IQGAP1 mediates the structure and dynamics of a novel multi-vesicular compartment

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ABSTRACT

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IQGAP1 is a master cytoskeletal regulatory protein that connects extracellular signaling to changes in cell polarity, motility, and adhesion with adjacent cells. IQGAP1 achieves these fundamental outcomes by acting as a scaffolding protein that coordinates a wide variety of signaling cascades in a highly spatially-dependent manner. This dissertation details the use of multiple imaging modalities to characterize localized, highly-dynamic IQGAP1-related processes in epithelial MCF-10A cells. This led to the discovery of a novel multi-vesicular compartment that is surrounded by an outer layer of IQGAP1-associated actin filaments. Further studies showed that this compartment participated in the internalization of cell-cell adhesion proteins via endocytic and recycling pathways. Live-cell imaging studies were conducted to correlate local cytoskeletal remodeling of this outer layer to various dynamic behaviors of the multi-vesicular core. These studies showed that IQGAP1 negatively correlated with actin polymerization during compartment formation and stabilization. During this time, high frequency actin assembly appeared to be constrained by a negative feedback mechanism. In contrast, IQGAP1 dissociation from the compartment’s surface was followed by a rapid, non-linear increase in actin polymerization that coincided with compartment disassembly and the release of multiple, high-motile intraluminal vesicles. Taken
together, these results suggest a potential role of IQGAP1 in regulating the trafficking of cell-cell adhesion proteins by promoting the stabilization of a novel multi-vesicular sorting compartment.
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Chapter 1

Background

This chapter provides background information on the roles cytoskeletal remodeling plays in regulating membrane dynamics and importance of IQGAP1 as a cytoskeletal regulator. This information is necessary for understanding the significance of a major component of this dissertation, the discovery and characterization of a novel IQGAP1-associated actin compartment and its role in mediating the structure, localization, and dynamics of an internal membranous core. This membranous core exhibits many interesting behaviors including a distinct intracellular organization, interactions with vesicular trafficking organelles, fusion, formation, and disassembly. Thus, it is an ideal platform to survey how IQGAP1-associated actin remodeling correlates to a wide variety of membrane dynamics.
1.1. Regulation of membrane dynamics via cytoskeletal remodeling

Cells use cytoskeletal remodeling in a variety of ways to influence membrane dynamics. For instance, cytoskeletal remodeling can positively regulate membrane interactions by providing the mechanical force necessary to overcome the electrostatic force repulsion between two lipid bilayers. This is the case in rat submandibular acinar cells, where actin microfilaments are recruited to secretory granules docked on the basal membrane to provide extrusion forces that increase secretion. On the other hand, cytoskeletal remodeling can be used to negatively regulate membrane interactions by blocking access to tethering and fusion sites. For example, during the resting phase of rat mast cells the actin cortex prevents exocytosis by forming a steric barrier between secretory granules and the basal membrane. Cytoskeletal remodeling can also lead to vesicular trafficking, either through the transport of vesicular cargo via motor proteins along cytoskeletal filaments or through the direct application of force via actin “comet tails”. Lastly, the actin cytoskeleton is important for the correct positioning and morphology of various membranous organelles, such as the Golgi complex in normal rat kidney cells.

Various cytoskeletal regulatory pathways are involved in the de novo assembly of actin on the surface of membranous organelles. Typically, this assembly is nucleated through a combination of WASp and Arp2/3. However, the upstream regulation of actin nucleation can vary. On isolated rat liver Golgi membranes, ARF-1 activation leads to downstream nucleation through the recruitment of mAbp1-bound Cdc42. In vacuoles purified from yeast, actin nucleation is regulated by Cdc42p. Another common regulator
of actin nucleation is PI(4,5)P2, a membrane marker that is important for identifying sites for vesicle targeting, docking in fusion.\(^9\) PI(4,5)P2 mediated nucleation is important for phagocytosis and the assembly of actin comet tails during vesicular transport to the apical membrane.\(^{10,11}\) Finally, activation of Protein Kinase C (PKC) in xenopus oocytes results in the assembly of actin filaments around endosomes, lysosomes, and secretory vesicles.\(^{12}\)

### 1.2. IQGAP1-mediated cytoskeletal regulation

IQGAP1 is a ubiquitous, master regulatory protein that links cytoskeletal remodeling to external cell signaling. It is found in a variety of eukaryotes such as humans, yeast and hydra.\(^{13,14}\) IQGAP1’s simultaneous involvement as a scaffolding protein in many diverse cytoskeletal regulatory pathways can lead to whole-cell phenotypic changes such as a change in motility, cell-cell adhesion, or polarization.\(^{15}\) A better understanding of IQGAP1 could lead to better therapeutic treatments for invasive cancers that overexpress IQGAP1.\(^{16,17}\)

IQGAP1 is perhaps best known for its role in the formation, maintenance and disassembly of adherens junctions.\(^{18-20}\) IQGAP1 bound to active (GTP-bound) forms of Rac1 and Cdc42 promotes strong adhesion through the assembly of an actin meshwork that prevents endocytosis of E-cadherin homodimers from cell-cell junctions.\(^{21,22}\) However, in response to TPA or HGF, IQGAP1 loses its junction stabilizing capabilities and forms associations with β-catenin.\(^{23}\) This regulatory behavior weakens and
ultimately dissassembles cell-cell contacts by breaking the bond between E-cadherin-mediated cell-cell contacts and their actin support structure.

Imaging studies have shown IQGAP1 is also important for developing or maintaining cell polarity. IQGAP anchors microtubules selectively to the basolateral membrane of Madin-Darby Canine Kidney Epithelial Cells (MDCK) in 3-D cultures.\textsuperscript{24} This function of IQGAP1 appears to be critical for mitotic spindle orientation, the controlled division of cells along a plane containing the apico-basal axis, and the resultant organization of an epithelial lumen. On the other hand, IQGAP1 also promotes polarity in non-epithelial cells through its recruitment of actin and microtubule plus ends to the leading edge of cells via Dia1 and CLIP-170 respectively.\textsuperscript{25,26} This behavior is activated through the binding of IQGAP1 to the Cdc42 and Rac1, and expression of constitutively active IQGAP1 forms multiple leading edges.\textsuperscript{27}

1.3. IQGAP1’s role in regulating the dynamics of membranous organelles

Many of IQGAP1s binding partners suggest an important role in the de novo nucleation of actin filaments at the surface membranous organelles. For instance, IQGAP1 directly interacts with neural-WASP to stimulate Arp2/3-dependent branched actin polymerization in vitro.\textsuperscript{28,29} Additionally, IQGAP1 stabilizes GTP-bound Cdc42, a known stimulator of N-Wasp and a likely means in which IQGAP1 enhances Arp2/3-mediated actin nucleation.\textsuperscript{30}
Unfortunately, not much is known about IQGAP1’s role in regulating membrane structure and dynamics. In one study, it was shown that IQGAP1 binds to Cdc42 and actin at the Golgi in CHO cells. In another study, it was revealed that binding of the N-terminus of IQGAP1 to the Exo70-SEPT2 complex promotes secretion in pancreatic beta cells. This IQGAP1-mediated secretion is down-regulated by activated Cdc42, providing a mechanism in which IQGAP1 acts as a switch between resting and secretory states. These limited studies and IQGAP1’s role as a master regulatory protein suggest that IQGAP1-mediated cytoskeletal regulation plays other roles in steering the dynamics of membranous organelles.

1.4. The need for comprehensive imaging studies of IQGAP1

IQGAP1 cytoskeletal regulation in response to extracellular signaling is highly spatially dependent. This is largely a result of its bidirectional role as an effector or regulator of small GTPases Cdc42 and Rac1 in response to extracellular signaling. In addition to this spatial dependence, IQGAP1’s role as a scaffolding protein in specific pathways may only occur in a small percentage of the total population of cells at a given time. These characteristics mean that techniques without sufficient spatial or temporal resolution, such as bulk proteomic studies or static imaging modalities, are limited in their ability to resolve individual regulatory behaviors of IQGAP1.

Our goal is to study IQGAP1 by primarily using a combination of live super resolution and live-cell imaging modalities. This combination of imaging gives the best combination of spatial information, such as nanoscale correlations between IQGAP1 and
filamentous actin structures, and temporal information, such as the evolution of IQGAP1 expression during dynamic cytoskeletal regulatory behaviors. Of particular interest is the study of IQGAP-associated actin remodeling at the surface of membranous organelles. Given that this regulatory process is highly dynamic and makes up only a small fraction of IQGAP1’s total activities in a given cell, it cannot be fully understood without sufficient spatial and temporal information.
Chapter 2

Multi-round STORM imaging with Dynamic DNA probes

This chapter details the development of a novel multi-round STORM microscopy approach that facilities multicolor super-resolution imaging with minimal crosstalk between markers. Multi-round STORM imaging is made possible through the use of dynamic DNA probes that mediate the sequential labeling and erasing of fluorescent tags on immunostained proteins. Previous dynamic DNA-mediated probe systems sacrificed labeling reaction speed for an increase in erasing efficiency, so a simplified probe system without this design constraint was developed in order to facilitate faster multi-round imaging experiments. This new probe system was used in conjunction with multi-round STORM microscopy to image nanoscale interactions between IQGAP1, a master cytoskeletal regulatory protein, and various filamentous actin structures at epithelial cell-cell junctions. These images revealed that IQGAP1 specifically correlates with a

1 The following individuals made contributions to this chapter: Duose, D. Y., Schweller, R. M., Zimak, J., Schweikart, V., and Diehl M. R.
subset of actin structures within ~1 μm of cell-cell contacts, and that the strength of this correlation increased as cells became more densely packed in a 2-D epithelial monolayer.

2.1. Introduction

Using standard fluorescent microscopes, non-overlapping point spread functions (PSFs) from individual fluorophores can be localized at super-resolution precision.\(^{37}\) This is accomplished by fitting each individual PSF to a 2-D Gaussian distribution whose peak is the predicted x, y localization of the fluorophore. Unfortunately, most biological samples are non-sparse, so individual PSFs from fluorescently-tagged biomarkers cannot be resolved from one another. This has led to the development of super-resolution imaging modalities that create sparse images from non-sparse marker distributions. Two sparsity-based super-resolution techniques are Stochastic Optical Reconstruction Microscopy (STORM) and Photoactivated Localization Microscopy (PALM).\(^{38,39}\) These two techniques are distinguished by the types of fluorophores they image, with STORM using photo-switchable cyanine dyes while PALM utilizes photo-activatable fluorescent proteins. Both STORM and PALM create sparsity though buffer conditions that cause most of the fluorophores in the field of view to be in a non-fluorescent dark state. Specific illumination conditions cause a small subset of those fluorophores to stochastically transition into the fluorescent light state. This randomly-changing sparse subset of the total distribution of fluorophores can be sequentially imaged until enough of the total dataset has been sampled to reconstruct a final, non-sparse image. STORM has been used to study nanoscale interactions between actin and proteins such as E-cadherin, spectrin, and septin.\(^{40-42}\) This chapter focuses on the use STORM microscopy to image nanoscale
associations between the actin and IQGAP1, a ubiquitous cytoskeletal regulatory protein (See Chapter 1 for more information on IQGAP1-mediated cytoskeletal regulation).

STORM has the capability to resolve associations between proteins with a resolution of ~25 nm. This resolution limit is largely dependent on the number of photons each dye emits while it is briefly in the fluorescent light state.\textsuperscript{43} Unfortunately, most of the dyes with the highest quantum yield per blinking event, such as Cy5 and Alexa 647, are in the red (647 nm) color channel.\textsuperscript{44} To get around this, STORM achieves multicolor imaging by pairing reporter dyes, the dyes that are actually imaged, with various activation dyes that increase rate on rate of reporter dyes in a proximity dependent fashion in response to being illuminated with a specific activation wavelength.\textsuperscript{45} This enables multicolor STORM to identify the “color” of specific STORM dimers, or pairs of activator and reporter dyes, by the color of wavelength used to activate the reporting dye. Unfortunately, non-specific activation of STORM dimers leads to cross-talk between different color channels that range from 10 to 20 percent in biological imaging applications.\textsuperscript{46} Using two different reporter dyes reduces the crosstalk to 5-10 percent, but has the added downsides of introducing chromatic aberrations and using lower quality, non-red reporting dyes. Cross-talk between color channels prohibits the measurement of high fidelity correlations between two different species of biomarkers. While image processing can remove some of the cross talk, computational cross-talk subtraction works best for applications in which the different markers do not significantly overlap with one another (i.e. microtubules and mitochondria), and it is not suitable for applications in which markers have a high degree of overlap (i.e. IQGAP1 and actin). Exacerbating this problem is the fact that different biomarkers can have
drastically different densities. For instance, a small fraction of misregistered actin can end up being a substantial percentage of the total signal measured in the relatively-sparse IQGAP1 channel.

To work around this issue, we have developed a novel variant of multicolor STORM that uses dynamic DNA probes to enable erasable immunofluorescence staining. Dynamic DNA probe systems typically consist of three major components: (1) A single-stranded target sequence that is conjugated to the biomarker of interest through the use of oligo-conjugated antibodies. (2) A probe complex that seeks out and labels a specific biomarker-conjugated target sequence though isothermal strand displacement. (3) An erasing complex that reverses the labeling reaction between the target sequence and the probe complex, producing an unlabeled target strand and a fully-hybridized fluorescent waste complex. These probes have previously been used to discretely amplify fluorescent labeling of proteins and to increase the multiplexing capabilities of standard immunofluorescence techniques. Using these probes, we have created a novel, multi-round STORM imaging approach in which different markers are imaged in sequential rounds to achieve “multi-color”, super-resolution imaging with drastically reduced inter-marker crosstalk. In this chapter, we will describe the development of a new, fast and efficient dynamic DNA probe system and its use in conjunction with STORM to image nanoscale associations between actin and IQGAP1 with minimal crosstalk. Additionally, we will present a brief study in which multi-round STORM is used to characterize spatial correlations between IQGAP1 and actin at adherens junctions.
2.2. Results

2.2.1. Dynamic DNA probe optimization

Past attempts at Dynamic DNA probe optimization sacrificed reaction kinetics for erasing efficiency.\(^5^0\) This trade-off emerged from the fact that probe systems with fast labeling kinetics between the probe complex and target strand also tended to have problematic reverse reactions during the erasing step in which dye-conjugated waste complexes would label free target strands. For example, a system containing a two-stranded probe complex, \(\text{PC}_{2s}\), could sufficiently label target sequences after just 30 minutes, but subsequent attempts to reverse said labeling resulted in a retention of \(\sim 33\%\) of the original signal level after 60 minutes. To prevent reverse reactions from occurring, an alternative system, \(\text{PC}_{3s}\), increased the unfavorability of potential reverse reactions at the cost of reducing the entropic gain of the labeling reactions from 6 bp to 2 bp. This change lowered the signal retention rate of the erase reaction to 5-10\% after < 30 minutes; however, it also increased the duration of the labeling reaction from 30 minutes to 60 minutes.

We have developed a modified probe system that has a 6 BP entropic gain in the labeling reaction and an erasing reaction that reduces the signal to 3-4\% of its original value after 30 minutes (See Figure 2-1Error! Reference source not found.). This was done through a simplified probe scheme in which the dyes on the probe complex and waste complex were no longer quenched by nearby IOWA Black molecules. The presence of a quencher in the previously designed probe complexes was found to be unnecessary, since washing the sample after labeling or erasing sufficiently removed any residual
fluorescent DNA strands/complexes that weren’t hybridized to fixed target sequences. Removing the design constraint of having a quencher in close proximity to dyes on the probe and waste complexes enabled the following probe system design (See Figure 2-1 A):

During the labeling step, a “toehold domain” on a probe complex (PC) initiates a strand-displacement reaction with an antibody-conjugated ssDNA target strand (TS). In addition to producing a fluorescently active reporting complex (IR), this reaction displaces a ssDNA from the target-binding domain of PC (W1). Prior to displacement, W1 had previously functioned as a means to reduce non-specific binding of the targeting region of PC. During the erasing step, a toehold region on IR reacts with a simple ssDNA erasing strand (E). This disassembles IR into a fully duplexed waste complex (W2) and a newly liberated TS.

The benefits of this system are two-fold. First, removing the quencher from the erasing strand (E) drastically reduces its cost. As a result, the erasing reaction can be stoichiometrically driven by increasing the concentration of E from 1 μM to 10 μM. Second, by removing the design constraint of having a quencher in PC, we were able to design a new PC in which each long strand (the complementary strand to TS) has a specific target-binding/toehold region of 30 bp followed by a generic 18 bp sequence that is complementary to a universal 18 bp dye-conjugated strand. Therefore, instead of ordering an expensive dye-conjugated long strand for every unique PC, a single dye-conjugated sequence can be ordered for each color channel and hybridized to any PC prior to labeling.
Figure 2-1: Characterization of an efficient Dynamic-DNA probe scheme. (A) A schematic of the quencher-free probe system. First, a probe complex (PC) labels a complementary target sequence (TS) via toehold-mediated DNA strand-displacement. This reaction outputs a fluorescently tagged biomarker (IR) and a ssDNA waste strand (W1). Fluorescent labels can be “erased from TS through the addition of a ssDNA erasing strand (E) that results in a fully duplexed waste complex (W2) and a newly liberated TS. Each TS is conjugated to a 2° antibody (Ab) which targets a primary antibody (not pictured) conjugated to a marker of interest (M). (B) Demonstration of DNA-mediated labeling and erasing of IQGAP1 and vimentin. Signal masks show the pixel values used to compute the mean intensity ratio of the labeled and erased images. The percentage of signal erased was 97% for IQGAP1 and 96% for vimentin. Scale bars = 10 μm.
2.2.2. Imaging nanoscale correlations between IQGAP1 and actin with negligible cross-talk with multi-round STORM

Multi-color STORM imaging of IQGAP1 and actin with minimal cross-talk was performed on fixed epithelial MCF10A cells through the use of DNA-mediated multi-round imaging (See Figure 2-2). Prior to staining for actin, dynamic DNA probes were used to stain IQGAP1 so that it could be imaged via direct STORM. Since no other markers have been stained, the only possible source of misregistered IQGAP1 is non-specific binding of the probe complex, which was found to be indistinguishable from autofluorescence during control experiments. After an erasing step of 30 minutes, STORM imaging confirmed that the number of STORM localizations was reduced to 0.844 ± 0.175% of the initial value at the start of round 1 (n = 3 separate multi-round imaging experiments). The fact that this value is lower than the 3-4% percent signal retention seen in epifluorescence erasing studies is likely a result of additional signal loss from photobleaching throughout STORM image acquisition of IQGAP1. After erasing, actin was labeled and imaged using phalloidin-Alexa 647. After a second round of direct STORM, high-fidelity 2-d cross correlation analysis could be used to examine associations between IQGAP1 and actin. The calculated local 2-d cross correlation, $\gamma$, gives a measure of the degree of similarity between nanoscale IQGAP1 and actin structures (See Equation 2-2). During STORM image rendering, the local value for $\gamma$ was calculated and mapped onto the color scale of the actin STORM image (See Figure 2-3). With this method of visualizing cross correlations at the local level we are able to compare IQGAP1 and actin correlations between distinct actin structures.
Figure 2-2 Multi-round storm imaging sequence. (A) Round 1: Direct STORM imaging is performed on MCF-10A cells stained for IQGAP1 using dynamic DNA probe complexes. (B) STORM imaging reveals that the majority of the fluorescent signal from round 1 was removed after the addition of ssDNA erasing strand. (C) Round 2: After the erasing step, the sample is stained for actin using phalloidin-Alexa 647. Subsequently, the same field of view as round 1 is imaged via direct STORM. (D) "Multicolor" STORM images are reconstructed by aligning the images from both rounds on top of one another using gold fiducial markers. Scale bars = 10 μm. In single marker images, color corresponds to the axial position of individual localizations.
Figure 2-3 Nanoscale correlations between IQGAP1 and actin. IQGAP1 (A) and actin (B) in epithelial MCF-10A cells were sequentially imaged using multi-round STORM. The color scale represents the axial position of single-molecule localizations. (C) A merged image showing IQGAP1-associates with distinct basal structures made of bundled actin filaments. (D) The normalized cross correlation, $\gamma$, between IQGAP1 and actin value was be mapped onto the actin image. The color of each pixel corresponds to the local cross correlation value between all IQGAP1 and actin localizations within 580 nm. Scale bar = 10 $\mu$m for whole cell images and 2 $\mu$m for zoomed in images.
2.2.3. IQGAP1 associates with specialized actin structures at cell-cell junctions

IQGAP1 localization patterns in fixed epithelial MCF-10A cells were evaluated using a combination of conventional, multi-color epifluorescence and 3-dimensional, super-resolution imaging procedures. Upon examination of high-throughput epifluorescence images, we observed that IQGAP1 co-localizes to lateral junctions that stain for actin and the epithelial markers E-cadherin and β-catenin (See Figure 2-4). Additionally, IQGAP1 is found at lower expression levels at junctions of a small subpopulation of cells expressing the mesenchymal marker N-cadherin. Overall, these observations are consistent with prior reports that IQGAP1 functions as a cytoskeletal regulator within E-cadherin-mediated junctions.

Multi-round STORM imaging was performed to gain insight into relationships between the ultrastructural organization of both IQGAP1 and actin cytoskeletal networks at the junctions of MCF-10A cells. We selected cells for STORM analyses that were organized into different structures ranging from small groups of spread-out cells possessing irregular morphologies, to much more compact, cobblestone-like epithelial cell communities. IQGAP1 and actin are found to be highly-concentrated within 1 μm of cell-cell junctions in nearly all cases (See Figure 2-5). Nevertheless, IQGAP1 localization behavior is dependent on the state of the multicellular community. While IQGAP1 associates with distinct filopodia-like structures
at the junctions of cells within small clusters or at the peripheries of larger clusters (See Figure 2-5 A & B), it tends to populate a single, often vertically-oriented sheet in cells that are incorporated into larger and presumably more polarized epithelial cell communities (See Figure 2-5 C & D). Additional actin filament structures are also found to run in parallel and in close proximity to cell-cell junctions in many of these cases. However, these structures are not decorated with appreciable amounts of IQGAP1 over background, indicating that IQGAP1 associates specifically with specialized actin structures at epithelial cell junctions while exhibiting little affinity for other nearby actin structures (See Figure 2-6). This selectivity is also clearly apparent in line intensity plots constructed by generating histograms of single-molecule localizations for both markers. Plots of IQGAP1 intensity contain a single peak centered at the point of cell-cell contact (See Figure 2-6 C). This peak displays significant overlap with a distinct band of actin in the same region. However, additional actin peaks that do not overlay with the IQGAP1 intensity plots are also found in the proximity of the junction.

The selectivity of IQGAP1 colocalization with junctional actin structures is also reflected in spatial maps of IQGAP1 / actin cross-correlations that depict the degree to which localizations of single IQGAP1 and actin probes overlap spatially in a cell (See Figure 2-6 Error! Reference source not found.D). These images display strong positive correlations ($\gamma$ up to 0.55) at cell-cell junctions, but much weaker correlations on other bundled actin filament structures (typically $\gamma < 0.1$, red arrow in Figure 2-6 D). Correlation values also vary appreciably along individual cell-cell junctions ($\gamma \approx 0.2-0.55$, yellow vs. green arrows in Figure 2-6 Error! Reference source not found.D), an effect
we attribute to the presence of distinct actin structures that branch out from the junctions at specific locations. Consistent with this hypothesis, branching frequency appears to be lower in junctions that exhibit high normalized cross correlation values (yellow vs. green arrows in Figure 2-6 D). Finally, IQGAP1/actin correlation values tend to be higher and more uniform along junctions of cells that exhibit properties that are indicative of a more established epithelial cell polarity, signified by more compacted actin at the cell periphery, straight and vertical junctions, and cobblestone-like multi-cell architectures (See Figure 2-6 C & D).
Figure 2-4: Epifluorescence image of a developing epithelial cell-cell junctions. Multi-color epifluorescence images of IQGAP1, F-actin, and adherens junction proteins (E-cadherin, β-catenin, N-cadherin) in epithelial MCF-10A cells. The intensity of IQGAP1 in cell junctions correlates with those of common epithelial markers, and with those of junction-associated actin and microtubule structures. By contrast, IQGAP1 is rarely observed at junctions exhibiting high expression of the mesenchymal marker N-cadherin. Scale bar = 40 μm.
Figure 2-5: IQGAP1/actin associations at adherens junctions vary with cell morphology. Multiplex STORM super-resolution images of epithelial MCF-10A cells. Each row contains stains images of IQGAP1, actin, and a normalized actin/IQGAP1 cross-correlation map. (A) In cells that contact each other only loosely, IQGAP1 is seen to colocalize with actin in filipodia-like structures in regions of cell-cell contact. (B) In cells that have established junctions but still exhibit spread-out, irregular morphologies, IQGAP1 is already present in the junctions while actin is distributed more broadly throughout the cell. (C, D) In relatively mature epithelial cell communities with cobblestone architectures, both actin and IQGAP1 are strongly localized to junction regions, and exhibit higher cross-correlation values than in less developed communities.
Figure 2-6: IQGAP1 associates with specialized actin structures at adherens junctions. STORM images of an epithelial MCF-10A show the ultrastructure of IQGAP1 (A) and actin (B). The color scale represents the axial position of single-molecule localizations. (C) Zoom-ins of IQGAP1 (1-3) and actin (1'-3') in the junction regions marked in (A, B), and corresponding localization histograms, demonstrating selective association between IQGAP1 and actin at cell-cell junctions. (D) A Map of cross-correlations reveals the distinct association behaviors between IQGAP1 and specific actin structures. Color map: Strong positive correlations (γ≈0.2-0.55) are seen in cell-cell junctions (green, yellow arrows) and in punctate cytosolic compartments (blue arrow), but not in filamentous actin structures (red arrow, γ<0.1) that run in close proximity to the junctions. Scale bars = 10 μm.
2.3. Discussion

In this chapter we have demonstrated a cost-effective system for DNA-mediated labeling of fixed cell samples with fluorescent dyes. Using this new system, target strand-conjugated biomarkers can be stained in 60 minutes (faster labeling times were not explored). Subsequent erasing reactions are capable of removing 96-97% of the existing fluorescent signal ~30 minutes. While these reaction times already make 3-4 round imaging in a single day practical, it is possible that after further characterization of the reaction kinetics the turnover time between imaging rounds could be further reduced.

This optimized DNA-mediated labeling scheme was applied to STORM imaging for the purpose of developing a method of multi-color STORM with reduced cross-talk between markers. The total cross-talk between markers was reduced to 0% for the first imaging round and < 1% for each subsequent round. This represents a significant decrease from previous multi-color STORM imaging modalities that achieved 5-10% crosstalk between different reporting colors and 10-20% crosstalk between different activation colors. With these improvements, direct STORM images with high-fidelity marker discrimination can be achieved, leading to a more exact means of characterizing nanoscale correlations between different species of proteins. Additionally, since multi-round imaging increases the multiplexing capacity of STORM in a temporal manner, it is compatible with any multi-color technique that spectrally separates each distinct biomarker. If traditional multi-color STORM methods were used to image 3 colors per
round in an imaging procedure similar to the one applied to traditional immunofluorescence imaging by Schweller et al., it is possible to achieve 6-12 marker super-resolution imaging after just 2-4 imaging rounds.\textsuperscript{49}

As a brief study, multi-round STORM of epithelial MCF-10A cells was used to image accurate correlations between IQGAP1 and actin at epithelial junctions. Super resolution images revealed that while IQGAP1 is correlated with actin within 1\,\mu m of E-cadherin mediated junctions, it does not correlate with the various bundled and branched actin structures that run parallel to areas of cell-cell contact. IQGAP1/actin cross correlation analysis revealed that IQGAP1’s association with actin in the vicinity of adherens junctions was heterogeneous. Brick-like cells closer to the center of densely-packed, multi-cellular clusters appeared to have a uniformly high correlation between IQGAP1 and actin along cell-cell junctions; however, the junctions of cells at the periphery of these clusters contained a mixture of high and low regions of IQGAP1/actin correlation. This suggests that IQGAP1 association at the junction may be positively correlated with junction maturation in epithelial tissues.

\textbf{2.4. Materials and methods}

\textbf{2.4.1. DNA probe design}

Probe sequences were designed using methods similar to those described by Duose et al.\textsuperscript{47} Every sequence involved the DNA-mediated labeling and erasing reactions was selected using a custom MATLAB script that generates random domains of specified
lengths with a pre-determined GC% range. This sequence generation process excludes previously generated domains, prohibitive structures (i.e. G quadruplexes), and domains the form secondary structures. The generated domains are ranked according to their normalized two-state hybridization energies with existing probe strands using mFold. The domains are then screened through the BLAST database to minimize probe sequence homology with the mRNA transcriptome. The final domain sequences are then selected manually from this list and concatenated with other domains to create full oligonucleotide sequences that will be incorporated into a probe complex. Other global criteria such as temperature, strand concentration, and salt concentration are specified prior to domain design. A table listing the oligonucleotide sequences can be found in Appendix B.

2.4.2. MCF-10A cell culture and fixation

MCF-10A human mammary gland cells (ATCC) were cultured in unfiltered MEGM without GA-1000 (Lonza) supplemented with penicillin, streptomycin, and ampicillin, and 100 ng/ml cholera toxin.

MCF 10A cells were trypsinized and then seeded onto fibronectin or matrigel coated coverslips at partial confluency and incubated for a period of 24-72 hours prior to fixation in 4 % paraformaldehyde in MEGM without GA-1000 and bovine pituitary extract (Lonza). After fixation, cells were washed PBS and then quenched in NaBH4 for 7 minutes. Finally, cells were washed with PBS again and subsequently permeabilized with 0.5 % Triton-X 100 for 10 minutes.
2.4.3. Antibody staining

Before primary antibody staining, fixed cells were first incubated in blocking buffer (BB) at least 15 minutes. Then, cells were incubated with primary antibodies in 10% blocking buffer diluted into PBS. Mouse primaries for β-catenin (Santa Cruz), E-cadherin (Santa Cruz), and N-Cadherin (BD Biosciences) at room temperature at a concentration of 5 µg/ml in blocking buffer. Rabbit anti-IQGAP1 antibodies were incubated in blocking buffer for 1-2 hours at 37°C at a concentration of 20 µg/ml. After washing 3 times with PBS, cells with incubated with secondary antibodies in blocking buffer for 1 hour at 37°C. IQGAP1 primaries were stained using goat anti-rabbit Cy3 (Life Technologies). Other primary antibodies were staining using goat anti-mouse Cy5 (Life Technologies). Cells were then washed 3 times with PBS prior to imaging.

2.4.4. DNA-mediated labeling and erasing procedures

Prior to labeling, fixed MCF-10A cells were stained with rabbit IQGAP1 or X vinculin primary antibodies using the previously described antibody staining protocol. Antibodies were conjugated to target strands (TS) by incubating cells with 10 µg/ml of goat anti rabbit secondary antibodies previously conjugated to TS using the Antibody-Oligonucleotide All-In-One Conjugation Kit (Solulink) for 1 hour at 37°C in BB + 1% Triton-X 100. After being washed three more times in PBS, the cells were labeled with 100 nmol/L of PC for 1 hour at 37°C in BB. Cells were washed 3x in PBS prior to imaging. After imaging, erasing was carried out by incubating the cell sample with 10 µmol/L of erasing strand in BB for 30 minutes at 22°C.
2.4.5. Epifluorescence microscopy

Images were acquired using a Nikon Eclipse TiE epi-fluorescent microscope using 60x NA 1.4 oil, 40x NA 0.95 air and 20x NA 0.5 air objectives and a 14-bit depth EMCCD (Luca, Andor), and processed using ImageJ.

2.4.6. Multi-round STORM imaging

DNA-mediated labeling and erasing of IQGAP1 with Alexa-647 was accomplished using the previously described DNA-mediated labeling and erasing procedures. Subsequent to the labeling, erasing, and staining of IQGAP1, actin was stained with phalloidin-Alexa 647 (Life Technologies) for 1 hours at 22°C, washed 3x in PBS, and then imaged using direct STORM.

All STORM imaging experiments were carried out in STORM image buffer, which was prepared fresh by combining 620 µl of 50 mM Tris-HCl (pH 8.0), 10 mM NaCl, 10 % glucose with 70 µl of 1M MEA dissolved in .25N HCl and 7 µl of 10 mM Tris-HCl (pH 8.0), 50 mM NaCl, 56 mg/ml glucose oxidase (Sigma Aldrich, 100 U/mg), 13.6 mg/ml catalase (Sigma Aldrich, 4966 U/mg). 10,000 to 30,000 frame STORM movies of IQGAP1 and actin were collected at a frame rate of 32 Hz. Multi-round STORM imaging experiments were conducted on commercially available coverslips with embedded gold nanoparticles (Hestzig). The PSF from the stable emissions of these nanoparticles were used as fiducial markers for the alignment of STORM reconstructions from each round using custom MATLAB software (Mathworks). See appendix A for a detailed description of the STORM microscope.
2.4.7. Quantification of erasing percentage

For epifluorescence quantification, the following formula was used to calculate the percentage of the fluorescence signal that remained after erasing reactions were completed, $\left( \frac{i_{OFF}}{i_{ON}} \right)$.

\[
\frac{\bar{i}_{OFF}}{\bar{i}_{ON}} = \frac{1}{n} \sum_{xy} \left[ i_{OFF}(x,y) - \bar{B}_{OFF} \right] 
\]

\[
= \frac{1}{n} \sum_{xy} \left[ i_{ON}(x,y) - \bar{B}_{ON} \right] 
\]

Equation 2-1: Calculation of epifluorescence signal retention after erasing

where $i_{ON}(x,y)$ and $i_{OFF}(x,y)$ represent the intensities of pixel $(x,y)$ before and after erasing respectively. For each image, each of the $n$ total pixels being considered for comparison were background subtracted and then averaged to calculate the mean signal before erasing, $\bar{i}_{ON}$, and after erasing, $\bar{i}_{OFF}$. These values were used to calculate the percentage of signal leftover upon completion of the erasing reaction $\left( \frac{i_{OFF}}{i_{ON}} \right)$. Background intensity values for each respective image, $\bar{B}_{ON}$ and $\bar{B}_{OFF}$, were calculated by taking the mean pixel value of regions containing no cells. Only pixels in which the background-subtracted signal $[i_{ON}(x,y) - \bar{B}_{ON}]$ was over a manually selected threshold value of $T$ were considered. The value was of $T$ was chosen such that background pixels and cell nuclei were excluded from analysis.
STORM erasing percentages were calculated by taking the ratio of the total number of localizations in the first 4082 frames of STORM movies acquired before and after the addition of the erasing probe.

2.4.8. Cross correlation maps

Using custom MATLAB software, reconstructed STORM images of IQGAP1 and actin were aligned on top of one another using either normalized cross correlation or by aligning the centroids of emissions from fiducial gold nanoparticles. Then, custom MATLAB software was used to generate a two-dimensional (x and y) normalized cross correlation heatmap, where each pixel value at each x,y value of the heatmap is the normalized cross correlation between the binned localizations of IQGAP1 and actin channels using only pixels within a 50 pixel (580 nm) radius of that x,y value. The normalized cross correlation value for every pixel of the source images was generated using the following equation,

$$\gamma(u, v) = \frac{\sum_{xy}[f(x, y) - \bar{f}_{uv}][t(x, y) - \bar{t}_{uv}]}{\left[\sum_{xy}[f(x, y) - \bar{f}_{uv}]^2 \sum_{xy}[t(x, y) - \bar{t}_{uv}]^2\right]^{0.5}}$$

**Equation 2-2 – Normalized 2-D cross correlation**

where $\gamma(u, v)$ is the normalized cross correlation heat map value at pixel $u,v$. Each cross correlation value was calculated using all pixels $x,y$ that are within a set radius of pixel $u,v$. $f(x, y)$ and $t(x, y)$ represent the total number of binned localizations at pixel $x,y$ in
the actin and IQGAP1 channels respectively. $f_{uv}$ and $\bar{f}_{uv}$ represent mean number of binned localizations for the subset of pixels $x,y$ in the actin and IQGAP1 channel respectively.
Chapter 3

IQGAP1 mediates the structure and dynamics of a novel multi-vesicular compartment

IQGAP1 has been known to both positively and negatively regulate local cytoskeletal remodeling. In this way IQGAP1 can act as switch that drives changes in cell motility, adhesion, and polarity. In this study we identified a novel class of membranous compartments that are surrounded by an outer shell of IQGAP1-associated actin filaments. The evolution of this compartment involves three distinct phases: formation, stabilization, and disassembly, and each phase correlates to distinct cytoskeletal remodeling behaviors in the outer IQGAP1-associated actin shell. During compartment formation and stabilization, actin polymerization appears to be constrained by a negative feedback mechanism that it correlated to an increase in IQGAP1 localization to the compartment’s surface. However, during disassembly IQGAP1 dissociates from the

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2 The following individuals made contributions to this chapter: Tsao, D. S., McLaughlin, R. T., D.Zimak, J., Schweikart, V., and Diehl M. R.
surface of the membranous compartment, leading to a rapid non-linear increase in actin polymerization that terminates with compartment deformation and disassembly. These results indicate that IQGAP1 is a potential role-player in a negative feedback pathway that promotes compartment stability through the prevention of rapid actin polymerization.

3.1. Introduction

The actin cytoskeleton long been known to both positively and negatively regulate membrane dynamics. Filamentous actin structures can promote membrane stability through the formation of a steric barrier that prevents interactions with membranous organelles. For instance, Dia1 acts downstream of RhoB activation to promote the assembly of bundled actin filaments on the surface of endosomes in HeLa cells. This actin barrier prevents long distance endosomal trafficking along microtubules and results in the preferential localization of endosomes to the cell periphery. The actin cortex also plays a major role in preventing membrane dynamics by acting as a barrier between secretory vesicles and the plasma membrane. On the other hand, actin dynamics have also been shown to promote membrane fission through forces exerted by actin polymerization. In mouse embryonic fibroblasts, F-actin assembly on mitochondria coincides with Drp1-mediated mitochondrial fission. Other studies have shown that Arp2/3 activation by WASP leads to branched actin polymerization that generates a pushing that drives endosome fission.
IQGAP1 is a master cytoskeletal regulatory protein that has been shown to interact with many different cytoskeletal regulatory pathways that lead to changes in cell motility and cell-cell adhesion. IQGAP1’s ability to switch between different cytoskeletal regulatory outputs is facilitated in part through the various ways in which it promotes or inhibits actin polymerization. By acting as a scaffolding protein, IQGAP1 has been shown to promote the polymerization of Arp2/3-mediated branched actin filaments in vitro and Dia1-mediated bundled actin filaments during macrophage migration and phagocytic cup formation. Alternatively, the c-terminus binding domain of IQGAP1 has been shown to negatively regulate actin polymerization by capping barbed ends of F-actin in a calmodulin-regulated manner. Given IQGAP1’s diverse cytoskeletal regulatory outputs and the multitude of ways in which its activities can be locally modulated, it is not hard to imagine a potential role for IQGAP1 as an important functional component of negative feedback loop that controls local cytoskeletal remodeling. In fact, a previous study has discovered that IQGAP1 plays a necessary role in directional cell migration by cyclically downregulating Rac1-mediated membrane protrusion. Briefly, a complex between IQGAP1, filamin-A (FLNa), and RacGAP1 forms to suppress Rac1-mediated membrane protrusion at sites of active β-integrin. The silencing of IQGAP1 results in unconstrained membrane protrusion and a loss of directional cell migration.

In this chapter we present a negative feedback model in which IQGAP1-associated actin filaments surround and mediate the dynamics of a novel class of multi-vesicular membranous compartments in epithelial MCF-10A cells. Live-cell imaging was used to
examine how changes in the amount IQGAP1 and F-actin localized to the compartment’s outer lumen correlated with changes in compartment structure and dynamics. These studies revealed that F-actin localization on the surface of compartments is partially regulated by a negative feedback mechanism that prevents periods of prolonged actin polymerization. The activity of this negative feedback pathway is positively correlated with an increase in IQGAP1 localization to the compartment’s surface. In the presence of low levels of IQGAP1 localization to the compartment’s surface, local actin polymerization increases in a non-linear fashion until catastrophic disassembly of the compartment occurs. Additional imaging studies found that these compartments shared similar characteristics with multi-vesicular bodies (MVBs) and potentially participate in the sorting of cell-cell adhesion proteins such as E-cadherin, N-cadherin, β-catenin, and CD44. Taken together, these results indicate that IQGAP1 association is correlated with periods of membrane stability and constrained actin polymerization, potentially providing a mechanism in which IQGAP1 regulates the structure and life cycle of a novel class of basal sorting compartments.

3.2. Results

3.2.1. IQGAP1 and actin localize to membranous structures on the basal actin cortex.

Live cell imaging studies were performed on epithelial MCF-10A cells transiently expressing CFP-LifeAct and YFP-IQGAP1 after incubation with the lipid membrane stain wheat germ agglutinin (WGA). These imaging studies showed that in addition to
colocalizing with F-actin along cell-cell contacts, presumably acting in its commonly known role as a regulator of the strength and stability of E-cadherin-mediated cell-cell junctions. IQGAP1 and actin colocalize with a novel class of membranous compartments (See Figure 3-1 A-C). 3-D confocal imaging studies of fixed, immunostained MCF-10A cells showed that these compartments were primarily localized to the basal actin cortex (See Figure 3-1 E-F). Furthermore, individual confocal z-slices of a basal compartment revealed that IQGAP1 and actin formed a hollow shell that completely ensconced an internal membranous core, suggesting that the membranous compartment is on top of the actin cortex and not an invagination of the underlying plasma membrane (See Figure 3-1 H). Particle tracking studies of IQGAP-associated basal compartments showed that compartments exhibit limited motility and do not directly interact with IQGAP1-associated actin structures at cell-cell junctions (See Figure 3-2). Notably, IQGAP1 and actin associated membranous structures were also observed in Sum-149 cells, an invasive breast cancer cell line with mixed basal and luminal epithelial phenotypes (See Figure 3-3 A). Lastly, changing cell growth substrates did not prohibit the formation of basal compartments (See Figure 3-3 B).
Figure 3-1: IQGAP1 and actin localize to basal membranous compartments. Live-cell epifluorescence images of YFP-IQGAP1 (A), CFP-LifeAct (B), and lipid membrane stained by WGA-647 (C) show that IQGAP1, F-actin, and membrane co-localize at compartment-like structures ~ 2 μm in diameter in epithelial MCF-10A cells. Confocal microscopy images of IQGAP1 and F-actin (phalloidin-647) in fixed MCF-10A cells show that these compartments are primarily localized on the basal actin cortex (D,E) and form a hollow shell around an inner membranous core (F-H). Scale bars 10 μm (A-C) and 5 μm (D-G) and 2 μm (H).
Figure 3-2: IQGAP1 and actin-associated membranous compartments are confined to a single position on the basal actin cortex. (A) Trajectories of compartments seen a 1 hour live-cell image of eGFP-IQGAP1. (B) Mean of the mean squared displacements from 38 particle trajectories with error bars representing the standard error of the mean. Scale bar = 5 μm.

Figure 3-3: Effect of changing cell type and substrate on compartment formation. (A) Compartments are present in fixed SUM-149 cells. An invasive breast cancer cell line with mixed basal and luminal epithelial phenotypes. (B) Compartments are present in fixed epithelial MCF-10A cells cultures on various cell growth substrates. Scale bars = 10 μm.
3.2.2. IQGAP1 and F-actin exhibit distinct membrane localization behaviors during compartment formation, stabilization, and disassembly

Intensity analysis of YFP-IQGAP1, CFP-LifeAct, and WGA within the boundaries of segmented compartments revealed that compartment formation begins with a simultaneous increase in the local amounts of IQGAP1, F-actin, and membrane (See Figure 3-4). The normalized total intensity of each marker was plotted as a function of time. Given that the compartment mask size did not vary with frame, this is equivalent to the normalized mean intensity. The localization of IQGAP1 and actin to compartments does not appear to be mediated by vesicular trafficking, suggesting that cytoplasmic IQGAP1 and actin are sourced locally. The average compartment formation lasted 40 ± 7.5 minutes (n = 5) and was typically followed by an extended period of compartment stabilization.

Prior compartment disassembly, a slow drop in IQGAP1 intensity is observed that occurs over a range of 10-50 minutes (n = 3) (See Figure 3-5). After IQGAP1 intensity at the basal compartment approaches the level of the cytoplasmic background, a rapid, non-linear increase in F-actin assembly is observed. This increase in actin polymerization is followed by the disassembly of the compartment and the release of multiple, diffraction-limited vesicular structures (See Figure 3-5 A). In limited observations of disassembly, the average duration of the F-actin increase lasted 9 ± 1 minutes (n = 3). During disassembly, compartment expansion primarily occurs along a single axis (See Figure 3-5 A (Actin, t = 43 min), B (Actin, t = 33 min) and C (Actin, t = 11.5 min)). Therefore, a possible explanation for compartment disassembly is strain induced by membrane
stretching as a result of polarized actin polymerization. The non-linear increase in F-actin assembly suggests that actin polymerization occurs in an Arp2/3-mediated, branch-like fashion rather than through the extension of long bundled filaments via formins such as Diaphanous-related formin-1,2, or 3 (Dia1/2/3). This effect could be similar to the extrusion forces generated by Arp2/3-mediated actin polymerization in budding yeast.55

Interestingly, membranous structures frequently reassembled in the regions previously occupied by the basal compartment in the absence of IQGAP1 and F-actin recruitment (See Figure 3-5 A (WGA, t = 56). In one instance, IQGAP1 and F-actin were subsequently recruited to this persistent membrane (See Figure 3-5 C (plot)). One potential explanation of the phenomenon is the reassembly of previously sheared membrane. Another possibility is the existence of a membrane tethering complex on the basal actin cortex that persists after disassembly.

While the intensity increases and plateaus of IQGAP1 and F-actin intensity during the formation and stabilization were correlated with one another on long timescales, we observed that higher frequency changes in IQGAP1 and actin during these phases exhibited varying degrees of anti-correlation (See Figure 3-4 C (plot); See Figure 3-6). After filtering out the low frequency changes in YFP-IQGAP, CFP-LifeAct, and WGA intensity changes within segmented compartment boundaries, it was found that the remaining high frequency changes in IQGAP1 and actin intensity were on average anti-correlated with one another (γ = -.19) (See Figure 3-7 A); Individual traces showed that this negative correlation was the result of alternating IQGAP1 and F-actin intensity peaks (See Figure 3-6). This data suggests a potential role for IQGAP1 as a negative regulator
of prolonged actin polymerization at the surface of basal compartments. This could potentially be a means of stabilizing the internal membranous core by reducing the stress applied by pulling forces on the outer lumen generated through contact with polymerizing actin filaments. The absence of this negative feedback after a loss of IQGAP1 at the compartment surface may explain observations of prolonged non-linear actin polymerization that terminates with compartment disassembly. This chain of events suggests that IQGAP1 may be a necessary component in a cytoskeletal regulatory pathway that constrains actin polymerization on the compartments surface. In this model, the absence of IQGAP1’s negative regulation enables F-actin assembly to eventually pass the slowly increasing initial phase and reach the point in which the rate of polymerization is large enough to impart a catastrophic shear stress on the internal membrane.

High frequency changes in CFP-LifeAct and WGA intensities were found to be positively correlated ($\gamma = .195$) on average (See Figure 3-7 B). Since WGA intensity presumably scales with membrane surface area, this suggests that high frequency changes in the surface area of the compartment’s outer lumen and the degree of F-actin assembly are correlated with one another. However, it is unknown whether or not F-actin assembly/disassembly drives or stabilizes the overall compartment morphology during compartment formation or stabilization. High frequency CFP-IQGAP1 and WGA intensities within segmented compartment boundaries showed no correlation with one another (See Figure 3-7 C). This suggests that while IQGAP1 and actin are anti-correlated
overall, these correlations are not significant over multiple degrees of separation 
\( \gamma_{\text{lag/gap}} \cdot \gamma_{\text{actin/wga}} \).
Figure 3-4: Formation of basal compartments. (A-C) Live cell imaging of epithelial MCF-10As transiently transfected with CFP-LifeAct and YFP-IQGAP1 and incubated with the lipid membrane stain WGA-647. Each row shows frames of interest during formation (Left) alongside plots of the normalized mean intensity changes (Right). Images reveal that IQGAP1, F-actin, and membrane intensity simultaneously increase during compartment formation. In some instances, IQGAP1 and actin intensities are noticeably anti-correlated (C). Scale bars = 2 μm.
Figure 3-5: Disassembly of basal compartments. (A-C) Live cell imaging of epithelial MCF-10As transiently transfected with CFP-LifeAct and YFP-IQGAP1 and incubated with the lipid membrane stain WGA-647. Each row shows frames of interest during disassembly (Left) alongside plots of the normalized mean intensity changes (Right). Images reveal that IQGAP1 slowly decreases prior to a non-linear increase in actin polymerization that culminates in compartment disassembly. Compartment disassembly leads to release of small, highly motile vesicles (A, t = 44). Scale bars = 2 μm.
Figure 3-6: Anti-correlation between IQGAP1/F-actin intensities in basal compartments. (A-C) Live cell imaging of epithelial MCF-10As transiently transfected with CFP-LifeAct and YFP-IQGAP1 and incubated with the lipid membrane stain WGA-647. Each row shows frames of interest displaying anti-correlation between IQGAP1 and F-actin (Left) alongside plots of normalized mean intensity changes (Right). Images reveal that IQGAP1 and F-actin intensity are anti-correlated during compartment stabilization (A,B) and compartment fusion (C). Scale bars = 2 μm.
Figure 3-7: Pairwise cross-correlations between IQGAP1, F-actin, and lipid membrane (WGA) intensity at basal compartments. (A) On average, IQGAP1 and actin are anti-correlated (A), Actin and WGA are correlated (B), and IQGAP1 and actin are uncorrelated (C) at delay = 0 minutes. Intensities traces were filtered using a Subtract Savitzky-Golay high pass filter with a 17 frame filter window.
3.2.3. Basal compartments are comprised of IQGAP1-associated actin filaments

Multi-color, super-resolution images of IQGAP1 and F-actin (phalloidin) in fixed epithelial MCF-10A cells were taken using multi-round STORM (See Figure 3-8). In these images we see that IQGAP1 associates at the single-filament level with basal F-actin compartments. This result indicates that IQGAP1 plays a functional role as a cytoskeletal regulator at the compartment’s surface and is not simply being trafficked to the membranous core for sorting or processing. Compartments display a wide range of morphologies, from smaller, spherical structures ~ 2 μm in diameter (See Figure 3-8 i) to larger, irregularly shaped ring-like or patch like actin structures (See Figure 3-8 i-iii). Live cell imaging of IQGAP1-eGFP showed that these diverse IQGAP1-associated structures were actually transient states of the same class of structures (See Figure 3-9). Larger, patch-like structures tend to condense over time into smaller, spherical structures that can subsequently be disassembled via a process that resembles ring-like expansion.

Local normalized 2-d cross correlation analysis were performed on the STORM reconstructions of IQGAP1 and actin to measure the degree of spatial similarity between the two markers (0 = uncorrelated, 1 = perfectly correlated, See Equation 2-1 on page 29 for more details). The local cross correlation values, represented as color in super-resolution STORM reconstructions of actin, show that high correlations between IQGAP1 and actin on the basal cortex are specific to basal compartments (See Figure 3-8). Surrounding actin stress fibers or mesh-like actin filaments, by contrast, are largely uncorrelated with IQGAP1. This indicates that IQGAP1 is specifically associating with
actin filaments on the basal compartments while displaying significantly less affinity for the actin cortex.
Figure 3-8: IQGAP1-associated basal actin compartments display a diverse set of transient structures. (A,B) Multi-round STORM super-resolution images of F-actin (phalloidin, cyan) and IQGAP1 (red) in fixed epithelial MCF-10A cells reveal a significant diversity of sizes and shapes of basolateral IQGAP1-actin structures. Insets: Zoom-ins of representative structures (baskets (i), rings (ii), and patchlike actin filament arrangements (iii-iv)) and corresponding IQGAP-actin cross-
correlation maps (color = normalized 2-D cross correlation coefficient). IQGAP1 localizes to filamentous actin in these basolateral structures (green in cross-correlation maps i-iv), whereas such co-localization is absent at other types of bundled actin filament structures (red in cross-correlation maps i-iv). See Scale bars 5 μm (A,B) and 1 μm (i-iv).

Figure 3-9: Morphological transitions of basal compartments. Live cell imaging of MCF-10A cells transiently transfected with eGFP IQGAP1 show basal compartments compacting into 2 μm diameter spherical compartments = and spherical compartments expanding into ring-like morphologies during disassembly (B). Scale bars = 10μm (whole cells) and 5 μm (insets).

3.2.4. IQGAP1-associated compartments have a multi-vesicular membranous core

Further live-cell imaging studies to identify the molecular identity of the membranous core of the compartment showed that the compartments stained for CFP-
CD63 and eGFP-CHMP6, markers known to associate with multi-vesicular bodies (MVBs) (See Figure 3-10 A). This is further evidenced by the fact that compartment disassembly leads to multiple, smaller, vesicles that also stain for CHMP6, as CHMP-6 is ESCRT-III complex component that has been shown to mediate the formation of small intraluminal vesicles (ILVs) in (MVBs) through membrane fission (See Figure 3-10 B). However, unlike traditional MVBs, which have a tightly regulated size of ~.5 μm in diameter, IQGAP1 and actin-associated compartments are typically > 2 μm in diameter and can have a wide variety of morphologies (See Figure 3-8).

Compartments also colocalized with eGFP-Exo70 (See Figure 3-10 A). Exo70 has been shown to be necessary in MCF-10A for adherens junction assembly and epithelial polarization. Therefore, rather than playing a compartment function, Exo70 may be localized to compartments as part of an adherens junction recycling or sorting pathway. Alternatively, Exo70 may play a role as part in the exocytosis of the compartment ILVs after disassembly of the outer lumen. Exo70 binds to the septin Sept2 as part of a vesicle-tethering exocyst complex during IQGAP1-regulated exocytosis in pancreatic β-cells, and a previous study has implicated the exocyst complex in the secretion of urinary exosomes from MVBs.
Figure 3-10: IQGAP1-associated compartments have a multi-vesicular core. (A) Live-cell imaging of epithelial MCF-10As shows that eGFP-CD63, eGFP-CHMP-6, and eGFP-Exo70 colocalize with IQGAP1-associated compartments. CD63 and CHMP-6 are known identifiers for MVBs. Exo70 is a known component of the exocyst complex. (B) After IQGAP1 dissociates from a CHMP-6 labeled compartment, multiple highly-motile CHMP-6 labeled vesicles are released. Scale bars = 10 μm (A) and 2 μm (B).
3.2.5. IQGAP1-associated compartments are destinations of highly-motile vesicular trafficking organelles

To assess whether or not IQGAP1-associated basal compartments participated in vesicular trafficking pathways, live-cell imaging experiments of IQGAP1 alongside various vesicular markers were conducted. Imaging was performed on epithelial MCF-10As transiently transfected with FusionRed-IQGAP1 alongside Rab11-eGFP, a common identifier of recycling endosomes (REs) (See Figure 3-11 A). Although tracking of individual REs was not possible at a 30 s framerate, preferential localization of diffraction-limited REs to the peripheries of every IQGAP1 compartment was discernible in individual frames, and was more pronounced when the pixel intensities of a time series were summed. The association of these compartments with REs is expected given the compartment’s possible function as a multi-vesicular sorting organelle. In addition to possible trafficking roles, Rab11 has been shown to be mediator of homotypic fusion of MVBs, and could therefore be involved in the fusion of IQGAP1 and actin-associated compartments. Rab11 has also been shown to regulate the exosome pathway in K562 cells, further suggesting that the ILVs liberated by compartment disassembly are secreted into the extracellular environment.

Live-cell imaging of IQGAP1-eGFP alongside mCH-Rab5, an identifier for early endosomes (EEs), could not confirm or deny potential interactions between basal compartments and either class of vesicular organelle (See Figure 3-11 B). While potential kiss-and-run interactions were commonly observed between Rab5-associated vesicles and basal compartments, transient localizations of EEs were so common throughout the
cell that it was not possible to assess which, if any, were actual interactions with basal compartments. Associations of basal compartments with dsRed-Rab7 labeled late endosomes and mCh-LAMP1 labeled lysosomes were not commonly observed, and summing the movie frames for either marker did not reveal any evidence of preferential localization to compartment peripheries (Figure 3-11 C-D).

Conventional immunofluorescence microscopy revealed that a number of cell-cell adhesion proteins, including CD44 and various adherens junction proteins (E-cadherin, N-cadherin, β-catenin), associate with basal compartments (See Figure 3-12). Moreover, E-cadherin and β-catenin were often localized to discrete puncta at the periphery of basal compartments (See Figure 3-12 A-B). Such behavior is consistent with the circumferential localization behaviors and vesicle kiss-and-run by Rab11-associated Res and Rab5-associated EEs. We note that both Rab5 and Rab11 have been identified as important regulators of endocytic E-cadherin recycling. An E-cadherin internalization assay was performed on fixed MCF-10A cells, and after 1 hour ~ 30% of the compartments had internalized E-cadherin that had been endocytosed from the plasma membrane (See Figure 3-13). Overall these results indicate that, although the IQGAP1 compartments do not appear to present classical endosomal marker identity, they appear to participate in the vesicular trafficking of cell-cell adhesion proteins via transient interactions with REs. Additionally, trafficking of cell-cell junction proteins to basal compartments via EEs cannot be ruled out, as potential transient interactions were observed between basal compartments and Rab5-associated vesicles.
Figure 3-11: IQGAP-1 associated compartments selectively interact with Rab11-associated recycling endosomes. IQGAP1 (1st Column) was imaged in live epithelial MCF-10A cells alongside various highly-motile vesicular trafficking organelles (2nd Column). (3rd Column) Summed frames of 30 minute movies of vesicular trafficking organelles reveal regions of preferential localizations. (4th Column) Merged images IQGAP1 (1st Column) and the summed frames of vesicular organelles (3rd Column). FusionRed-IQGAP1 was imaged alongside eGFP-Rab11 (A) while eGFP-IQGAP1 was imaged alongside mCh-Rab5 (B), dsRed-Rab7 (C), and Lysotracker-Red (D) in separate imaging experiments. Of these vesicular markers, only Rab11 (A) shows specific localization to the IQGAP1-associated compartments. Scale bars = 10 μm.
Figure 3-12: IQGAP-1 associated compartments co-stain with cell-cell adhesion markers. IQGAP1 (1st Column) is imaged alongside various cell-cell adhesion markers (2nd Column). (3rd Column) Merged images IQGAP1 and various cell-cell adhesion markers. (4th column) Expanded images of insets from Column 3 show that cell-cell adhesion markers co-stain in punctate spots with IQGAP1-associated compartments. Scale bars = 10 μm.
Figure 3-13: E-cadherin internalization assay. Live epithelial MCF-10A cells were stained for HECD-1, the extracellular domain of E-cadherin. After allowing various amounts of time for antibody internalization, cells were washed with acetic acid to strip residual surface bound antibodies, fixed in paraformaldehyde, and then immunostained for IQGAP1. (A) Fixed cells that were not washed in acetic acid. (B) Cells that were washed and fixed immediately after staining with HECD-1 antibodies, allowing no time for E-cadherin internalization. (C) Cells that were washed and fixed 60 minutes after staining with HECD-1 antibodies, at this time E-cadherin internalized to ~30% of IQGAP1-associated compartments. Scale bars = 10 μm.
3.2.6. Growth Factors TGF-β and TNF-α induce an EMT-like transition in which compartment formation is downregulated.

To examine the selectivity of the IQGAP1 compartment localization within a polarized epithelial state, we also characterized IQGAP1/actin spatial correlations in MCF-10A cells that were cultured in media supplemented with the growth factors TGF-β1 and TNF-α to disrupt epithelial cell junction complexes and induce an epithelial to mesenchymal (EMT) like transition (See Figure 3-14). Basal IQGAP1 compartments are virtually non-existent in these conditions compared to those using standard cell culture media (See Figure 3-14 B). On the other hand, a greatly increased incidence of small, diffraction-limited IQGAP1 and actin containing structures can be observed via confocal microscopy (See Figure 3-14 D-F). These structures localize to the apical cell cortex and tend to associate with actin stress fibers. Taken together, these results suggest that the apical associations between IQGAP1 and actin induced by TGF-β1 and TNF-α belong to a different class of structures than the previously observed basal IQGAP1 and actin-associated compartments. Thus, the existence of basal IQGAP1 and actin-associated compartments appears to be dependent upon growth conditions conducive to the establishment and stabilization of adherens junctions.
Figure 3-14: The addition of TGF-β and TNF-α downregulates the number of multivesicular basal compartments. (A) Fixed MCF-10A cells were incubated with TGF-β and TNF-α for 48 hours prior to being fixed and immunostained for IQGAP1 using dynamic DNA probes. Select cells from epifluorescence images were then imaged using multi-round STORM. (B) A STORM image of IQGAP1 in round 1 shows the absence of IQGAP1 basal compartments. (C) A STORM image from round 2 shows the increased presence of polarized F-actin stress fibers and an increased irregularity of cell-cell junction morphology. (D,E) Further 3-D confocal imaging studies of IQGAP1 and F-actin show that IQGAP1 and actin colocalize in punctate structures on the apical membrane. (F) A life profile draw through images D and E shows overlapping peaks of IQGAP1 and F-actin intensity corresponding to IQGAP1/F-actin correlations at punctate spots on the apical membrane. Scale bars = 10 μm (A,B,C) and 5 μm (D,E).
Figure 3-15: A proposed model for a novel multi-vesicular sorting compartment with dynamics that are regulated by an outer IQGAP1-assocaited actin shell. (A) At the onset of compartment formation, IQGAP1 and actin are simultaneously localized to a growing membranous organelle on the basal actin cortex. (B) During stabilization, the compartment matures into a multi-vesicular sorting compartment that participates in the recycling of cell-cell adhesion proteins (N-cadherin, CD44, and a cadherin complex made of E-cadherin and α/β catenin) by way of Rab11-associated recycling endosomes. Also during this stage, proteins are sorted between the outer lumen and newly formed intraluminal vesicles (ILVs). The budding of ILVs is mediated by an ESCRT-III complex containing CHMP-6. During formation (A) and stabilization (B), high frequency changes in IQGAP1 and actin localization to the outer lumen are anti-correlated, suggesting that the recruitment of IQGAP1 to the outer lumen stabilizes the compartment’s structure through negative regulation of rapid actin polymerization. (C) However, after the disociation of IQGAP1 from the outer lumen, actin is recruited in a prolonged, non-linear fashion. (D) This leads to compartment disassembly via stress from polarized actin polymerization and the subsequent release of highly-motile ILVs.
3.3. Discussion

Our results indicate that IQGAP1-associated actin filaments surround and mediate the dynamics of a special class of membranous compartments on the basal actin cortex (See Figure 3-15). During formation and stabilization, rapid increases in F-actin assembly appear to be held in check by a negative feedback mechanism that downregulates actin polymerization. A likely role-player in this negative feedback pathway is IQGAP1, a cytoskeletal regulatory protein whose localization to the surface of basal compartments is anti-correlated with local F-actin polymerization. Dissociation of IQGAP1 from the surface of basal compartments is correlated with a prolonged, non-linear increase in actin polymerization on the surface of basal compartments. This non-linear increase in actin polymerization indicates branched actin assembly mediated through WASP-activated Arp2/3. Actin polymerization on the surface of basal compartments is directional, as during compartment disassembly the outer actin shell appears to expand primarily along a single axis. One possible explanation for IQGAP1-mediated negative feedback is that the c-terminus of IQGAP1 binds to the barbed ends of actin filaments to prevent prolonged polymerization. Another possible explanation is that IQGAP1 acts as a negative regulator of a pathway responsible for actin nucleation and polymerization. Future experiments with IQGAP1 mutants missing various binding domains could be used to examine which binding domains are necessary to facilitate the anti-correlative behavior observed between IQGAP1 and F-actin on surface of basal compartments.

Negative feedback, and the subsequent lack thereof, may provide a mechanism in which an outer layer of IQGAP-associated actin filaments mediates the structure and
dynamics of an internal multi-vesicular sorting compartment. During the stabilization phase, observed kiss-and-run interactions between REs and EEs, identified by Rab11 and Rab5 respectively, may be responsible for trafficking proteins endocytosed from the plasma membrane to these multi-vesicular compartments. Supporting this model is the fact that basal IQGAP1-associated compartments have been shown to co-stain with cell-cell adhesion proteins such as β-catenin, E-cadherin, N-cadherin, and Cd44. IQGAP1-associated compartments also appear to form ILVs in a CHMP-6-associated manner, therefore displaying a behavior similar to classic sorting compartments like MVBs. CHMP-6, an ESCRT-III component known to facilitate intraluminal budding, has also been shown to stain the high-motile vesicular organelles that are released after compartment disassembly. Taken together these results suggest that proteins trafficked to basal compartments are sorted between the compartment’s outer lumen and CHMP-6-mediated ILVs. After a set period of time, compartments are disassembled via actin-polymerization-mediated shear stress and the ILVs are released. The destination of these ILVs is currently unknown, as their increased speed relative to the dynamics of the larger basal compartments make them difficult to track. One possible explanation is that the ILVs are released as exosomes into the extracellular environment. Multi-vesicular compartments stain for CD63 and CHMP-6, markers known to stain both MVBs and exosomes in the MVB-exosome pathway. Compartments also stain for Exo70, a key component of the exocyst complex. Previous studies have suggested that the mammalian exocyst, which is partially comprised of Exo70, is involved in the secretion of urinary exosome-like vesicles.
In conclusion, this study provides evidence for a new role of IQGAP1 in a negative feedback pathway that stabilizes basal multi-vesicular compartments by preventing prolonged non-linear actin polymerization on the compartment’s outer lumen. This mechanism provides a potential link between IQGAP1-mediated cytoskeletal regulation and the sorting of cell-cell adhesion proteins in epithelial MCF-10A cells.

3.4. Materials and methods

See chapter 2 for materials and methods for: DNA probe design, MCF-10A cell culture and fixation, Antibody staining, DNA-mediated labeling and erasing procedures, Epifluorescence microscopy, Multi-round STORM imaging, and Cross correlation maps.

3.4.1. Nucleofection

For all transfections, 1 ug of plasmid DNA per construct were added to 100 µL of Nucleofector solution T (Lonza). 500 x 10^6 MCF-10A cells were dissolved into Nucleofector solution and DNA mixture and cells were electroporated using a Nucleofector 2 (Amaxa) set to program T-024. Nucleofected cells were allowed to grow in MEGM media for 1-2 days before being seeding onto coverslips for live cell imaging.

3.4.2. Live-cell imaging

Lysotracker Deep Red (Molecular Probes) and Wheat Germ Agglutinin Alexa-647 (Molecular probes) were used to stain for lysosomes and generic membrane, respectively. GFP-rab11 WT and DsRed-rab7 WT were gifts from Richard Pagano.
mCh-Rab5 was a gift from Gia Voeltz (Addgene plasmid # 49201). CHMP6-GFP was a gift from Daniel Gerlich (Addgene plasmid # 31806). pEGFP-C3-Exo70 was a gift from Channing Der (Addgene plasmid # 53761). CD63-pEGFP C2 was a gift from Paul Luzio (Addgene plasmid # 62964). eGFP-IQGAP1 and FusionRed-Lifeact were amplified by PCR from plasmids gifted by David Sacks (Addgene plasmid # 30112) and Michael Davidson (Addgene plasmid # 54778), respectively, and inserted into a vector specifically designed for potential later assembly of multigene constructs developed by Weiss et al. This procedure was used to create vectors for eGFP-IQGAP1, FusionRed-IQGAP1, YFP-IQGAP1, and CFP-LifeAct.

3.4.3. Generation of intensity vs. time traces of marker intensities at basal compartments

Each of the three color channels acquired during live cell images of MCF-10As transfected with CFP-LifeAct, YFP-IQGAP1 and incubated with WGA were processed separately using the following methods:

Individual frames were corrected for drift using the Linear Stack Alignment with SIFT ImageJ plugin and background subtracted using a 10 pixel (2.3 micron) rolling ball radius (ImageJ). Then, the total intensity of each background-subtracted image was normalized to correct for photobleaching.

After image processing, individual compartments were segmented and tracked in the IQGAP1 channel using the Squassh and Particle Tracker 2D/3D utilities of the Mosaic
Then aggregate masks for each trajectory were constructed from every pixel in each individual segmentation in that trajectory. This represents the total area explored by a particular compartment. For each background-subtracted marker image, summed intensity levels within each compartment’s aggregate mask were calculated on a per frame basis.

### 3.4.4. Live-cell cross correlation analysis

Time-traces of marker intensities (IQGAPI, Actin, WGA) in individual compartments were created by integrating signal intensity within segmented compartments in each frame of live-cell time-lapse movies. Time traces were high-pass filtered with a ~35 minute timescale cutoff, by subtracting a Savitzky-Golay low-pass filtered trace. Normalized cross-correlations between the remaining high-frequency fluctuations of the three markers were computed, and averaged over 36 traces originating from compartments in 4 distinct cells.

### 3.4.5. E-cadherin Internalization

Cells were passaged 24 h prior to experiments and seeded to yield sub-confluent layers. To label surface-bound E-cadherin was labeled with monoclonal antibody HECD-1 against the extracellular domain of E-cadherin, cells were incubated for 1 hour at 4 °C in HBSS+Ca+Mg + 50 mg/ml BSA + 5 microgram/ml HECD1. Cells were then washed with PBS to remove any remaining unbound antibody, and transferred to HBSS+Ca+Mg at 37 °C to allow for internalization of surface-labeled E-cadherin. After varying amounts of time, cells were washed with with 0.5 M Acetic acid + 0.5 M NaCl (3x 5’ washes) to strip
any residual antibodies remaining on the cell surface. Cells were then fixed, permeabilized and stained with fluorescent secondary antibody to detect the internalized E-cadherin. Samples were also stained to visualize IQGAP1 via indirect immunofluorescence.

3.4.6. Generation of MSD for particles

Live cell images of IQGAP1 were drift corrected using the Linear Stack Alignment with SIFT ImageJ plugin. Then trajectories of regularly shaped particles were created using the TrackMate ImageJ plugin. Lastly, the average MSD was calculated using the msdanalyzer MATLAB function developed by Tarantino et al.91
Chapter 4

**Future Directions**

The primary focus of this dissertation is the characterization of a novel multivesicular sorting compartment that acts as an intermediate destination in adherens junction recycling. The current hypothesis is that the compartment’s structure and dynamics are mediated by an outer shell of actin filaments. During periods of compartment stability, the polymerization of these filaments is partially controlled by an IQGAP1-mediated negative feedback mechanism. Eventually, upstream signaling triggers the dissociation of IQGAP1 from the compartment’s surface, which subsequently promotes unconstrained actin polymerization. This results in compartment disassembly and the release of highly-motile CHMP-6 mediated intraluminal vesicles (ILVs).

This model is supported by measurements of IQGAP1 and actin localization to wheat germ agglutinin (WGA) stained basal compartments as function of time. However, while super-resolution images show clear colocalization between IQGAP1 and actin filaments on the surface of basal compartments, currently there is no direct evidence of IQGAP1s role in stabilizing actin polymerization. Therefore, the primary focus of future
experiments will be to perturb IQGAP1 and its upstream regulators in order to better understand IQGAP1’s specific role in regulating the structure and dynamics of multi-vesicular basal compartments. In addition to experiments involving IQGAP1, the roles of other cytoskeletal regulatory proteins in mediating compartment structure and dynamics will be explored. Finally, future studies will also explore the function of these multi-vesicular compartments and their potential role in epithelial maturation.

4.1. Characterization of IQGAP1’s involvement in the regulation of membrane dynamics

IQGAP1 is a master cytoskeletal regulatory protein with at least six distinct protein-binding domains. Starting at the N-terminus they are: (1) A calponin homology (CH) domain that directly binds to actin, (2) A coiled-coil (CC) domain with no known interacting partners, (3) Two conserved tryptophan-containing (WW) domains that bind to Erk1/2, (4) 4 IQ motifs that modulate bind to calmodulin, an important regulator of IQGAP1 function, and calmodulin-related proteins, (5) A GTPase activating protein related domain (GRD) that binds to small GTPases such as Cdc24 and Rac1, and (6) a distal C-terminus (dCT) with diverse cytoskeletal regulatory functions. By examining the behaviors of IQGAP1 mutants with modified or deleted binding domains, one can better understand which domains mediate IQGAP1’s regulatory behavior at multi-vesicular compartments. More specifically, which binding domains are sufficient and/or necessary for IQGAP1’s anti-correlative behavior with actin localization at the compartment’s surface? In general, the type of experiment carried out would be to
transiently transfect epithelial MCF-10A cells with wild type IQGAP1, LifeAct, and a specific IQGAP1 mutant or fragment. This would enable a direct comparison between the dynamics/localization patterns of IQGAP1 mutants and wild type IQGAP1.

The significance of IQGAP1-mediated actin cross-linking on the compartment’s surface could be examined using IQGAP1 mutants lacking the n-terminal CHD. Previous studies have shown that the CHD (amino acids 1-216) is both necessary and sufficient for direct actin binding, which mediates actin cross-linking. In addition to examining the effect of direct actin binding on IQGAP1’s localization to basal compartments, the connection between compartment dynamics and IQGAP1-mediated actin cross-linking could be further analyzed by perturbing the affinity between IQGAP1 and actin in a Ca^{2+} dependent manner.

Additional IQGAP1 mutants could be studied in order to examine IQGAP1’s potential role as an actin capping protein on the surface of multi-vesicular compartments. A previous study has shown that a c-terminally truncated fragment of IQGAP1 (amino acids 744-1502) is sufficient for capping of barbed ends of actin filaments. This study also showed that the absence of Ca^{2+} downregulated IQGAP1-mediated capping. Therefore, adding EGTA, a calcium chelator, to live-cell samples during imaging could potentially lead to a change in multi-vesicular compartment dynamics via extending the length of associated actin filaments.

To investigate whether or not multi-vesicular compartments secrete ILVs via the exocyst complex upon degradation, mutant IQGAP1 unable to bind to Exo70, Sec3 and
Sec8, components of the exocyst complex, could be examined. Previous studies have shown that amino acids 1347-1562 are necessary for exocyst-mediated tumor cell invasion.\(^{84}\)

Various IQGAP1 mutants could be used to test for potential IQGAP1-mediated connections between the actin-filaments on the surface of multi-vesicular compartments and microtubules. Connections between microtubules and actin have been proposed as a mechanism to induce membrane fission and may play a role in the polarized expansion that occurs during multi-vesicular compartment disassembly.\(^{85}\) A previous study has shown that amino acid residues 678-863 are necessary for IQGAP1's activity in capturing microtubule plus ends to select cortical actin regions via SKAP.\(^{86}\) Another study showed that the c-terminal fragment of IQGAP1 (amino acids 1503-1657) bound to APC and CLIP-170 to form a complex that links microtubules and actin filaments during polarized cell migration.\(^{87}\)

Lastly, the effect of IQGAP1's activation via interactions with small GTPases could be examined using IQGAP1 mutants with modified or deleted GRD domains. One study proposed a constitutively active mutant of IQGAP1 that was defective in Cdc42/Rac1 binding and promoted lamellipodia formation in fibroblasts.\(^{27}\) IQGAP1 mutants missing the Cdc42 binding site (amino acids 1056-1077) could also be considered, but a previous study has shown that this causes IQGAP1 to mislocalize to the cell periphery.\(^{88}\)
4.2. Quantification of cytoskeletal regulatory protein localization to basal compartments as a function of time

Two major classes of actin nucleators that have been shown to associated with membranous organelles are the Arp2/3 complex and formins (i.e. Dia1/2/3).\textsuperscript{11,89,90} While Arp2/3 promotes brached actin assembly, formins promote the assembly of unbranched actin structures. In addition to functioning independently, Arp2/3 and Dia1 have been shown to function cooperatively to regulate processes such as E-cadherin-mediated cell-cell contacts and phagocytosis.\textsuperscript{91–94} Live-cell imaging of common actin nucleators, alongside actin and IQGAP1 could uncover which pathways are responsible for actin polymerization on the surface of multi-vesicular compartments. More specifically, does the type of nucleator recruited to the surface of multi-vesicular compartments vary with different actin-associated membrane dynamics? Perhaps during stabilization formins are recruited to stimulate unbranched polymerization, leading to linear actin filaments. On the other hand, during compartment disassembly Arp2/3-mediated branched actin assembly could be responsible for the non-linear increase in actin polymerization. If the same actin nucleator(s) are recruited to multi-vesicular compartments during both processes, it would suggest that the primary determining factor as to whether actin polymerization stabilizes or disassembles compartments is the activation, or lack thereof, of a negative feedback loop that curtails actin assembly.
4.3. Further characterization of the structure of multi-vesicular basal compartments

While the IQGAP1-associated actin filaments that surround basal compartments have been thoroughly imaged at the nanoscale using multi-round STORM, little is known about the nanoscale structure of the multi-vesicular membranous core. STORM microscopy has been used in previous studies to image lipid membranes stained with WGA-Alex 647 in fixed mouse retina and isolated Xenopus laevis oocyte nuclear envelopes. Using similar methods, STORM imaging could be used to visualize nanoscale structure of multi-vesicular basal compartments. This would enable a better understanding of the shape of the outer lumen and the formation of ILVs.

4.4. Revisiting population level studies of compartment incidence as a function of epithelial maturation

By imaging IQGAP1-associated multi-vesicular structures in fixed epithelial MCF-10A populations at various levels of confluency and times after seeding, statistical correlations between epithelial maturation and compartment formation could be made. Previous studies revealed potential positive correlations between compartment appearance and the maturation of epithelial MCF-10As into a 2-D monolayer. However, these studies were compromised by poor image resolution, which made accurately quantifying compartment numbers difficult. Revisiting these images at a higher resolution and imposing strict guidelines for counting compartments could yield more reliable results.
References


Appendix A

Figure A-1 – Diehl Lab STORM Microscope Schematic.
A.1. STORM optical train description

A.1.1. Excitation pathway

When active, a 600-800 nm acousto-optic tunable filter (AOTF$_{\text{NIR}}$, Gooch and Hsuego 97-0295-31) selectively diffracts a 600 nm imaging laser (L$_{640}$, Crystalaser DL640-100) onto the excitation pathway in response to radio wave frequencies from a single channel direct digital synthesizer (DDS, Gooch and Hsuego 97-0295-31). Since AOTF refraction is a polarization dependent process, a half-wave plate (HWP$_{633}$, Thorlabs WPH05-633) was inserted between the imaging laser and the AOTF to control the percentage of light that is refracted onto the optical train. Light refracted into the excitation pathway first passes through a half-wave plate (QWP$_{633}$, Thorlabs WPQ05M-633) to rotate the polarization of the excitation laser for molecules whose absorption is highly polarization dependent, such as gold nanoparticle fiducial markers. Then, light passes through a 10x telescope formed through a combination of achromatic doublets (AC$_{50}$ and AC$_{500}$) with focal lengths of 50 and 500 mm that are separated by a total distance of 550 mm. Finally, light is focused onto the back focal plane of the imaging objective (OBJ$_{60}$, Nikon Plan Apo Tirf 60x, oil immersion, numerical aperture (NA) = 1.45) to create a collimated sample illumination of 2 kW/cm$^2$. To achieve oblique illumination, light enters back focal plane of OBJ$_{60}$ at a position slightly off the center axis of the objective.

A.1.2. Activation pathway
A.1.3. Imaging pathway

The imaging pathway, or the path of fluorescent image exiting the back of the imaging objective, is separated from the excitation and activation lasers using the Cy5 channel of a three channel dichroic (DC3CH). Additional filtering of the image from the laser light sources is performed by a combination of a long pass filter (LP665, Chroma ET665lp), a Cy5 band pass filter (BP692, Semrock FF01-692/40), and a green laser line filter (SL532, Semrock NF01-532U-25). The image is focused onto the chip of an EMCCD (Andor DU-897E-CS0-#BV) using an achromatic doublet with a focal length of 400 mm (AC400). In order to achieve the astigmatism desired for three dimensional STORM imaging, a cylindrical lens (CL1000) is placed just before the EMCCD chip.

A.1.4. Focus lock pathway

An 830 nm laser (L830, Point Source) was combined with the Excitation pathway using a long pass dichroic mirror with a cutoff of 800 nm (LP800). This laser was focused onto the back focal plane of the imaging objective at a distance from the center axis of the objective lens such that the 830 nm laser beam hits glass/sample interface at an angle greater than the critical angle for TIRF illumination. Under this circumstance, 100% of the beam is reflected back through the objective, where it reverses back through the focus lock pathway until it is captured and focused by an achromatic doublet (AC500') onto a quadrant photodiode (QPD, New Focus 2901). While imaging, any changes in the distance between the objective and sample due to drift will result in a change in the 830 nm laser position along the x-axis of the photodiode. This change in position changes the voltage
of the x output of the QPD, which can be input into a PID loop that controls a piezo stage (Piezosystems Jena MIPOS100PLSG, Piezosystems Jena NV40/1CLE)) that moves the imaging objective to compensate for sample drift.

A.2. STORM acquisition guide

A.2.1. Turning on the STORM microscope

1. Power on all appropriate lasers. Allow each laser to warm up for ~1 minute before lasing.
2. Turn on the piezo stage controller.
3. Turn on QPD and QPD power supply.
4. Plug the EMCCD camera into back of acquisition computer.
5. Turn on the digital controller for the optical table.
6. Turn on the oscilloscope to monitor the QPD voltage from the x output. This gives the user a means to track the focus lock function.
7. For each AOTF in use, plug a 12 V DC power supply into back of the corresponding direct digital synthesizer.

A.2.2. Configuring an AOTF for direct STORM

1. Ensure that the AOTF for excitation laser is connected to the acquisition computer via USB.
2. Run the program “AODS Controller”.
3. Enable modulation.
4. Load saved spectrum 1.

5. Click the send button in the leftmost panel under the list of channels

**A.2.3. Configuring AOTFs for multicolor STORM**

**Brief explanation:** Gooch and Hsuego AOTFs can be used to switch between color channels with a two bit binary signal. The switching of colors is performed by the LabVIEW program *multicolor-storm-master.vi*, which sends the binary signal in the form of two digital outputs to the AOTF pins FSK+/ and BLANK+/-. However, the AOTF has to be configured for multicolor use before computer control over color switching can be achieved.

1. Switch on the power supply connected to analog input of AOTF for the excitation laser. Set the output voltage to 10 V. This only needs to be done for the excitation AOTF as the AOTF for the activation lasers uses a LabVIEW-controlled analogue output on the DAQ card.

2. Connect the AOTF for the excitation laser to the acquisition computer via USB.

3. **IMPORTANT:** Under the modulation tab, near profile tab, click on the asterisk so that it is highlighted. Initially finding this out was a major source of frustration.

4. Click the “Send Script File” button and select the file *imaging-script.txt*.

5. Close the AODS Controller program.

6. Connect the AOTF for the activation lasers to the acquisition computer via USB (I have not yet figured out how to simultaneously connect two AOTFs without getting an error, so each AOTF has to be set up sequentially).
7. **IMPORTANT**: Under the modulation tab, near profile tab, click on the asterisk so that it is highlighted. Initially finding this out was a major source of frustration.

8. Click the “Send Script File” button and select the script *activation-script.txt*.

**A.2.4. Setting the illumination cycle for multicolor STORM**

1. Open *multicolor-storm-master.vi*.

2. Set parameters for illumination cycle
   
   a. **Color select** - Selects activation colors to use.

   b. **Activation/Imaging frames** - Sets how many consecutive camera frames to expose the sample to activation and excitation illumination respectively. When the EMCCD is taking images, if the program is running it will automatically begin alternating between activation and excitation illumination. If multiple activation colors are selected, then the program will automatically rotate through the colors after every completed activation and imaging cycle.

   c. **Activation Power** - Controls output to AOTF in the activation laser optical train. In most cases, it is probably easier to control sample illumination intensity by rotating the half wave plates in front of each laser source.

   ASIDE: Controls associated with multicolor storm only work if the AOTFs are configured for multicolor imaging. If AOTFs are configured for direct storm then the previously stated controls can be disregarded as they are no longer functional.
A.2.5. Configuring camera acquisition

1. Run Andor SOLIS.
2. Open the Setup acquisition window.
3. In the Setup camera tab set Acquisition mode to kinetic and select Frame transfer on. Set the Kinetic series length to the number of frames you wish to image (Typical STORM images take around 10,000 frames to reconstruct an image).
4. In the Spooling tab select Enable spooling and set the file type to Tiff. Specify the File stem and Location and then click OK.

A.2.6. Performing STORM image acquisition

1. Add fresh STORM imaging buffer to sample.
2. Oil the imaging objective and place the 10-well into the recess of the microscope stage. Gently tighten the set screw in the sample stage to hold the sample in place. Do not over tighten or unwanted strain will be placed on the 10-well.
3. If it is not open already, open multicolor-storm-master.vi.
4. Run multicolor-storm-master.vi.
5. Set position to ~ 20.
6. Manually adjust the sample focus using the micrometer on the three axis sample stage. If fine focus is necessary, adjust the focus by changing the position value in LabVIEW.
7. At this stage it is recommended to turf off or block all activation and excitation laser illuminations from the activation and/or excitation lasers to prevent
photobleaching during further acquisition setup. Alternatively, the one can move to an unimportant field of view.

8. Using an oscilloscope to monitor the QPD output voltage, center the TIRF reflection of the 830 nm focus lock laser on the QPD such that the x output is ~0 V.

9. Turn on excitation laser or move to the desired field of view to refocus on sample.

   Then, flip the Get setpoint switch to set PID focus lock setpoint. After this step, turn off or block the excitation and/or activation lasers to prevent photobleaching.

10. If necessary, set parameters for the acquisition of the transfer function between the QPD x/sum output and the z position of the imaging objective.

   a. *Transfer step size* - controls step size of objective motion. At each step, the voltage of the QPD will be read and stored alongside the corresponding objective z position. This will be used to interpolate the corresponding objective/sample separation from a given QPD voltage.

   b. *Transfer half range* - Controls how far on either side of the set point the transfer function is sampled.

   c. *Time between write and read* - Adds a delay between each step in the objective z position and the subsequent QPD voltage readout. This given the peizo stage time to stabilize after moving the objective.

11. Flip the Get transfer switch to acquire the QPD voltage to z position transfer function.

12. Flip the Run PID switch to automatically focus on sample.
13. Turn on or unblock the laser illuminations from the activation and/or excitation lasers.

14. Begin the image acquisition process by clicking the Take signal icon in Andor SOLIS. Make sure the camera is not acquiring for a few seconds before you start image acquisition so that *multicolor-storm-master.vi* can automatically reset the frame tracker to 0 in response to going > 1 s without receiving a TTL pulse from the EMCCD camera.

15. After image acquisition is over, turn off PID by flipping the Run PID switch.

16. You can save the depth per frame and color per frame data after image acquisition for potential later use during image processing by switching the Save frame data or Save color data controls respectively.

17. For multi-round imaging experiments, you can refocus onto the PID setpoint after performing any erasing and relabeling steps by switching the Run PID control and repeating the imaging process. Be sure to save any color or frame data prior to restarting the PID focus lock, since any unsaved data will be lost at this time.

**A.3. STORM calibration curve generation guide**

**A.3.1. Creating a calibration sample of precipitated fluorescent beads on a glass slide**

1. If necessary, create a stock bead suspension by adding 1 μl of 0.22 μm flash red fluorescent microspheres (Bangs Laboratories FCO2F) to 100 μl of water.
2. Create a final bead suspension for use during calibration by adding 1 μl of the stock dye suspension to 100 μl of 200 mmol MgCl₂.

3. Construct a flow chamber from a 1 mm thick, 18x18 mm square coverslip attached to a glass slide by two parallel pieces of double-sided tape that are separated by a distance of ~ 10 mm.

4. Pipette the final bead suspension into the flow chamber. Seal off the flow chamber edges with nail polish. Allow the nail polish seal to air dry for 10 min before imaging.

A.3.2. Taking a calibration image stack

1. Follow protocol for turning on the STORM microscope.

2. Configure the excitation AOTF for direct storm imaging, aka constant illumination.

3. Oil the objective and place the fluorescent-bead-containing flow chamber onto the microscope stage.

4. Open calibrate.vi in LabVIEW

5. Run Calibrate.vi

6. Bring dyes to focus by adjusting the “Position” control

7. Switch on “Create setpoint” to set position for the center of the calibration curve

8. Switch off or block the excitation illumination to prevent photobleaching of fluorescent beads.

9. If necessary, set parameters for the acquisition of the transfer function between the QPD x/sum output and the z position of the imaging objective.
a. **Transfer step size** - controls step size of objective motion. At each step, the voltage of the QPD will be read and stored alongside the corresponding objective z position. This will be used to interpolate the corresponding objective/sample separation from a given QPD voltage.

b. **Transfer half range** - Controls how far on either side of the set point the transfer function is sampled.

c. **Time between write and read** - Adds a delay between each step in the objective z position and the subsequent QPD voltage readout. This given the peizo stage time to stabilize after moving the objective.

10. Flip the **Get transfer** switch to acquire the QPD voltage to z position transfer function. The **Have transfer?** button will turn green when the transfer function acquisition process is complete.

11. If necessary adjust the parameters for calibration curve acquisition

   a. **Cal curve step size** - resolution of z steps in calibration curve (Default = .05 μm)

   b. **Cal curve half range** - Distance stepped away from zero point of the calibration curve (Default = .6 μm)

   c. **Delay before measure** - Time between objective steps before prior to measuring the astigmatism. Gives the focus lock time to settle at new set point (default = 500 ms).

   d. **Measurement time** - How many frames to image each calibration point in stepwise imaging protocol (default = 1000 ms).
12. Prior to acquisition of calibration curve, if the camera is on, turn camera off using Andor SOLIS. Switch the Camera reset control to set the Frame number back to 0 if necessary.

13. Turn on the Camera on control. The control readies the Labview software to begin counting camera frames.

14. Turn on or unblock the laser illumination from the activation excitation laser.

15. Begin acquiring and saving images for stepwise calibration curve using the Take signal icon in Andor SOLIS. Make sure that the Andor SOLIS software is set to acquire at least 1000 frames.

16. When calibration process is complete, a dialogue box will pop up for you to save two files sequentially. File one is the calibration data, which denotes which frames are used for calculating the calibration curve. File two is the z depth of the focal point of the objective. These files will be necessary later on when generating the calibration curve parameters in MATLAB.

17. Stop camera acquisition in Andor SOLIS.

**A.3.3. Fitting the x and y widths of dyes in a calibration image**

1. This step requires the use of mufit_analysis.py, part of the storm analysis software package 3d_daostorm developed by the Zhuang lab. Additional information can be found in the README.txt file in the 3d_daostorm installation folder.

2. Save a copy of the calibration_settings.xml file to the folder of your STORM file. This file will be referenced by mufit_analysis.py. Prior to use saving, make sure that the fit model is 3d.
3. In the command window, change the current directory to the directory of the calibration image tiff stack.

4. Run the following code in the command window, replacing file paths and filenames when appropriate:

   ```
   PYTHONPATH=~.../mufit_analysis.py calibration_image_name.tif
   output_file_name.bin calibration_settings.xml
   ```

5. The output binary file contains the fitted points in the calibration image.

**A.3.4. Fitting the x and y widths of dyes in a calibration image**

1. Run the function `my_calibration(my_bounds)` in MATLAB. The input argument `my_bounds` is the max and min z-extents that will be used in your non-linear fit. To reliably have data points at all values of the z range, the magnitude of `my_bounds` should be slightly smaller than the “Cal curve half range’ set in LabVIEW. A `my_bounds` input of [-400,400] usually works well and gives a sufficiently large range of z values.

2. Respond to the following the prompts to tell `my_calibration` the following:
   a. The location of the output binary from mufit_analysis.py
   b. The calibration data file that was saved after running Calibrate.vi
   c. The file containing the frame by frame z depth of the focal plane. This is another file that was saved after running Calibrate.vi.
3. After running the function `my_calibration`, it will output the parameters for the defocus curve used to solve the z depth of a fluorophore based on its x and y widths. It will also automatically save the parameters as a `.mat` file in the same directory as the fitted points binary file. Rows 1 and 2 correspond the parameters of the curve for the x width and y width as a function of z respectively. Each row contains five solved parameters (w0, wc, wd, A, and B). These parameters are entered into the `.xml` files required by `mufit_analysis` when the value `Z` is selected as the fitting model. Calibration curves should be stable over time and new ones only need to be generated when the position of the cylindrical lens or imaging lens is changed.

**A.4. STORM image analysis guide**

**A.4.1. Fitting STORM peaks**

1. Generate a command script for the peak fitting analysis of spooled tiff files by running `my_write_command_script.m`. Navigate to the folder containing the spooled tiff files when prompted. A text file of commands for running `mufit_analysis.py` on each tiff stack will be automatically saved in the same directory as the tiff files.

2. Paste a copy of `zfit_settings.xml` in the folder containing the spooled tiff files. If necessary modify the variable values to adjust fitting parameters. For 3-D storm imaging, set the fitting `model` to `Z` and make sure that the `wx_vs_z` and `wy_vs_z` parameters match those generated by the most current calibration curve.

3. In the command window, change the current directory to the directory containing the spooled tiff files.
4. Run the following commands to begin image acquisition:

   
   sudo chmod u+x ./my-command-script.txt
   /my-command-script.txt

5. You can check the accuracy of the fitting algorithm at any time by running the zhuang lab's visualizer.py program. For more information see the README.txt file in the 3d_daostorm directory.

6. After fitting is completed, corresponding binary files for each spooled tiff image will appear in the current directory.

**A.4.2. Reconstructing STORM image files in MATLAB**

1. Run my_storm_filter.m and navigate to the directory containing the mufit_analysis binary output files when prompted. This program filters out bad STORM localizations, strings individual blinking events spanning multiple frames into a single localization, and identifies potential fiducial markers. See commented MATLAB code for further details.

2. Run my_storm_render.m. This program uses the output file from my_storm_filter.m to reconstruct STORM images. If multiple files from different markers are selected to be rendered, then my_storm_render.m automatically renders a multicolor STORM image. The stacking of different markers can be performed by either fiducial marker alignment or normalized 2-D cross correlation. If only one marker is selected to be imaged, my_storm_render.m automatically renders the image with the color corresponding to z position. See commented MATLAB code for further details.
A.4.3. Performing normalized cross correlation analysis between two markers

1. Run my_xcorr_map_4.m. This requires the input file my-marker-data.mat that is automatically saved upon completion of my_storm_render.m. The program my_xcorr_map_4.m will only run successfully if my-marker-data.mat was generated in the process of rendering exactly two markers. See commented MATLAB code for further details.

A.5. Troubleshooting Guide

A.5.1. Poor image quality

Problem: Low intensity point spread functions

Solution A: Increase the excitation laser power to increase the on rate such that the average total on time is less than or equal to the camera frame rate.

Solution B: Reduce the concentration of the primary thiol in the STORM imaging buffer to the increase quantum yield of blinking events.

Problem: Signal density is too high

Solution: Increase the primary thiol concentration.

Problem: Signal density is too low

Solution: Increase the activation laser intensity. In the absence of an activator dye in close proximity to the reporter dye, 405 nm activation light can be used to directly increase the on rate of Cy5 or Alexa 647.
**Problem:** Dye blinking kinetics are a function of xy position

**Solution:** Ensure that laser illumination is centered in the field of view.

**Problem:** High background fluorescence

**Solution A:** Move the activation/excitation laser beams farther away from the center axis of the objective as they enter its back focal plane. This reduces the depth of the oblique illumination on the sample.

**Solution B:** Reposition the cylindrical lens farther away from the camera, this increases the rate in which PSFs astigmatism as a function of depth. This causes out of focus PSFs to expand more rapidly to the point where their emission is too diffuse to be measurable.

**Problem:** Fiducial markers are too bright

**Solution:** Change the polarization of the excitation laser by rotating HWP$_{633}$.

**A.5.2. PID feedback loop errors**

**Problem:** At the start of round 2, the focus lock does not return to the round 1 setpoint

**Solution:** Replace the imaging buffer, ensuring that no blocking buffer or PBS from the erasing step remains in the sample well. Slight variations in the NA of the imaging buffer can reposition the TIRF reflections of the 830 nm laser and offset the QPD feedback voltage.
Problem: PID moves away from setpoint

Solution: Change the sign of the proportional gain

Problem: PID overcorrects drift or does not correct drift fast enough

Solution: Tune the PID gains. Typically integral time and derivative time are set to 0 and the proportional gain is set very low.

Problem: PID does not engage

Solution: Check that the QPD x and sum signals are correctly reaching the inputs on the DAQ card using the Measurement and Automation Explorer (National Instruments). Occasionally inputs can saturate due to voltage leaks and the only solution is to reposition the BNC cables of the inputs.

A.5.3. Laser alignment errors

Problem: Illumination field moves while putting microscope into TIRF or oblique illumination

Solution: Ensure that the imaging laser is going straight up through objective.

Problem: Activation laser illumination is too low

Solution: Re-align the activation lasers through the single-mode fiber. Use a photodiode to measure the output of the single mode fiber and track the alignment progress.
A.5.4. Calibration Errors

**Problem:** Dyes bleach during calibration curve acquisition

**Solution A:** Decrease the imaging laser intensity and increase camera gain.

**Problem:** Calibration curve is too steep/flat

**Solution A:** If the curve is too steep move the cylindrical lens toward camera, if the curve too flat move the lens away from camera.

**Problem:** Astigmatism isn't oriented along x or y axis of camera

**Solution A:** Rotate the cylindrical lens.

**Problem:** Irregular PSF astigmatism

**Solution A:** Check alignment of AC400 or the cylindrical lens.
Table 1: List of oligonucleotide sequences used in design of DNA-circuits. To assemble probe complex (PC), anneal equal amounts of PS8_L + PS8_QNQ + D647.