Distinct patterns of phosphatidylserine localization within the Rab11a-containing recycling system

Nicholas W Baetz1 and James R Goldenring1,2,3,4,*

1Section of Surgical Sciences and the Epithelial Biology Center; Vanderbilt University Medical Center; Nashville, TN USA; 2Department of Cell & Developmental Biology; Vanderbilt University School of Medicine; Nashville, TN USA; 3Vanderbilt-Ingram Cancer Center; Vanderbilt University Medical Center; Nashville, TN USA; 4Nashville Veterans Affairs Medical Center; Nashville, TN USA

Keywords: Rab11-FIP, Rab11a, Rab5, Rab7, Rab8a, live cell microscopy, phosphatidylserine, structured illumination

Introduction

Cell membranes form a permeable barrier to extracellular elements as well as a dynamic network of internal compartments whose function is determined by the combination of proteins and lipids that associate with these organelles. In mammalian cells, endosomes are commonly characterized by the presence of one or more Rab GTPases that regulate the passage of membranes and proteins throughout the cell.1,2 Furthermore, visualization of cargo passage through endosomal compartments has added insight to the organization of these intracellular organelles.3,4 Recent efforts have focused on the characterization of the lipids within endosomes to contextualize the growing network of proteins that regulate intracellular traffic.5

Membrane trafficking involves transport of proteins and lipids between intracellular organelles and the cell surface. This process is regulated by small Rab GTPases that switch between GTP "active" and GDP "inactive" states.6 Rab11 proteins organize recycling of membranes and proteins to and from a pericentriolar, recycling compartment.7 Furthermore the presence of effector proteins known as the Rab11-family interacting proteins (Rab11-FIPs) coordinate transitions through the Rab11 containing compartments of the recycling pathway.8-12 Rab11-FIP1B, Rab11-FIP1C, Rab11-FIP2, and Rab11-FIP5 all contain N-terminal C2-domains.13 The C2 domain was originally characterized as one of the four conserved regions of Protein Kinase C (PKC), which was responsible for PKC-lipid interactions.14-16 Previous work has demonstrated an affinity of Rab11-FIP C2 domains for phospholipids, most notably phosphatidic acid, PI(3,4,5)P3, and phosphatidylserine (PS).17 Additionally, induction of phosphatidic acid production leads to translocation of FIP1C, FIP2, and FIP5 to the periphery of A431 cells,17 and diacylglycero kinase activity is required for translocation of FIP1C to pseudopods of A2780 cells.18 All these data point to an inherent role for phospholipid regulation of Rab11 and Rab11-FIP-dependent membrane recycling.

Phospholipids make up approximately 70% of the lipid composition within cells with the majority being phosphatidylcholine...
and phosphatidylethanolamine whereas phosphatidylserine (PS) is as much as 10% of the lipid composition within cells.\textsuperscript{19,20} Commonly known to serve as a negatively charged interface with which positively charged amino acids interact, PS is found widely throughout cells in a variety of compartments and organelles.\textsuperscript{21} In recent years, multiple groups have developed fluorescently labeled phospholipid binding domains as markers for specific compartments and lipid domains.\textsuperscript{22} Of particular interest is a PS binding probe made from the C2 domain of lactadherin (LactC2) that serves as a marker for discrete intracellular compartments.\textsuperscript{23} Previous studies have shown that the distribution of this probe overlaps with transferrin receptor, as well as a group of Rab small GTPases including Rab7, Rab23, and Rab35.\textsuperscript{23}

Since Rab11-FIP proteins can potentially associate with not only phosphatidic acid, but also phosphatidylserine, we have utilized LactC2 to examine the distribution of PS with Rab11 and Rab11-FIP containing compartments. Overall, we have found that Rabs and Rab11-FIPs exhibit differential patterns of overlap with the expressed LactC2 as an indicator of PS distribution. The inhibition of trafficking through the Rab11-containing recycling system with expression of carboxyl terminal regions of Rab11-FIP1C or Rab11-FIP2 or the tail of Myosin Vb caused accumulation of the LactC2 probe. However, the presence of a C2 domain in Rab11-FIPs did not necessarily correlate with overlap between Rab11-FIPs and LactC2. The data suggest that domains within the recycling system defined by Rab11-FIPs show distinct patterns for phosphatidylserine distribution.

**Results**

Rab GTPases overlap with Lactadherin C2 (LactC2) domain in live HeLa cells

A combination of studies have suggested specificity in the phospholipid content of various endosomal compartments and other intracellular organelles.\textsuperscript{24} To elucidate differences in phosphatidylserine (PS) distribution within Rab compartments, we used live cell fluorescence microscopy to visualize the overlap between the PS probe, EGFP-Lactadherin C2 domain (EGFP-LactC2) and mCherry-chimeras of five Rab GTPases associated with endosomal compartments (Fig. 1). We found distinct separation between Rab5a and LactC2 in HeLa cells, especially in the periphery of cells. Of note we observed overlap between LactC2 and Rab7a in the pericentriolar region, as previously reported.\textsuperscript{23} However we also observed distinct separation of these two proteins in peripheral regions of the cell, which may be a result of cell-specific differences in lipid composition of endosomal compartments. Overlap was more readily visible between LactC2 and Rab8a or Rab10a in both the pericentriolar recycling compartment and vesicles in the periphery of cells, although overlap between Rab10a and LactC2 was inconsistent over multiple experiments. Rab11a also displayed consistent

---

**Figure 1.** EGFP-LactC2 (PS) and mCherry-Rab GTPases in live HeLa cells show distinct patterns of overlap. HeLa cells expressing EGFP-LactC2 and mCherry-Rab proteins were imaged with live cell deconvolution microscopy every 2 s for at least 1 min. Rab5a and Rab7a showed limited overlap with LactC2 in the periphery of cells while Rab8a and Rab11a display consistent overlap with mCherry-LactC2 throughout the cell. Rab10 overlap with LactC2 was present but inconsistent over multiple experiments. Data represent at least 3 independent experiments. Bars, 10 μm
overlap with LactC2, supporting previous results that LactC2 is an efficient marker of transferrin-containing compartments particularly in the pericentriolar recycling endosome.\(^3\) We observed coordinated movement of compartments labeled for Rab11a and LactC2 over time and evidence of Rab11a localization to distal tips of PS-containing tubes (Vid. SV1). The data demonstrate differential labeling of various Rab-containing endosomal compartments by LactC2 and suggest that PS is enriched in the Rab11a-containing recycling system.

**Rab11-FIP1 proteins consistently overlap with LactC2-labeled PS in live HeLa cells**

Based on the specific differences we observed in the overlap between the LactC2 PS probe and a select group of endosomal Rab GTPases, we next examined overlap between the PS probe and Rab11-FIPs using live cell deconvolution microscopy to visualize the overlap between Rab11-FIP1 proteins and LactC2 (Fig. 2). Rab11-FIP1A consistently overlapped with the LactC2 probe along endosomal tubules in live HeLa cells and both probes moved concurrently within cells, indicating occupation of the same compartment (Vid. SV2). Additionally, we found that Rab11-FIP1B and Rab11-FIP1C also overlapped with LactC2 in the pericentriolar region of HeLa cells and that LactC2 appeared to be accumulated in this area in the presence of overexpression of either of these two Rab11-FIP1 proteins. Of interest, Rab11-FIP1B and Rab11-FIP1C have C2 domains, while Rab11-FIP1A does not, suggesting that Rab11-FIP1A associates with PS-containing membranes through a mechanism other than a C2 domain binding to phospholipid. We further confirmed our findings with structured illumination microscopy (Fig. 3A) and quantitative analysis of the overlap between LactC2 and Rab11-FIP1 proteins revealed similar correlation coefficients (Rab11-FIP1A = 0.472 ± 0.029, Rab11-FIP1B = 0.491 ± 0.044, Rab11-FIP1C = 0.462 ± 0.045) that were not statistically different (\(p > 0.05\)) indicating Rab11-FIP1 proteins exhibit similar levels of association with LactC2 positive membranes (Fig. 3B). Of note, Rab11-FIP1A overlapped with LactC2 in pericentriolar and peripheral endosomes, while Rab11-FIP1B and Rab11-FIP1C overlapped primarily with LactC2 in the pericentriolar region only.

**Figure 2.** EGFP-Rab11-FIP1 proteins consistently overlap with mCherry-LactC2 (PS) in live HeLa cells. EGFP-Rab11-FIP1 proteins and mCherry-LactC2 overlapped in peripheral and pericentriolar compartments during imaging of live HeLa cells. FIP1B and FIP1C induced a partial accumulation of LactC2 in the pericentriolar compartments. Cells were imaged every 2 s for at least 1 minute. Data represent at least 3 independent experiments. Bars, 10 \(\mu\)m.

In addition to fluorescence microscopy, we isolated GFP-Rab11-FIPs from HEK293 cell lysates in the presence and absence of detergent and analyzed recovered mCherry-LactC2 by SDS-PAGE and western blot. We found that mCherry-LactC2 was not recovered with any GFP-Rab11-FIPs when detergent was included in the preparation. However, mCherry-LactC2 was recovered with Rab11-FIP1A, Rab11-FIP1C, and Rab11-FIP2 when the detergent was omitted (Fig. S1). The lower band present in the detergent-free preparation is likely a degraded form of mCherry-LactC2 that retains the ability to bind Rab11-FIP containing membranes. These data suggest that mCherry-LactC2 is specifically associated with Rab11-FIPs in the presence of membranes, but that these associations do not result from GFP-Rab11-FIPs binding directly to the LactC2 probe. The combined data demonstrate that Rab11-FIP1 proteins associate with LactC2 containing membranes and that...
these associations may be mediated by domains other than the N-terminal C2 domains.

**Distinct pattern of LactC2 association with compartments containing Rab11-FIP2, Rab11-FIP3, or Rab11-FIP5**

We next examined the overlap of mCherry-LactC2 co-expressed with other GFP-Rab11-FIPs (FIP2, FIP3, and FIP5) in HeLa cells (Fig. 4). We observed distinct separation between mCherry-LactC2 and either GFP-Rab11-FIP2 or GFP-Rab11-FIP5 in the periphery of cells. Similarly, we observed points of separation between mCherry-LactC2 and GFP-Rab11-FIP3. However, we did note considerable overlap between mCherry-LactC2 and GFP-Rab11-FIP3 in the pericentriolar area along elaborate branching tubules, suggesting that the GFP-Rab11-FIP3 and mCherry-LactC2 were present along coincident

**Figure 3.** The Rab11-FIP1 proteins are within 100–200nm of LactC2 by SIM. (A) HeLa cells transfected with EGFP-Rab11-FIP1 proteins and mCherry-LactC2 were imaged on coverslips using structured illumination microscopy. Each Rab11-FIP1 protein displayed overlap with LactC2. Images were collected over a 1 μm stack of individual HeLa cells. Bars, 10 μm. (B) Pearson’s correlation coefficients were analyzed for each condition. Rab11-FIP1A (0.472 ± 0.029, n = 14 cells), Rab11-FIP1B (0.491 ± 0.044, n = 9 cells), and Rab11-FIP1C (0.462 ± 0.045, n = 8 cells) had statistically similar overlap with LactC2 (p > 0.05) Results were analyzed using an unpaired, two-tailed, Student’s t test and presented as Mean ± SEM.
membranes. Additionally, we further imaged these conditions using structured illumination microscopy and found that the patterns of overlap and separation observed with deconvolution microscopy were consistent with that of structured illumination microscopy (Fig. 5A). Analysis of the correlation between LactC2 and the Rab11-FIPs studied in this experiment showed that Rab11-FIP2 (0.268 ± 0.033) and Rab11-FIP5 (0.308 ± 0.022), while having similar levels of overlap with LactC2 (p > 0.05), each had significantly lower correlation coefficients (p < 0.05) than either Rab11-FIP1 proteins or Rab11-FIP3 with LactC2 (0.435 ± 0.041) (Fig. 3B). Rab11-FIP3 overlap with LactC2 was not statistically different from the overlap between LactC2 and the Rab11-FIP1 proteins (p > 0.05) (Fig. 3B). These combined data indicate that additional Rab11-FIPs outside the Rab11-FIP1 group also occupy PS-containing membrane domains, but not with the same uniformity as the Rab11-FIP1 proteins. Furthermore these data confirm that the association of Rab11-FIPs with PS-containing membranes is not solely dependent upon the presence of the C2 domain found in Rab11-FIP2 and Rab11-FIP5, yet absent in Rab11-FIP3.

Phosphatidylserine is enriched within tubular compartments of Rab11-FIPs

We next examined the co-expression of multiple Rab11-FIPs in HeLa cells in the presence of LactC2 to discern whether PS is enriched along tubular endosomal compartments that contain multiple Rab11-FIPs. We used live cell deconvolution microscopy to image HeLa cells expressing Cerulean-Rab11-FIP3, Venus-Rab11-FIP1A, and mCherry-LactC2. Previous studies have shown that Rab11-FIP3 and Rab11-FIP1A form a series of subdomains along tubular endosomal compartments that move in concert throughout the pericentriolar region.25 Therefore, we used this same combination of proteins to determine whether LactC2, as an indicator of PS-containing membranes, is enriched along specific tubular recycling system compartments. We found that mCherry-LactC2 was visible along tubular endosomes co-labeled with both Rab11-FIP1A and Rab11-FIP3 (Fig. 6). The movement of these compartments together suggests that tubular endosomal compartments are triple labeled for Rab11-FIP3, Rab11-FIP1A, and PS (Vid. SV3). Neither Rab11-FIP1A nor Rab11-FIP3 has a C2 domain, yet they were associated with the membranes enriched in PS. Thus Rab11-FIP1A and Rab11-FIP3 both associate with PS-containing membranes through a mechanism independent of integral C2 domains.

Inhibition of trafficking through the recycling system induces an accumulation of PS

Previous studies have determined that N-terminal truncations of Rab11-FIP1C and Rab11-FIP2 that delete the C2-domains (∆C2-Rab11-FIP1C or ∆C2-Rab11-FIP2, respectively) inhibit transferrin trafficking through the Rab11a-containing recycling compartment.12 Similarly, expression of the motorless tail of Myosin Vb also potently inhibits recycling of transferrin.26 We therefore evaluated whether these inhibitory constructs could influence the localization of LactC2 as a reflection of PS. ∆C2-Rab11-FIP1C or ∆C2-Rab11-FIP2 were expressed with mCherry-LactC2 and we used deconvolution microscopy to visualize the
Both ΔC2-Rab11-FIP1C and ΔC2-Rab11-FIP2 induced an accumulation of LactC2 in the pericentriolar recycling compartment. We also examined these transfected cells using structured illumination microscopy and found that, while we detected overlap between ΔC2-Rab11-FIP1C and mCherry-LactC2, we noticed distinct separation of ΔC2-Rab11-FIP2 and mCherry-LactC2 even within the tight tubular network in the pericentriolar recycling compartment (Fig. 8). When we expressed the Myosin Vb tail, we also observed an accumulation of LactC2 (Figs. 7 and 8). Measurement of the Pearson’s correlation coefficient between LactC2 and the ΔC2-Rab11-FIPs or Myosin Vb tail indicated that ΔC2-Rab11-FIP2 (0.383 ± 0.052) had significantly lower correlation with LactC2 (p < 0.05) than ΔC2-Rab11-FIP1C (0.629 ± 0.086) and Myosin Vb tail (0.616 ± 0.051) (p > 0.05) (Fig. 8b). Together these results suggest that inhibition of trafficking through the recycling system leads to accumulation of PS in inhibited membrane cisternae.

**Discussion**

Our investigations have demonstrated that Rab GTPases and Rab11-FIPs associate with LactC2-labeled (PS-containing) membranes at select locations within HeLa cells as determined by...
live cell and structured illumination microscopy. Co-expression of Rab GTPases with LactC2 in HeLa cells highlighted distinct overlapping patterns between LactC2 and Rab8a, Rab10, and Rab11a, while Rab5a and Rab7a displayed distinct separation from the PS probe in the periphery of cells. Rab11-FIP1 proteins each appeared to overlap with LactC2 especially in the pericentriolar region of the cell despite the differences in the presence or absence of C2 domains. Similarly other members of the Rab11-FIPs displayed selective association with LactC2, but Rab11-FIPs with C2 domains did not necessarily overlap with PS-containing membranes consistently. We did find that LactC2 was enriched along tubular compartments containing multiple Rab11-FIPs that do not possess C2 domains, supporting our earlier findings and suggesting that C2 domains are not the sole mechanism for Rab11-FIPs to associate with PS-containing membranes. Finally we found that truncated Rab11-FIP chimeras induced an accumulation of PS containing membranes in the pericentriolar region, suggesting that perturbation of Rab11a dependent vesicle recycling influences the distribution and movement of PS-containing membranes within the recycling system. Overall, this study indicates that the plasma membrane recycling system contains multiple discrete domains, which are defined by Rab11-FIPs and selective associations with PS containing membranes.

The diverse associations between Rab11-FIPs and LactC2 positive membranes observed in the current study may result from multiple potential modes of interaction for these effector proteins with PS-containing membranes. Rab11-FIPs primarily associate with lipids and membranes via their interaction with Rab11 or an N-terminal phospholipid-binding C2 domain. Purified C2 domains of Rab11-FIPs bind a variety of acidic lipids and C2 domains in general bind a variety of phospholipids often in a calcium dependent manner. Thus, the overlap we observed among Rab11-FIPs and LactC2 may be a result of binding between Rab11-FIPs and multiple phospholipids on the same membrane as LactC2. The removal of the C2 domains from Rab11-FIPs induced an accumulation of LactC2 in the pericentriolar compartment, yet we observed a clear separation of LactC2 from ΔC2-Rab11-FIP2 in the pericentriolar cisternae. The lack of co-localization between LactC2 and either Rab11-FIP2 or Rab11-FIP5 in the cell periphery also suggested that Rab11-FIPs might associate with phospholipids other than PS, different subpopulations of PS, or perhaps even compete for the same pool of PS as LactC2. Heterodimerization among Rab11-FIPs and interactions with Myosin Vb to form protein complexes that regulate membrane trafficking might also explain the diverse patterns of localization described in the current study.

Furthermore, the assembly of Rab11-FIP1A and Rab11-FIP3 along LactC2 membrane domains indicated that additional binding motifs exist and that the formation of Rab11-FIP complexes may facilitate associations between Rab11-FIPs and phospholipids. Finally, interactions of Rab11-FIPs with other Rabss such as Rab4 or Rab14 and previously characterized interactions of Rab11-FIP3 (also known as Arphophilin-1) with Arf-GTPases may also promote associations with a variety of phosphoinositides. The current data confirm the previous reports of PS as a component and potential target lipid in the Rab11-containing recycling system and indicate a range of possibilities for these associations to occur.

The occupation of membranes by Rab11-FIPs and LactC2 highlights potential sorting domains that have important implications for membrane trafficking. Membranous compartments within cells are continuous yet distinct based on their composition and the development of reliable lipid probes has allowed for visualization of Rab11-FIPs in the context of a dynamic lipid environment. While lipids are commonly thought of as substrates for proteins to bind and associate with various compartments, the current technology permits visualization of the coordinated movement of Rab11-FIPs with PS-containing compartments. We observed coordinated movement among the Rab11-FIP1 proteins with LactC2 that was more apparent than we observed between LactC2 and Rab11-FIP2 or Rab11-FIP5, indicating differences in association with lipids and potential mechanisms for sorting cargoes based on the lipid content of...
membrane domains. The LactC2 probe permitted visualization of inhibited PS movement in the presence of truncated forms of the Rab11-FIPs. Similarly, the patchwork assembly of Rab11-FIP1A and Rab11-FIP3 along PS-containing domains coupled with the selective nature of Rab11-FIP associations with LactC2 membrane domains indicates that multiple pathways may be at work in the same recycling system. Previous reports have indicated that the formation of such complexes is required to construct defined domains. Furthermore, the assembly of these complexes promotes critical events such as fusion of vesicle membranes. Each of these findings argues in favor of dynamically pleomorphic endosomal compartments, as proteins and lipids are recycled through the cell. Endosomal compartments change in protein composition, lipid framework, as well as internal cargo and pH during trafficking. Here we observed both location and time-dependent associations of Rab11-FIPs with PS-containing membranes that are likely mediated by both direct and indirect interactions. The selective nature of Rab11-FIP associations with PS-containing compartments supports the concept that dynamic associations between Rab11-FIPs and phospholipid-containing domains regulate cargo sorting in the Rab11-dependent recycling system.

The current study has demonstrated selective associations among Rab11-FIPs with LactC2-labeled membranes that have important implications in Rab11-dependent vesicle recycling. The data presented here confirm previous findings that PS is likely an integral component of endosomal compartments occupied by Rab11 and Rab11-FIPs during vesicle recycling. The development of molecular probes that detect specific lipids including PS, PI(3)P, PI(3,5)P, and cholesterol have made it possible to visualize these dynamic trafficking processes in the context of a lipid framework. The presence of selective associations and discrete domains indicates potential sorting points within the Rab11-containing recycling system, as well as intersections among multiple pathways. Future investigations regarding the specific mechanisms that permit Rab11-FIP interactions with various phospholipids and the modes by which they regulate the cargoes that pass through related compartments will help determine the progression of these processes. All of these findings implicate a dynamic set of interactions among protein and phospholipid regulators to define the complexities of trafficking through the recycling system.

**Materials and Methods**

**Plasmids and expression vectors**

The preparation of the following plasmids has previously been described: mCherry-Rab5a, mCherry-Rab8a, EGFP-Rab11a, EGFP-Rab11-FIPs (FIP1A, FIP1B, FIP1C), EGFP-Rab11-FIP2 and EGFP-Rab11-FIP3, EGFP-Rab11-FIP2ΔC2, EGFP-MyosinVb-tail. EGFP-Rab11-FIP5 was a gift from R. Prekeris at the University of Colorado. Preparation of mCherry Rab7a was prepared by cloning Rab7a from EGFP-Rab7a, a gift from A. Wandinger-Ness at the University of New...
Isolation and Western Blotting of EGFP-Rab11-FIPs
Preparation of HEK cell lysates for western blot analysis was conducted as previously described. Briefly, HEK-293 cells co-transfected with EGFP empty vector or EGFP-Rab11-FIP chimeras (200 ng) and mCherry-LactC2 (200 ng) were scraped on ice into 1 mL of 30 mM Tris, 150 mM sodium acetate, and 20 mM magnesium acetate (Buffer A). Cells were sheared open by passing cells 50 times through a 27 gauge needle and lysates for detergent preparation had 1% Triton X-100 added at this time for 30 min at 4 °C. Resulting supernatant was added to GFP binding protein (GBP) on agarose beads equilibrated in Buffer A. Equal amounts of protein from each condition were added to beads and rotated at 4 °C overnight then beads were resuspended in 30 µL Laemmli buffer. All samples were boiled and resolved on 10% SDS-PAGE gel, transferred to nitrocellulose membrane (Whatman 10 401 196) and exposed to and development of Kodak film (864–6770). Membranes were probed a second time using a primary rabbit anti-EGFP (ab290 Abcam) antibody at 1:2500 in 0.1% TBS-Tween-20 and a Trueblot anti-rabbit IgG HRP (eBioscience 18–8816–33) rabbit secondary at 1:5000 in 5% milk in 0.1% TBS-Tween-20 and a Trueblot anti-rabbit IgG HRP (eBioscience 18–8816–33) rabbit secondary at 1:5000 in 0.1%TBS-Tween-20. Membranes were washed and developed as above.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Acknowledgments
This work was supported by NIH National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) Grant RO1 DK48370 to JRG and the NWB was supported by T32 CA106183. Live cell imaging studies were performed in the Vanderbilt Digital Histology Shared Resource and the Epithelial Biology Center. Confocal fluorescence imaging was performed through the use of the VUMC Cell Imaging Shared Resource supported by the Vanderbilt Digestive Disease Research Center and the Vanderbilt-Ingram Comprehensive Cancer Center and National Institute of Health (NIH) Grants CA68485, DK20593, DK58404 and HD15052. The authors would like to thank Dr. Rytis Prekeris at the University of Colorado for providing the EGFP-Rab11-FIP5/Rip11 construct and Dr. Wandinger-Ness from the University of New Mexico for the gift of the EGFP-Rab7a construct.

Supplemental materials may be found here:
www.landesbioscience.com/journals/cellularlogistics/article/28680

www.landesbioscience.com Cellular Logistics e28680-9
**A**

| Cherry-LactC2 (PS) | Merged |
|--------------------|--------|
| GFP-FIP1C-ΔC2      |        |
| GFP-FIP2-ΔC2       |        |
| GFP-MyosinVb-tail  |        |

**B**

LactC2 correlation with ΔC2-Rab11-FIPs and MVb-tail domain

![Graph showing Pearson's Correlation Coefficient](image)
Figure 8 (opposite). The mCherry-Lactc2 and Rab11-FIP-2 proteins exhibit selective overlap in the pericentriolar region. (A) EGFP-Rab11-FIP-2 constructs coexpressed with mCherry-Lactc2 on coverslips and imaged using structured illumination microscopy demonstrated distinct associations between Lactc2 and Rab11-FIP1c or Myosin Vb, but visible separation between Lactc2 and Rab11-FIP2. Bars, 10 μm. (B) Rab11-FIP2 (0.383 ± 0.052, n = 9 cells) and Lactc2 (p < 0.05) had significantly lower correlation than C2-Rab11-FIP1C (0.629 ± 0.086, n = 3 cells) or Myosin Vb tail (0.616 ± 0.051, n = 9 cells; p < 0.05). Results were analyzed using an unpaired, two-tailed, Student’s t-test and presented as Mean ± se M.
40. Lemmon MA. Membrane recognition by phospholipid-binding domains. Nat Rev Mol Cell Biol 2008; 9:99-111; PMID:18216767; http://dx.doi.org/10.1038/nrm2328

41. Araç D, Chen X, Khant HA, Ulbach J, Ludhke SJ, Kikkawa M, Johnson AE, Chiu W, Stidhof TC, Rizzo J. Close membrane-membrane proximity induced by Ca(2+)-dependent multivalent binding of synaptotagmin-1 to phospholipids. Nat Struct Mol Biol 2006; 13:209-17; PMID:16491093; http://dx.doi.org/10.1038/nsmb1056

42. Huotari J, Helenius A. Endosome maturation. EMBO J 2011; 30:3481-500; PMID:21878991; http://dx.doi.org/10.1038/emboj.2011.286

43. Gillooly DJ, Morrow IC, Lindsay M, Gould R, Bryant NJ, Gaulier JM, Parton RG, Stenmark H. Localization of phosphatidylinositol 3-phosphate in yeast and mammalian cells. EMBO J 2000; 19:4577-88; PMID:10970851; http://dx.doi.org/10.1093/emboj/19.17.4577

44. Li X, Wang X, Zhang X, Zhao M, Tsang WL, Zhang Y, Yau RG, Weissman LS, Xu H. Genetically encoded fluorescent probe to visualize intracellular phosphatidylinositol 3,5-bisphosphate localization and dynamics. Proc Natl Acad Sci U S A 2013; 110:21165-70; PMID:24324172; http://dx.doi.org/10.1073/pnas.1318641110

45. Maxfield FR, Wüstner D. Analysis of cholesterol trafficking with fluorescent probes. Methods Cell Biol 2012; 108:367-93; PMID:22325611; http://dx.doi.org/10.1016/B978-0-12-386487-1.00017-1

46. Ducharme NA, Ham AJ, Lapiere LA, Goldenring JR. Rab11-FIP2 influences multiple components of the endosomal system in polarized MDCK cells. Cell Host 2011; 1:57-68; PMID:21686255; http://dx.doi.org/10.1016/j.chom.2011.02.010

47. Roland JT, Kenworthy AK, Peransen J, Caplan S, Goldenring JR. Myosin Vb interacts with Rab8 on a tubular network containing EHD1 and EHD3, Mol Bad Cell 2007; 18:2828-37; PMID:17507647; http://dx.doi.org/10.1010/mmc.e07-02-0169

48. Jin M, Goldenring JR. The Rab11-FIP1/RCP gene codes for multiple protein transcripts related to the plasma membrane recycling system. Biochem Biophys Acta 2006; 1759:281-95; PMID:16920206; http://dx.doi.org/10.1016/j.bbapap.2006.06.001

49. Roland JT, Lapiere LA, Goldenring JR. Alternative splicing in class V myosins determines association with Rab10. J Biol Chem 2009; 284:1213-23; PMID:19088234; http://dx.doi.org/10.1074/jbc.M805957200

50. Rizzo MA, Springer GH, Granada B, Piston DW. An improved cyan fluorescent protein variant useful for FRET. Nat Biotechnol 2004; 22:445-9; PMID:14990965; http://dx.doi.org/10.1038/nbt945

51. Gustafsson MG. Surpassing the lateral resolution limit by a factor of two using structured illumination microscopy. J Microsc 2000; 198:82-7; PMID:10810003; http://dx.doi.org/10.1046/j.1365-2818.2000.00710.x

52. Manders EEM, Verbeek FJ, Aten JA. Measurement of co-localisation of objects in dual-colour confocal images. J Microsc 1993; 169:375-82; http://dx.doi.org/10.1111/j.1365-2818.1993.tb03313.x

53. Rothbauer U, Zolghadr K, Muyldermans S, Schepers A, Cardoso MC, Leonhardt H. A versatile nanotrap for biochemical and functional studies with fluorescent fusion proteins. Mol Cell Proteomics 2008; 7:282-9; PMID:17951627; http://dx.doi.org/10.1074/mcp.M700342-MCP200

54. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970; 227:680-5; PMID:5432063; http://dx.doi.org/10.1038/227680a0