45 would regulate the apical secretion of PLAP-sec. Would it be possible that the intracellular cluster of PLAP-sec that we monitor, being Cab45-independent, are relying on high calcium concentration as it has been postulated in the protein aggregation model in the professional secretory cells (Borgonovo et al, 2006; Chanat et al. 1991; Gerdes et al. 1989)? The ectodomain of PLAP has a calcium binding domain (Millán 2006), therefore it is important now to evaluate the clustering state of PLAP-sec upon ionomycin treatment for instance. It is plausible that both Cab45 and PLAP-sec requires high calcium levels for their clustering and that this is essential for the apical Cab45-dependent secretion of PLAP-sec.

Another open question is whether PLAP-sec is using the conventional secretory pathway? PLAP-sec is localized in the ER and its apical sorting is relying of Cab45 that localizes intracellularly in the Golgi. However, Cab45 has various splicing variants: 1) Golgi resident Cab45 (Cab45- G) and its splice isoforms 2) secreted Cab45 (Cab45-S) and 3) cytosolic Cab45 (Cab45-C). Cab45-S is only 14 amino acids shorter than Cab45-G, but it lacks the C-terminal HEEF signal. Would it be possible that PLAP-sec is bypassing the Golgi? In order to answer this question, we performed the temperature block assay that allows to accumulate protein in the Golgi but we could not monitor PLAP-sec in the Golgi (**Figure II A**). It is important to note that not all proteins undergoing classical secretory pathway accumulate well in the Golgi upon temperature block as exemplified by GPI-AP PLAP. In order to address this question, I will perform classical cell fractionation (Dunphy et al. 1981; Fries and Rothman 1981; Imjeti et al. 2011; Paladino et al. 2008; Yamamoto et al. 2013) in order to define whether PLAP-sec is found in the Golgi fraction of polarized MDCK cells. Regarding the role of N-glycosylation in the apical sorting of PLAP-sec, it appears from our data that N-glycosylation regulates the exocytosis of PLAP-sec but not the polarity of its secretion. Another important experiment I have to perform is to analyze the co-localization of PLAP-sec upon tunicamycin treatment with KDEL marker and define its clustering state intracellularly upon N-glycosylation impairment.

How Cab45 regulates the apical exocytosis of both GPI-APs and soluble cargoes is still unsolved, therefore, I further analyzed Cab45 expression levels intracellularly but also secreted. Interestingly, I revealed that Cab45 is glycosylated in MDCK cells as shown by the two specific bands at 70kD and 45kD. A treatment of protein extraction or cellular medium with an O- and N- glycosylated mix deglycosylation enzymes, allowed to observe the disappearance of the 70kD bands and revealed only the 45kd bands (**Figure III A**), indicating that Cab45 is glycosylated. Next, I

monitored the levels of Cab45 secreted both in non-polarized and polarized MDCK cells and found that Cab45 is equally secreted in both conditions (**Figure III B**). Since Cab45 is regulating the polarity of exocytosis of apical soluble protein and GPI-APs, I also monitored the polarity of its secretion through establishment of polarity by collecting apical and basolateral medium of MDCK cells plated on filters for 1 Day, 2 Days, 3 Days, 4 Days and 5 Days. Surprisingly, I monitored that Cab45 from day 1 to day 5 is more secreted in the basolateral medium compared to the apical medium as show in (**Figure III D).** Of interest, we could not monitor any correlation between the glycosylation state of secreted Cab45 and the polarity of its secretion at the different time points. Then, we monitored by Native PAGE the clustering state of secreted Cab45 in non-polarized and polarized MDCK cells and found that in both conditions Cab45 is forming cluster of roughly 720kD (**Figure III E**). In addition, we also found that both apical and basolateral secreted Cab45 in 1day or 4days MDCK cells is forming cluster (**Figure III F**).

In conclusion all secreted Cab45 is clustered and Cab45 is more secreted basolaterally than apically while we revealed a role of Cab45 in the apical exocytosis of GPI-APs and soluble PLAP-sec. This let us think that Cab45 could have an additional role in cells although we also established that Cab45 expression is not required for the basolateral sorting of 1) transmembrane E-cadherin and the cleaved APP protein and 2) basolateral GPI-AP (GFP-PrP FL and GFP-PrP anchor only) (Lebreton et al. under revision in PNAS). Remarkably, some cancer cells express high amounts of Cab45-S such as pancreatic cancer cells (Grønborg et al. 2006). And the expression level of Cab45-S seems to be correlated with the cervical carcinoma grade (Chen et al. 2016), indicating that secretion and polarity of secretion of Cab45 might contribute to tumorigenesis.

I would like to end this discussion by underlying the choice of PLAP-sec as soluble cargoes. Although it is a truncated protein, it was reported that the

hyperphosphatasia mental retardation syndrome (HPMR), an autosomal recessive disease characterized by mental retardation and elevated serum alkaline phosphatase (ALP) levels is caused by mutations in the coding region of the GPI-anchor biosynthesis, class V (PIGV) gene. The experiment in CHO cells showed that the release of ALP in the medium was the result of cleavage at the ω-site of PLAP by the GPI transamidase in the ER which is the same position of PLAP-sec devoid GPI-anchor (Murakami et al. 2012), suggesting the exocytosis of PLAP-sec in the non-polarized cells or the polarized secretion in polarized cells have significant meaning physiological relevance, therefore the study of the mechanism of secretion of PLAP-sec in our study appears relevant.

Finally, it is obvious that a better understanding of the variety of the different anterograde routes in polarized epithelial cells is needed as it is required 1) for establishment and maintenance of epithelial polarity, 2) it is challenged in many human diseases and 3) could help to identify selective compounds to block the arrival of specific receptor at the cell surface for instance in case of overexpression in cancer cells. Therefore, the next future of this work is to use in fully polarized MDCK cells the RUSH (Retention upon selective hook) assay (Boncompain et al. 2012) in order to characterize the kinetic of different proteins TM, GPI-APs, soluble apically or basolaterally sorted to 1) confirm the existence of several anterograde routes in polarized MDCK cells by monitoring Golgi residency time, arrival time to the apical or basolateral surface; 2) characterize the morphology of the cargoes budding from the Golgi in order to reveal also existence of several machineries enabling the sorting of these different proteins; 3) determine the site of segregation of different apical (TM or GPI-APs or soluble) and basolateral cargoes (Golgi, post-Golgi); 4) decipher the role of the molecular factors identified so far as lipids (cholesterol, sphingomyelin), actin, calcium, SPCA1 and Cab45. Ultimately, we will need to analyze whether these molecular machineries are altered in MDCK cells undergoing EMT, or by comparing healthy epithelial and breast cancer cells for instance.

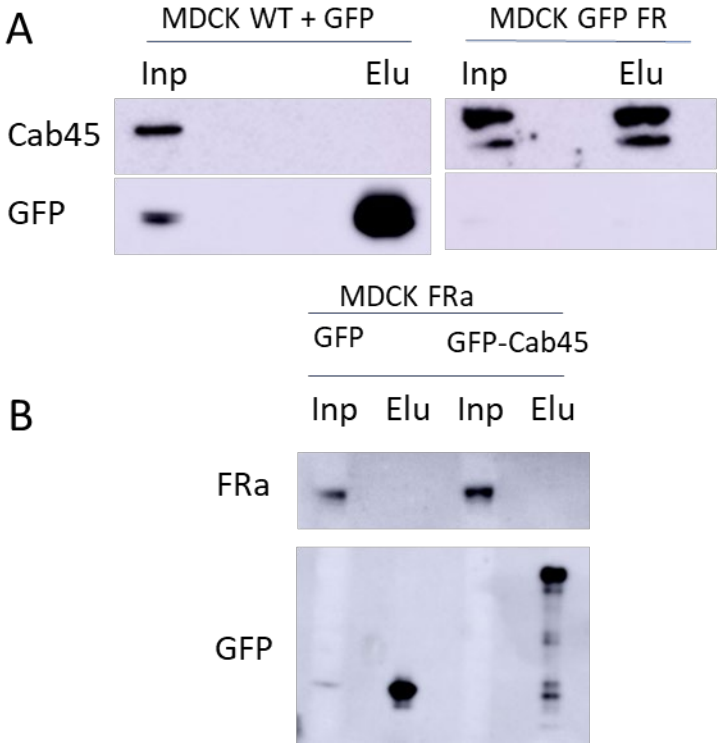

Figure I. Cab45 do not interact with GPI-APs in polarized MDCK cells.

(A)(B) Generate the GFP-Trap of MDCK WT cells, MDCK GFP FR cells which stable express GFP FR (A), of MDCK Fra full length cell which already stable express Fra full length protein (B) and transfected GFP Cab45 and GFP as negative control. The cells lysates were extracted by using NP-40 for 1H. Perform the SDS page after pulldown, loading lysates contain 20ug proteins before immunoprecipitating with beads labelled input, loading the whole lysates collected on one 10cm dish after immunoprecipitation as pulldown. Inp: Input; Elu: Pulldown.

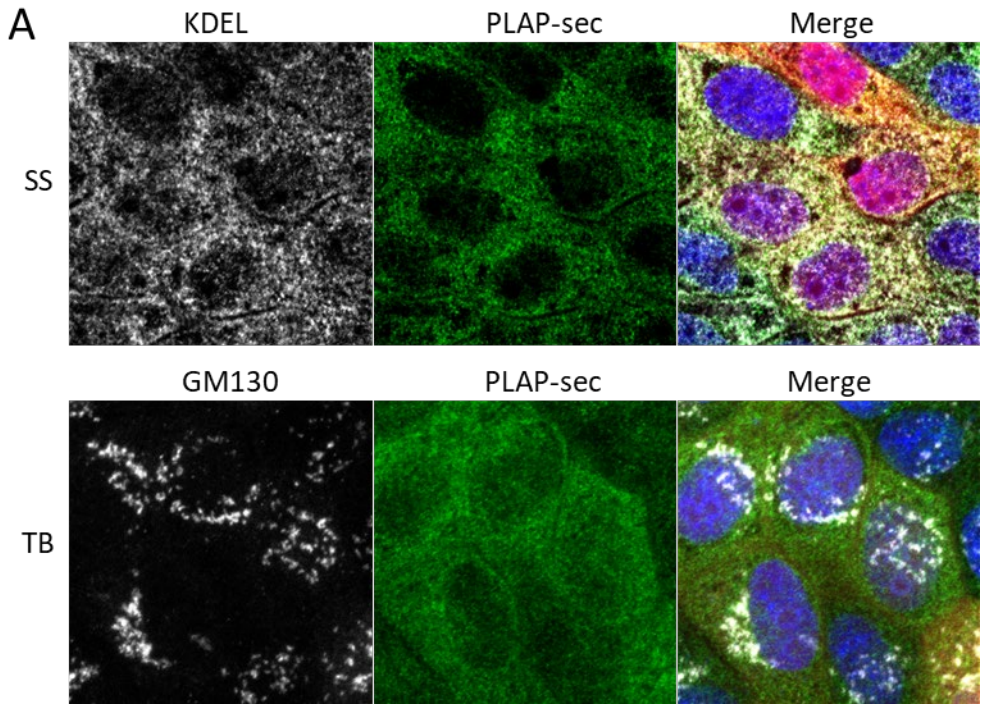

Figure II. PLAP-sec cannot be accumulated in the Golgi by temperature block.

(A) MDCK PLAP-sec were plated on coverslip for 4 days for immunostaining of PLAP-sec and KDEL, an ER marker in steady state or PLAP-sec and GM130, a cis-medial Golgi marker in temperature block 19 ℃. SS: steady state; TB: temperature block.

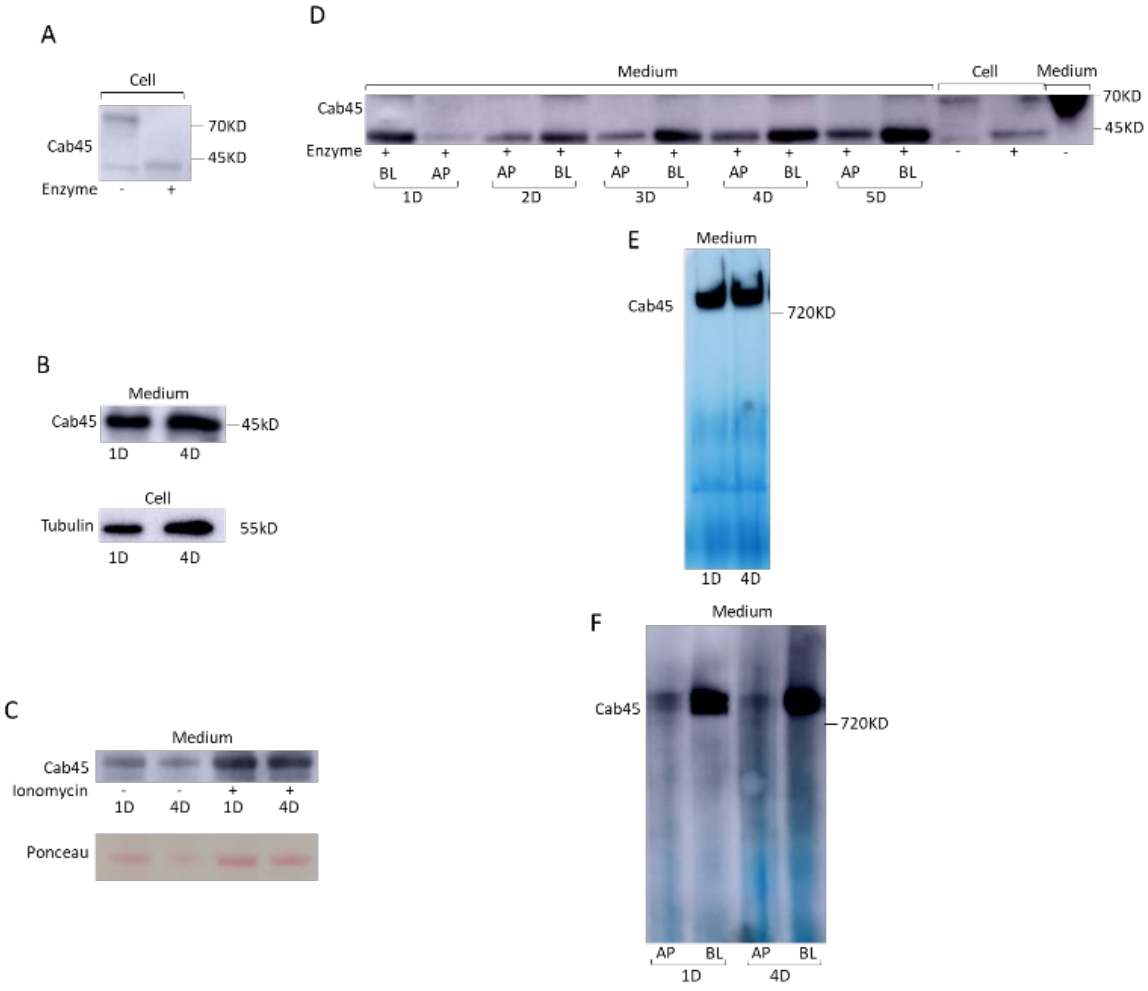

Figure III Cab45 clusters secreted from basolateral more than from apical domain of MDCK cells.

(A) MDCK cells was treated (+) or not (-) O and N mixture deglycosylation enzymes as showed in lane 2 and 1 before performing the western blot. The experiment was repeated 3 times. (B) MDCK cells were grown for 1 or 4 days and the secreted medium was collected, proteins were precipitated by vacuum and perform Western blot to immunoblot Cab45 antibody. Tubulin from protein lysates was used as normalizer. (C) The medium was collected with (+) or without (-) ionomycin treatment. After concentrate by vacuum and load the same volume of supernatant. The membrane stain with ponceau before incubating with the Cab45 antibody. (D) Time course of secreted Cab45 in apical vs basolateral during establishment of epithelial polarity. Seed the

MDCK GFP FR cells on the filters and collected the medium from apical and basolateral chamber each day from the first to the fifth days. The proteins were resuspended with the same volume of medium after precipitated by vacuum. Perform the SDS PAGE after treated all the samples by mixture deglycosylation enzymes and reveal the secretory Cab45 by immunoblotting the Cab45 antibody. The cell lysates and medium of basolateral were treated (+) or not (-) with deglycosylation enzymes were load into the SDS gel. AP: medium of apical chamber. BL: medium of basolateral chamber. PE: protein extraction of cells lysate. (E) MDCK GFP FR cells were seeded on the dish for 1 day and 4 days. The cell culture medium was collected and concentrated by vivaspin, then loaded the same volume to perform the Native PAGE. The secretory Cab45 were revealed by Cab45 antibody. (F) MDCK cells were grown on the filters and collected the medium from apical and basolateral for 1 or 4 days, proteins were precipitated by vivaspin until the same volume of medium and perform Native PAGE to immunoblot Cab45 antibody. AP: medium of apical chamber. BL: medium of basolateral chamber.

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