THE ROLE OF AN ARF1 AND ARF6 SIGNALING PATHWAY IN HGF-STIMULATED EPITHELIAL CELL MIGRATION

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by

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Abstract

Hepatocyte growth factor (HGF) is a potent signaling factor that acts on epithelial cells, causing them to dissociate and scatter. This migration is coordinated by a number of small GTPases, such as ARF6 and Rac1. Active ARF6 is required for HGF-stimulated migration and intracellular levels of ARF6-GTP and Rac1-GTP increase following HGF treatment. During migration, cross talk between ARF6 and Rac1 occurs through formation of a multi-protein complex containing the ARF-GEF Cytohesin-2, the scaffolding protein GRASP/Tamalin, and the Rac1-GEF Dock180. Previously, the role of ARF6 in this process was unclear. We have now found that ARF6 and ARF1 regulate trafficking of GRASP and Dock180 to the plasma membrane following HGF treatment. Trafficking of GRASP and Dock180 is impaired by blocking ARF6-mediated recycling pathways and is required for HGF-stimulated Rac1 activation. In addition, HGF treatment stimulates association of GRASP and Dock180. Inhibition of ARF6 trafficking pathways traps GRASP and Dock180 as a complex in the cell. Finally, we found that the interaction between GRASP and Dock180 is required for efficient HGF function. These data elucidate a role for active ARF6 and ARF1 in cytohesin-induced Rac1 activation and demonstrate the importance of GRASP and Dock180 recycling in the HGF signaling pathway.
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<td>AP1, 3, and 4</td>
<td>Adaptor protein 1, 3, and 4</td>
</tr>
<tr>
<td>ARF</td>
<td>ADP-ribosylation factor</td>
</tr>
<tr>
<td>ARL</td>
<td>ARF-like</td>
</tr>
<tr>
<td>ARNO</td>
<td>ARF nucleotide binding site opener</td>
</tr>
<tr>
<td>Arp 2/3</td>
<td>Actin-related proteins 2/3</td>
</tr>
<tr>
<td>BiFC</td>
<td>Bimolecular fluorescence complementation assay</td>
</tr>
<tr>
<td>BRAG</td>
<td>Brefeldin resistant ARF GEF</td>
</tr>
<tr>
<td>CHO</td>
<td>Chinese hamster ovary</td>
</tr>
<tr>
<td>Dock</td>
<td>Dedicator of cytokinesis</td>
</tr>
<tr>
<td>DHR</td>
<td>Dock homology region</td>
</tr>
<tr>
<td>EFA6</td>
<td>Exchange factor for ARF6</td>
</tr>
<tr>
<td>EHD1</td>
<td>Eps-15 homology domain-containing protein 1</td>
</tr>
<tr>
<td>ELMO</td>
<td>Engulfment and cell motility</td>
</tr>
<tr>
<td>EMT</td>
<td>Epithelial-mesenchymal transition</td>
</tr>
<tr>
<td>FBX8</td>
<td>F-box only protein 8</td>
</tr>
<tr>
<td>FRAP</td>
<td>Fluorescence recovery after photobleaching</td>
</tr>
<tr>
<td>FRET</td>
<td>Fluorescence resonance energy transfer</td>
</tr>
<tr>
<td>GAB1</td>
<td>GRB2 associated binding protein 1</td>
</tr>
<tr>
<td>GAP</td>
<td>GTPase activating protein</td>
</tr>
<tr>
<td>GBF/BIG</td>
<td>Golgi BFA-resistance factor 1/BFA-inhibited GEF</td>
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<tr>
<td>GDP</td>
<td>Guanosine diphosphate</td>
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<td>GEF</td>
<td>Guanine nucleotide exchange factor</td>
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<tr>
<td>Term</td>
<td>Description</td>
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<tr>
<td>GRASP</td>
<td>General receptor for phosphoinositides 1 associated scaffolding protein</td>
</tr>
<tr>
<td>GRB2</td>
<td>Growth factor receptor-bound protein 2</td>
</tr>
<tr>
<td>GRP1</td>
<td>General receptor for phosphoinositides-1</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine triphosphate</td>
</tr>
<tr>
<td>HGF</td>
<td>Hepatocyte growth factor</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen activated protein-kinase</td>
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<tr>
<td>MEK1</td>
<td>MAPK/ERK kinase 1</td>
</tr>
<tr>
<td>MDCK</td>
<td>Madin-Darby canine kidney</td>
</tr>
<tr>
<td>mGluR</td>
<td>Metabotropic glutamate receptor</td>
</tr>
<tr>
<td>PDK1</td>
<td>3’-phosphoinositide dependent kinase 1</td>
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<tr>
<td>PDZ</td>
<td>PSD-95/discs-large/ZO-1</td>
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<tr>
<td>PH</td>
<td>Pleckstrin homology</td>
</tr>
<tr>
<td>PI-3K</td>
<td>Phosphoinositide 3-kinase</td>
</tr>
<tr>
<td>PKB</td>
<td>Protein kinase B</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein Kinase C</td>
</tr>
<tr>
<td>PtdIns(3,4,5)P&lt;sub&gt;3&lt;/sub&gt;</td>
<td>Phosphatidylinositol 3,4,5-trisphosphate</td>
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<tr>
<td>PtdIns(4)P</td>
<td>Phosphatidylinositol 4-phosphate</td>
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<tr>
<td>PtdIns(4,5)P&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Phosphatidylinositol 4,5-bisphosphate</td>
</tr>
<tr>
<td>PIP-5K</td>
<td>Phosphatidylinositol 4-phosphate 5-kinase</td>
</tr>
<tr>
<td>PLD</td>
<td>Phospholipase D</td>
</tr>
<tr>
<td>PTEN</td>
<td>Phosphatase and tensin homologue</td>
</tr>
<tr>
<td>RAF</td>
<td>Rapidly accelerated fibrosarcoma</td>
</tr>
<tr>
<td>Ras</td>
<td>Rat sarcoma viral oncogene homolog</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>SH3</td>
<td>SRC homology 3</td>
</tr>
<tr>
<td>SOS</td>
<td>Son of sevenless</td>
</tr>
<tr>
<td>VSV-G</td>
<td>Vesicular stomatitis virus-glycoprotein</td>
</tr>
<tr>
<td>WASp</td>
<td>Wiskott-Aldrich syndrome proteins</td>
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</table>
Acknowledgements

In August 2012 I moved to State College and I had no idea what the upcoming years would bring. During my graduate career, I made friends, learned new lab techniques, met my husband, spent hours looking at fluorescent cells, got married, went to conferences, adopted two cats, commuters between Hershey and State College, learned even more new lab techniques, and became a scientist. As I look back, I know I would not be writing this dissertation today if it weren’t for the many family, friends, and colleagues who have encouraged and helped me along the past five years.

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Chapter 1

Introduction

Epithelial tissue

Functions of epithelial tissue

Epithelial cells are arranged as single layer sheets that line the inside of organs and play a number of important roles. First, epithelial cells act as a protective barrier between the underlying specialized cells and dangers such as toxins, pathogens, or physical damage. They also regulate the absorption and transportation of nutrients and chemicals into the blood supply or intestines. Some epithelial cells contain cilia that help create currents and push substances through the lumen. For example, ciliated epithelial cells aid in the movement of the ovum to the uterus through the fallopian tubes [1]. Finally, epithelial cells assist in digestion, excretion, and other metabolic processes through the secretion of certain fluids. For instance, epithelial goblet cells located in the trachea secrete mucus that aids in the removal of dust and bacteria from the lungs [2,3].

Epithelial cell polarity

In order to carry out their particular functions, epithelial cells form polarized sheets containing distinct apical and basolateral domains. The apical plasma membrane faces the lumen or outside environment. The basolateral region of the cell membrane faces away from the lumen and contacts the basement extracellular matrix. The two domains differ in behavior as well as protein composition [4]. In the small intestine, the apical membrane contains multiple microvilli that increase the surface area of the
membrane and aid in nutrient absorption [5]. On the other hand, the basolateral membrane removes metabolic waste and secretes nutrients into the bloodstream that had been absorbed from the lumen. In order to perform their separate functions, both domains contain a distinct set of membrane proteins that are distributed and kept apart by polarized trafficking machinery [6]. Cell junctions (which will be discussed below) physically prevent the diffusion and mixing of membrane proteins between the two domains [7].

*Epithelial cell junctions*

Epithelial cell monolayers are held together by four important cell-cell junctions: adherens junctions, tight junctions, desmosomes, and gap junctions (Fig. 1.1). These junctions allow a cell to interact and communicate with adjacent cells or the extracellular matrix. First, adherens junctions are composed of trans-membrane cadherin proteins and the catenin family of proteins. These protein complexes interact with the actin skeleton and form cell-to-cell contacts with neighboring cells, thus anchoring the two cells together [8]. Second, tight junctions are composed of the trans-membrane proteins occludin, claudin, and other scaffolding proteins. These junctions join the membranes of two adjacent cells. The formation of multiple tight junctions between cells preserves the polarity of the cell sheet by preventing the movement of ions from the apical to the basolateral side [9]. Third, desmosomes link extracellular adhesion proteins to intracellular intermediate filament cytoskeleton. They are not as rigid as tight junctions and thus help the sheets of cells withstand mechanical stress [10]. Lastly, gap junctions, composed of connexin proteins, directly connect the cytoplasm of neighboring cells.
This allows for the regulated exchange of solutes and ions between the two connected cells [11]. Epithelial cells typically maintain these junctions and exist as immobile sheets. However, there are circumstances in which cells lose some of their contacts and adopt a migratory phenotype.

**Figure 1.1 Epithelial cell junctions.** Cell-cell junctions organize epithelial cells into a tightly packed polarized monolayer. Adherens junctions anchor neighboring cells through actin interactions. Tight junctions are composed of trans-membrane proteins that join the plasma membranes of neighboring cells. Desmosomes help the cell withstand mechanical stress through interactions with cytoskeleton filaments. Gap junctions directly connect the cytoplasm of neighboring cells to allow the exchange of ions and solutes between the cells.

**Epithelial cell migration**

*Examples of epithelial cell migration*

When epithelial cells do migrate, they can migrate as sheets or separate and migrate individually [12]. During gastrulation, epithelial cells shift to a migratory phenotype and travel to the primitive streak where they mature into the primary germ layers: endoderm, ectoderm, and mesoderm [13]. Similarly, mammary gland
development involves the migration of cell as a multilayered collective to form branching ducts [14]. Following injury, inflammatory factors signal for cells surrounding the wound to migrate into the injured area and proliferate; creating a new epithelial layer. Movement occurs as sheets from all edges of the wound and stops when the migrating cells meet in the center of the injury [15,16].

**Mechanism of epithelial cell migration**

Regardless of its occurrence during development or injury recovery, individual cell migration can typically be broken down into four major steps (Fig. 1.2). First, actin polymerizes to form protrusions, known as lamellipodia and filopodia, at the leading edge that push the cell forward. Second, adhesions linking the cell to the substratum form at the leading edge. Third, stress fibers located in the rear of the cell contract and this contraction pushes the nucleus and cell body towards the front of the cell. Finally, cell adhesions located along the rear of the cell are broken down and the trailing edge is able to retract [12,17].

In addition to changes in the cytoskeleton and adhesions that occur during migration, cells also lose their apical-basolateral polarity and instead establish a front-rear axis. This new polarity allows cells to maintain persistent and directional migration [12]. The localized activation of the Rho family of small GTPases plays a critical role in the establishment of this polarity [18]. At the leading edge, Cdc42 and Rac activity promotes formation of actin filaments and microtubule membrane protrusions [19,20]. Cdc42 also regulates the orientation of the cell during migration [21]. At the trailing rear of the cell,
Rho activity stimulates rear-end retraction and forward motion through actomyosin contractions [12]. In addition, polarity complexes located at the leading edge are tightly

Figure 1.2 Epithelial cell migration. Epithelial cell migration occurs via a four-step process. 1) Extension: Actin protrusions push the cell forward. 2) Adhesion: New adhesions form at the leading edge. 3) Translocation: Contraction of stress fibers at the back of the cell pushes the nucleus and cell body forward. 4) De-adhesion: Adhesions at the rear are broken down and the trailing edge retracts.
coupled to activity of the Rho family small GTPases during establishment and maintenance of the front-rear axis of migrating cells. The polarity protein Par6 binds to Cdc42 and promotes aPKC activation, which leads to polarized microtubule organization [22,23]. At the leading edge, Par6 can also recruit the ubiquitination regulatory factor Smurf1. Recruitment of Smurf1 promotes RhoA degradation and limits RhoA activity to the rear of the cell [24]. The polarity protein Scribble controls cell orientation by interaction with the Cdc42-GEF βPIX that in turn promotes Cdc42 activation [25]. Loss of Scribble results in random non-directional cell migration [26]. Finally, recruitment and localization of the small GTPases and polarity complexes to the leading edge is regulated by the plasma membrane phosphoinositide composition of the migrating cell. Phosphatidylinositol-3,4,5-triphosphate (PtdIns(3,4,5)P₃) typically accumulates at the leading edge and phosphatidylinositol-3,4-bisphosphate (PtdIns(3,4)P₂) distributes through the rest of the membrane. The distinct localization of these two phosphoinositides is believed to be due to the spatial activity of phosphoinositide 3-kinase (PI-3K) at the leading edge and phosphatase and tensin homologue (PTEN) everywhere else [27,28].

Cells within a migrating group also establish a front-back orientation, but individual cells in the collective do so to varying extents. During collective migration, finger-like protrusions extend out from the cell monolayer and are directed by a single leader cell [29]. Cells located in the migration fingers exhibit strong directional polarity. However, the extent of polarization in cells decreases from the fingers to the epithelium [30]. It is also important to note that polarity during collective migration is induced and promoted by cell-cell adhesions that are often
cadherin-mediated [31,32]. Whether in an individual cell or groups of cells, the underlining theme is that establishment of polarity is essential for persistent directed cell migration.

*Collective cell migration*

Unlike individual cell migration, collective cell migration involves groups of cells that remain connected as they migrate. Collective migration is characterized by the following three traits. First, the cell-cell junctions between the cells remain physically and functionally intact [33,34]. Second, the actin cytoskeleton is polarized throughout the migratory cells in order to generate traction and protrusion force [35]. Third, collective migration leads to changes in the tissue as cells clear the path ahead and modify the extracellular matrix [36,37]. Cells can migrate collectively as a two-dimensional sheet, a three-dimensional strand, or as an unorganized mass [36,38]. Understandably, collective migration is complex and requires collective cytoskeletal organization, coordinated polarization, and cellular communication in order to produce additive and productive force. In order to create and maintain this force, cells pull on each other as well as exert force on the basement membrane. As one study explained, collective migration can be thought of as a tug-of-war with the leading cells acting as the anchor at the end of the rope [39,40].

*Three-dimensional cell migration*

Whether a cell is migrating in a collective or individually, the nature of the surface on which the cell is traveling affects the type of movement the cell adopts. Cell
migration on two-dimensional surfaces primarily follows the four-step cycle outlined above (Fig. 1.2). Cells spread out and form broad, flat lamellipodia. However, migration through a three-dimensional matrix requires slightly different processes due to the environment and space constrictions. In a three-dimensional matrix, migrating cells can adopt either thin lamellipodium- or bleb/amoeboid-driven movement depending on the nature of the extracellular matrix [41]. In addition, cells do not undergo the typical extension-retraction cycle like that seen in cells migrating on a two-dimensional plane [42]. Instead, cells undergo a pulling mechanism in which the leading cell pulls on collagen fibers to create a tensile force that propels the cell forward. This creates a track through the matrix that follower cells can utilize [43]. One common form of migration through a three-dimensional environment is amoeboid movement. During amoeboid migration, cells compact their shape to the surrounding matrix and form pressure driven bleb-like protrusions that investigate the surrounding area [44]. One study found that cells adopt an amoeboid form of migration when they sense a softer matrix substrate. Malignant cancer cells, in particular, often adopt a form of amoeboid movement when migrating through a three dimensional environment [45]. In this study we are primarily interested in individual cell migration that follows the four-step process described above (Fig. 1.2). However, it is important to keep in mind that other forms of migration do occur and can be pathologically significant.

*Epithelial-mesenchymal transition*

While it understanding the processes involved in normal migration is crucial, it is also important to understand the occurrences of abnormal migration. Epithelial cell
migration is tightly controlled, however, mis-regulated migration can occur and is often detrimental to the organism. The primary example of abnormal cell migration is cancer metastasis. During the initial steps of metastasis, epithelial cells undergo a process known as epithelial-mesenchymal transition (EMT). Cells undergoing EMT shift their polarity and lose their cell-cell adhesions. This allows them to dissociate and move away from their neighboring cells. The cells secrete proteases to break down the basement membrane and extracellular matrix. They can then enter the bloodstream and make their way to a secondary site [46,47]. Delineating the mechanisms of both normal and abnormal cell migration is critical, as cancer metastasis is associated with a poor prognosis [48]. The American Cancer Society estimates there will be 1,685,210 new cases and 595,690 cancer-related deaths in 2016. Of those new cases, the four most common types are epithelial cancers: Prostate, breast, lung/bronchus, and colon/rectum [49].

During mesenchymal migration, cells can migrate individually or collectively. In fact, one study found that populations of cells undergoing EMT exhibited a collective migratory front from which individual cells broke off and scattered away from. These individual cells migrate faster and have straighter paths than their collective relatives. This allows them to efficiently disperse away from the migrating group. Interestingly, they also observed that the migratory phenotype was relatively dynamic as some individually migrating cells switched back to collective migration over the time course and vice versa [50].
Migration Cues

Just as there are multiple forms of and reasons for migration, there are also multiple pathways and factor that signal for cells to move. Migration cues can include physical stress (such as injury), the characteristics of the extracellular matrix (such as stiffness or steepness), adhesion molecules, or soluble growth factors/ligands [51,52]. The interaction of these extracellular cues with receptors on the plasma membrane results in the activation of intracellular signal transduction pathways and eventually leads to actin re-organization [53-55]. Growth factors, in particular, can act as guidance cues that trigger and direct migration by way of a concentration gradient (chemotaxis) [51,56]. This is known to play an important role during development and tumor angiogenesis [56,57]. There are multiple growth factors that promote migration such as Platelet-derived growth factor, Epidermal growth factor, and Transforming growth factor-α [58-60]. Many of these growth factors transduce their signals through binding with their associated tyrosine kinase receptors [61].

Hepatocyte growth factor (HGF)

HGF structure and function

Another growth factor that promotes migration is Hepatocyte growth factor (HGF). HGF is a potent signaling molecule that is produced by mesenchymal cells, but primarily targets epithelial and endothelial cells. Stimulation with HGF affects both cell motility and morphogenesis [62,63]. HGF is encoded as a single polypeptide that is inactive when secreted. Serine proteases cleave the inactive polypeptide into a 69 kDa alpha chain and a 34 kDa beta chain. These two chains are bound by a di-sulfide bond to
create the active heterodimeric protein [64,65]. HGF signals through binding with its receptor, c-Met [66]. The c-Met receptor is a tyrosine kinase receptor that consists of an extracellular alpha subunit and a trans-membrane beta subunit connected by a disulfide bond. Binding of HGF results in phosphorylation of two tyrosine residues on the c-Met receptor [67,68]. This phosphorylation allows the receptor to interact with signal transducer proteins and leads to the activation of a number of pathways such as Ras, STAT-3, and PI-3K. (Fig. 1.3) [69-72].

HGF signaling plays an important role during both embryogenesis and wound healing. The involvement of HGF in embryogenesis is not limited to a single developmental stage, but it is essential throughout the whole process. HGF is critical for the development of a number of epithelial tissues such as the liver, placenta, and lung [73-75]. *In vitro*, Madin-Darby canine kidney (MDCK) cells form branching tubules when grown in collagen and treated with media containing HGF [76]. *In vivo*, mice

![cMet signaling pathway](image)

**Figure 1.3 cMet signaling pathway.** Binding of HGF to cMet results in the phosphorylation of key tyrosine residues on cMet’s trans-membrane β subunit. Other tyrosines on the C-terminal tail are phosphorylated and act as binding sites for substrates of other signaling pathways, such as STAT3 and PI3K. Activation of the cMet signaling cascade leads to changes in cell survival, migration, proliferation, and differentiation.
unable to produce HGF exhibit impaired development and die in utero [73]. In adult organisms, cMet is globally expressed and helps regulate normal functions such as blood glucose levels and blood cell production [77-80].

HGF also plays a critical role regenerating epithelium following injury and has been shown to drive the recovery of a number of epithelial tissues such as the kidney, lung, and gastro-intestinal organs [81-83]. After injury, levels of HGF mRNA and protein increase [84,85]. HGF promotes the survival of injured tissue by activating AKT/PI 3-Kinase and MAPK signaling pathways that in turn block apoptosis [70]. HGF also signals for healthy cells to proliferate and migrate to the damaged regions in order to re-cover the injured area [86-88]. Finally, HGF promotes re-differentiation of remaining cells to restore function [89-91].

**HGF and cancer**

Due to its role in so many important processes, HGF signaling is under tight control. However, loss of this control can lead to major problems. Both HGF and its receptor cMet are overexpressed in some epithelial cancers and this overexpression is associated with a poor prognosis [92,93]. Overexpression of HGF results in metastasis by promoting dissociation of cancerous cells from their neighbors, breakdown and invasion through the basement membrane, and angiogenesis [94,95]. Familial and sporadic forms of papillary renal carcinomas are often linked to missense mutations in the MET gene [96]. These mutations typically affect the tyrosine kinase domain of cMet and result in constant activation of the receptor. Continued activation of cMet leads to over-activity of its downstream pathways that regulate migration and proliferation [97]. In addition to
increased receptor activity, an inability to properly recycle the cMet receptor is also linked to tumorigenesis [98].

**HGF and small GTPases**

Under normal conditions, the signals downstream of HGF are primarily regulated by small GTPases in the Rho family. The best studied and most conserved members of the Rho family are RhoA, Rac1, and Cdc42 [99]. Rac1 regulates actin polymerization at the leading edge of the cell to produce fan-shaped lamellipodia protrusions [100]. This actin polymerization is promoted by interaction of Rac1 with the WAVE complex and Actin-related proteins 2/3 (Arp2/3) [101]. Cdc42 directs cell polarity with the Par-3/Par-6 polarity complex and localizes Rac1 activity at the leading edge through processes such as microtubule capture [102,103]. Cdc42 also regulates the protrusion of thin, F-actin rich filopodia at the leading edge [104]. RhoA can be found at the rear of the cell and mediates contraction of the cell body and detachment of the trailing edge [105]. Interactions between RhoA and p160Rock lead to increased phosphorylation of myosin and crosslinking of actin fibers [106]. This in turn results in contractile forces at the rear of the cell that push the cell forward [107]. RhoA activity at the trailing edge also results in disassembly of focal adhesions and release of the rear of the cell from the basement membrane [108].

**Endocytosis and recycling pathways**

Proper small GTPase function is dependent on its spatial and temporal localization in the cell. Therefore, endocytosis and recycling pathways play a critical role
in processes such as HGF signaling and initiation/maintenance of migration. There are a variety of mechanisms by which extracellular material and membrane proteins can be internalized, however, these mechanisms are typically separated into clathrin-dependent and clathrin-independent routes [109]. During clathrin-dependent endocytosis, adaptor proteins recognize signal sequences on the cytoplasmic domain of membrane proteins and package them into clathrin-coated vesicles for internalization [110]. Proteins that don’t contain an adaptor recognition sequence can be internalized by a variety of different routes that are all considered forms of clathrin-independent endocytosis [111]. Following initial internalization, cargo from either clathrin-independent or –dependent endocytosis is delivered to the early endosome for sorting. The early endosome is marked by the small GTPases Rab5a and has a mildly acidic lumen that facilitates the release of ligands from receptors [112]. One possible route of cargo from the early endosome is to the lysosome for degradation. The generation of tubular membranes from the early endosomes provides another route directly back to the plasma membrane and is known as the “fast recycling” pathway [113,114]. A final route cargo can take from the early endosome is to the early recycling endosome. The early recycling endosome emanates from the early endosome and lies along microtubules. Cargo transported to the recycling endosome travels via a “slow recycling” pathway back to the plasma membrane (Fig. 1.4) [115,116].

Transport between endocytic compartments is regulated by small GTPases in the Rab family. There are around 70 Rab proteins that regulate vesicle formation, movement, and docking during protein trafficking. Different Rabs associate with different organelles throughout the endomembrane system. Thus, the identity of an organelle or trafficking
pathway can often be determined by examining the associated Rabs (Fig. 1.4) [117]. Based on their nucleotide-bound state, Rabs cycle between association with an organelle membrane or existing free in the cytosol [118].

**Figure 1.4 Endocytic and recycling pathways.** Internalized cargo is transported to a Rab5-positive early endosome. From there, cargo is either: 1) transported to Rab7-positive lysosomes for degradation. 2) recycled directly back to the plasma membrane (fast recycling pathway). 3) transported to the plasma membrane by way of the Rab8/Rab11-positive early recycling endosome (slow recycling pathway).

**Rab8 and Rab11**

Rab8 and Rab11 are well-studied members of the Rab family and are known to localize to recycling endosomes. Rab8 was initially found to regulate trafficking of vesicular stomatitis virus-glycoprotein (VSV-G) and other newly synthesized membrane
proteins from the trans-Golgi network to the plasma membrane in [119]. Later, it was discovered that Rab8 also co-localizes with the recycling endosome marker transferrin and collaborates with AP-1B during transport from the recycling endosome to the basolateral membrane [120]. In addition, Rab8 is the predominant Rab found at the primary cilium. There, it interacts with cenexin and ODF, basal body and microtubule binding proteins, and regulates cilia formation through polarized trafficking [121]. In fact, Rab8 and Rab11 coordinate their activity during cilia formation as Rab11 directly interacts with and stimulates Rabin8 which in turn activates Rab8 [122]. Rab11 is the predominant marker used for identifying recycling endosomes and regulates trafficking of transferrin (another recycling endosome marker) from these organelles [123,124]. Overexpression of Rab11 leads to accumulation of e-cadherin in recycling endosome structures and changes in cell shape [125]. In MDCK cells, Rab11 and the motor protein myosin VB interact through the adapter protein Rab11-FIP2 to regulate the recycling of membrane proteins such as transferrin and IgA [126]. Rab8 is also able to interact with the tail region of myosin VB and localizes with myosin VB to EHD1-positive tubules [127]. Rab8 and Rab11 have become important experimental tools for visualizing recycling endosomes and confirming localization of proteins to these structures [4,109]. In addition, expression of mutant forms of either small GTPase disrupts recycling pathways and thus can be used to identify the trafficking routes of proteins of interest [120,124].
**ADP-ribosylation factors (ARFs)**

*ARF family and functions*

Another group of small GTPases that regulates endocytic recycling and HGF signaling is the ARF family. The ADP-ribosylation factor (ARF) family of proteins is a group of proteins that belong to the Ras superfamily. There are six ARF isoforms separated into three classes: Class I (ARF1-3), Class II (ARF4, 5), and Class III (ARF6) [128]. Class I ARFs are the most conserved and Class III the most divergent [129,130]. There are also a number of ARF-like (ARL) small GTPases that are believed to be involved in a broader range of processes [131]. ARF proteins cycle between a guanosine triphosphate (GTP) bound state or a guanosine diphosphate (GDP) bound state. The ARF family primarily regulates membrane trafficking and actin remodeling by modifying the lipid make-up of membranes, recruiting coat proteins, and interacting with other regulatory proteins [132]. ARFs modulate the lipid composition of membranes through direct activation of Phospholipase D (PLD) and Phosphatidylinositol 4-phosphate 5-kinase (PIP-5K). PLD hydrolyzes phosphatidylcholine to phosphatidic acid and PIP-5K phosphorylates phosphatidylinositol 4-phosphate (PtdIns(4)P) to PtdIns(4,5)P₂ [133].

ARF family members have both an amphipathic helix and a co-translationally added myristoyl group tail attached to the amino terminus. These features mediate membrane binding when the ARF is GTP bound and result in close association of the ARF with the membrane [134]. This is in contrast to Ras and Rho proteins which are anchored to the membrane by a long c-terminal linker [135]. Evidence suggests that ARF1 and 3 are released from membranes when their bound GTP is hydrolyzed, but
ARF6 (potentially ARF4 and 5, as well) remains bound to membranes in its GDP or GTP bound state [136,137].

**ARF localization and trafficking**

The various ARF isoforms function at different locations throughout the cell. ARF1, 3, 4, and 5 primarily regulate trafficking of vesicles in the secretory pathway originating from the endoplasmic reticulum and during intra-Golgi transport [138,139]. At the Golgi, the ARFs recruit COP1 as well as the clathrin adaptor proteins AP1, AP3, and AP4 to sort and transport vesicles to their necessary locations. Interestingly, the Golgi ARFs appear to be redundant and possibly work in pairs as knockdown of a single ARF isoform does not impair Golgi function [140]. ARF6, on the other hand, is predominantly found at the plasma membrane where it regulates endosomal recycling, actin cytoskeleton rearrangements, and membrane lipid composition [141]. Lipid composition of the plasma membrane is critical for activation of other small GTPases, membrane protein sorting, and clathrin-mediated endocytosis [142,143]. In addition, ARF6 regulates the trafficking of endosomal membrane from recycling endosomes to the plasma membrane. Tubular recycling endosomes are located around the microtubule-organizing center and are dynamic regions of trafficking that connect the endocytic and exocytic pathways [123,144]. ARF6-regulated trafficking from these structures is critical for the formation of focal adhesions and maintenance of polarization through trafficking of E-cadherin [125,145,146]. ARF6 has also been implicated in HGF-stimulated cell migration as levels of active ARF6 increase following HGF treatment [147]. In order for ARF proteins to properly direct their associated recycling pathways, their spatial and
temporal activation must be tightly controlled. Thus, it is key to also understand how ARF activity is regulated.

**Guanine nucleotide exchange factors (GEFs)**

*GEF overview and function*

ARFs, like all small GTPases, cycle between a GTP-bound and a GDP-bound state (Fig. 1.5). The GTP-bound conformation is generally considered the “active” state, as it is able to interact with membranes, while the cytosolic GDP-bound conformation is considered the “inactive” state. Differences between these two states are primarily observed in what is known as the Switch 1 and Switch 2 regions. Switch 1 and 2 tightly interact with the gamma phosphate of GTP, but not GDP [148]. ARFs in either conformation are able to bind downstream effectors, however, the affinity for various binding partners differs based on the nucleotide status [149,150]. GTPase activating proteins (GAPs) catalyze the hydrolysis of GTP to GDP while guanine nucleotide exchange factors (GEFs) assist in the exchange of GDP to GTP. There are 15 known human ARF GEFs that are separated into five families based on their domain organization and conserved sequence. These five families include: Exchange factor for ARF6 (EFA6), Brefeldin resistant ARF GEF (BRAG), F-box only protein 8 (FBX8), Golgi BFA-resistance factor 1/BFA-inhibited GEF (GBF/BIG), and Arf nucleotide binding site opener (ARNO)/cytohesin [151]. All ARF GEFs contain a highly conserved 200 amino acid Sec7 domain that catalyzes the release of bound GDP [152]. The catalytic domain is composed of 10 α helices and two of these helices form a ‘glutamic finger’ [153]. The Sec7 domain pulls the switch 1 and switch 2 regions open and the glutamate
residue is pushed into the nucleotide-binding region. Insertion of the glutamate residue competes with binding of GDP and leads to its displacement [154,155].

![Small GTPase regulatory cycle](image)

**Figure 1.5 Small GTPase regulatory cycle.** Small GTPases cycle between binding GTP (active form) or GDP (inactive form). GAPs stimulate the hydrolysis of GTP to GDP. GEFs activate small GTPases by assisting in the exchange of GDP to GTP. GTP-bound small GTPases interact with effectors, which results in the activation of downstream signals.

**Cytohesin family of ARF GEFs**

Our lab is particularly interested in the cytohesin family of ARF GEFs. The cytohesin family is composed of four members: Cytohesin-1, Cytohesin-2/ARNO, Cytohesin-3/GRP1 (General receptors for phosphoinositides-1), and Cytohesin-4 [156]. These ARF GEFs are approximately 45-50 kDa and have a highly similar domain structure. At the amino-terminus, approximately 60 amino acids make up a coiled-coil domain that facilitates homodimerization and interacts with other binding partners. The central catalytic Sec7 domain contains the GEF activity, as described above. The
pleckstrin homology domain (PH domain) is located at the carboxy terminus. The PH domain interacts with inositol phospholipids and is believed to regulate membrane recruitment and association [151,157,158]. Cytohesins can exist as splice variants with the PH domain containing either two or three glycines in a loop situated between two β-sheets [159-161]. This difference in glycine count leads to differential phosphoinositide affinity. The diglycine form binds tightly to PtdIns(3,4,5)P$_3$ while the triglycine form binds PtdIns(3,4,5)P$_3$ as well as PI(4,5)P$_2$ [159]. The number of glycines in the PH domain effects cytohesin localization, cell adhesion and spreading, and integrin recycling [162]. The cytohesin family is involved in a number of signaling pathways, such as integrin, EGFR, and PI-3K-dependent insulin signaling [163-165]. However, cytohesins do not appear to be involved in trafficking within the Golgi apparatus as inhibition of cytohesin activity has no affect on Golgi integrity [166].

Regulation of cytohesin activity

In order to activate ARFs at the correct time and in the correct place, cytohesin activity is tightly regulated. Protein Kinase C (PKC) phosphorylates a serine located in the carboxy-terminus of the polybasic domain. When un-phosphorylated, this region is highly positively charged due to the presence of many basic amino acids and assists in association with the plasma membrane [167,168]. The introduction of the negatively charged phosphate group thus leads to membrane dissociation [169]. Cytohesin membrane association is blocked by treatment with PI-3K inhibitors, suggesting that membrane recruitment is also facilitated by the local production of PtdIns(3,4,5)P$_3$ [170,171]. In addition, Cytohesin-2 exhibits two distinct auto-inhibitory functions. First,
a linker region between the Sec7 and PH domains acts as a pseudosubstrate to inhibit GEF activity [172]. Second, binding of the coiled-coil domain to the PH domain prevents membrane association. Phosphorylation of a conserved threonine residue within the PH domain, possibly by AKT, relieves this inhibition [173]. Cytohesin-2 also contains conserved binding elements in its PH domain that interact with members of the ARF-related GTPase family (ARL4, ARL4c, and ARL4d) and ARF6. This binding promotes association of Cytohesin-2 with the plasma membrane [174,175].

*Cytohesin-2 and cell migration*

As already mentioned, cytohesins are involved in a number of signaling pathways. Cytohesin-2, in particular, is known to play an important role during the regulation of cell migration, typically through activation of ARF6. For example, neurite outgrowth is blocked by inhibition of Cytohesin-2 or knockdown of ARF6 [176]. Cytohesin-2 also regulates migration of preadipocytes by binding with the focal adhesion adaptor protein paxillin and activating ARF6 [177]. In epithelial cells, Cytohesin-2 activates and co-localizes with ARF6 at the plasma membrane [178]. SecinH3 is an inhibitor of cytohesins and is known to block cytohesin GEF activity. Treatment with SecinH3 impairs HGF-stimulated migration [161]. MDCK cells scatter when treated with HGF and this scattering phenotype can be mimicked by overexpression of Cytohesin-2 [178,179].
Rac1 GEF Dock180

Dock180 structure and function

Other small GTPases, such as Rac1, have their own associated GEFs that regulate their activity. It is not unusual for cross talk and other interactions to occur between GEFs that then connect their target small GTPases within signaling pathways. Our lab has been investigating the relationship between the ARF-GEF Cytohesin-2 and the Rac1-GEF Dock180. Previous studies indicate that Dock180 co-localizes with Cytohesin-2 at the plasma membrane. Dock180 belongs to the Dedicator of cytokinesis (DOCK) family of GEFs and is an ortholog to the protein CED-5 found in C. elegans [180]. Currently, eleven human DOCK proteins have been identified [181]. Typical Rac1 GEFs contain a Dbl homology domain that harbors the GEF activity and a PH domain that regulates membrane association [182]. The DOCK family, however, consist of a Dock homology region 1 (DHR1) domain that interacts with phospholipids and a catalytically active DHR2 domain [181,183]. The DHR2 domain contains an essential valine residue within an α-helix insert that detects GDP-bound GTPases. This valine destabilizes bound GDP by forcing out the Mg2+ ion in the nucleotide pocket [184]. Initial studies found that overexpression of Dock180 led to plasma membrane ruffling and activation of Rac1 [185,186]. The phosphatidyl DHR1 binding domain of Dock180 interacts with PtdIns(3,4,5)P3 at the front of the cell and leads to polarized Rac1 activation at the leading edge. Continued Rac1 activation results in persistent, directed cell movement and formation of actin protrusions at the cell front [183,187].
Dock180 and ELMO interactions

It is difficult to describe the function of Dock180 in the cell without also mentioning the adaptor protein Engulfment and cell motility (ELMO). There are three isoforms within the ELMO family (ELMO1-3) with ELMO1 being the most studied [188]. A C-terminal proline-rich region and an extended α-helix of ELMO interact with the N-terminal SH3 domain of Dock180 [189]. This interaction appears to enhance binding of the complex to Rac1 and increases levels of Rac1 nucleotide exchange [190,191]. Interestingly, overexpression of Dock180 alone does not lead to noticeable cellular effects. However, co-expression of Dock180 and ELMO induces membrane ruffling, cell movement, and phagocytosis [187,188,190]. There are three proposed mechanisms for this relationship between ELMO and Dock180. First, ELMO may stabilize the interaction between Dock180 and nucleotide-free Rac1 by forming a ternary complex with the two proteins through its PH domain [191]. Second, the N-terminal SRC homology 3 (SH3) domain of Dock180 binds to Dock180’s Docker domain to negatively regulate its activity. ELMO relieves this steric inhibition by interacting with Dock180’s SH3 domain [192]. Finally, the presence of 550 amino acids in the N-terminal region of ELMO is necessary for recruitment of Dock180 to the plasma membrane. This suggests that ELMO may also be responsible for targeting of Dock180 [187,193].

ARF-to-Rac1 crosstalk

Formation of a multi-protein complex

While exploring the relationship between Dock180 and Cytohesin-2, our lab found that Cytohesin-2 stimulates Dock180-induced Rac1 activation and migration [194].
Figure 1.6 ARF6 to Rac1 crosstalk. The scaffolding protein GRASP binds the coiled-coil domain of Cytohesin-2 and the SH3 domain of Dock180 during formation of the multi-protein complex.

Interestingly, both inhibition of cytohesin GEF activity and expression of a dominant negative form of Dock180 impair HGF-stimulated Rac1 activation and migration. These results suggest that Cytohesin-2 and Dock180 are also involved in the HGF signaling pathway. Our lab was curious if Cytohesin-2 and Dock180 interact in the cell. We found that we could only co-immunoprecipitate Cytohesin-2 with Dock180 if cells were treated with the protein cross-linker DSP [195]. These results suggest that, while these two proteins do interact, the interaction is indirect and intervening proteins may be present. The small scaffolding protein General receptor for phosphoinositides 1 associated scaffolding protein (GRASP, also known as Tamalin) is known to interact with the
coiled-coil domain of Cytohesin-2 [196]. Knockdown of GRASP decreases the interaction between Cytohesin-2 and Dock180 in MDCK cells, suggesting that GRASP forms a multi-protein complex with the two proteins [195]. Further co-immunoprecipitation experiments with various truncation mutants revealed that GRASP’s leucine-rich region interacts with the coiled-coil domain of Cytohesin-2 and GRASP’s proline-rich region binds the SH3 domain of Dock180 [161]. Thus, GRASP acts a bridge between the two GEFs (Fig. 1.6).

**Role of active ARF6**

Expression of a Cytohesin-2 mutant lacking its coiled-coil domain is unable to promote HGF-stimulated Rac1 activation [161]. This suggests that formation of the multi-protein complex is essential for the signaling pathway from HGF stimulation to Rac1 activation. Interestingly, expression of a Cytohesin-2 mutant lacking its GEF activity, and therefore unable to active ARF6, also fails to stimulate Rac1 activation [178]. Thus, it appears that both formation of the Cytohesin-2/Dock180 complex and the GEF activity of Cytohesin-2 are required for Rac1 activation. These results suggest that active ARF6 is necessary for cytohesin-induced Rac1 activation following HGF stimulation. However, it is not clear how crosstalk between ARF6 and Rac1 occurs. We hypothesize that ARF6 regulates trafficking of GRASP and Dock180 from recycling endosomes to the plasma membrane following HGF stimulation. This positions Dock180 to activate the membrane localized Rac1 (Fig. 1.7).
Aims and Hypotheses

In my thesis I set out to explore why activation of ARF6 is required for cytohesin-induced Rac1 activation. I did this by first determining the cellular localization of GRASP and Dock180. Recycling endosomes are a subpopulation of endosomes that are dynamic centers of trafficking. ARF6 is known to direct recycling between recycling endosomes and the plasma membrane. Therefore, we hypothesized that GRASP and Dock180 would localize to recycling endosomes. Next, I wanted to explore how cytohesin GEF activity promotes Rac1 activation. Our lab has previously shown that both the GEF activity and scaffolding function of Cytohesin-2 is required for cytohesin-induced Rac1 activation. However, Cytohesin-2 is not able to activate Rac1 directly. We hypothesized that Cytohesin-2 activates ARF6 and ARF6 then regulates trafficking of GRASP and Dock180 from recycling endosomes to the plasma membrane. This would position Dock180 to activate a plasma membrane-localized Rac1. Finally, I wanted to determine the importance of the GRASP and Dock180 interaction in HGF function. Within that aim, I wanted to determine if the interaction between GRASP and Dock180 is stable or dynamic and whether the interaction is required for HGF function. We hypothesized that HGF stimulation would affect association of GRASP and Dock180. GRASP has been found to regulate the trafficking of receptors to the plasma membrane. Thus, we hypothesized that the interaction between GRASP and Dock180 would be required for HGF function, such as Rac1 activation.
Figure 1.7 Introductory model. We will test our hypotheses by exploring the following three aims. 1) Determine where GRASP and Dock180 localize in the cell. Hypothesis: GRASP and Dock180 localize to recycling endosomes. 2) Determine how Cytohesin-2 promotes Rac1 activation. Hypothesis: Cytohesin-2 activates ARF6. ARF6 regulates trafficking of GRASP and Dock180 to the plasma membrane. 3) Determine the importance of the GRASP/Dock180 interaction in HGF function. 3a) Determine if the interaction is stable or dynamic during HGF signaling. Hypothesis: HGF stimulation will affect GRASP and Dock180 association. 3b) Determine if the interaction is required for HGF function. Hypothesis: The interaction between GRASP and Dock180 will be required for processes downstream of HGF.

Hypotheses Summary:

1. GRASP and Dock180 localize to recycling endosomes.

2. ARF6 regulates trafficking of GRASP and Dock180 to the plasma membrane.

3a. HGF stimulation affects association of GRASP and Dock180.

3b. The interaction between GRASP and Dock180 is required for HGF function.
Main Finding 1: GRASP and Dock180 localize to recycling endosomes in resting cells.

ARF6 is known to regulate trafficking between recycling endosomes and the plasma membrane. In addition, Cytohesin-2 localizes to EHD1-positive recycling endosomes and promotes trafficking of integrin and R-Ras to the plasma membranes. We show that GRASP and Dock180 co-localize with recycling endosome markers. Treatment with HGF results in movement of GRASP and Dock180 off of these structures.

Main Finding 2: ARF1 and ARF6 regulate trafficking of GRASP and Dock180 from recycling endosomes to the plasma membrane.

GRASP and Dock180 form a multi-protein complex that is required for HGF-stimulated Rac1 activation. We show that HGF stimulation promotes trafficking of GRASP and Dock180 to the plasma membrane. This movement is dependent on functional ARF6 and ARF1 trafficking pathways. Inhibiting ARF associated pathways impairs HGF-stimulated Rac1 activation. This suggests that trafficking of Dock180 to the plasma membrane positions Dock180 to activate the membrane localized Rac1.

Main Finding 3a: HGF stimulation promotes GRASP and Dock180 association.

The proline-rich region of GRASP binds the SH3 domain of Dock180. We show that GRASP and Dock180 association increases following stimulation with HGF. We also determine that inhibiting trafficking traps GRASP and Dock180 together in resting cells.
Main Finding 3b: The interaction between GRASP and Dock180 is required for HGF function.

The proline-rich region of GRASP contains two potential SH3 binding domains. We mutated both SH3 binding sites to create a mutant form of GRASP that is unable to bind Dock180. We show that this mutant is mis-localized and it appears that GRASP recruits Dock180 to recycling endosomes. Expression of the mutant impairs HGF-stimulated trafficking of GRASP and Dock180 and Rac1 activation.
Chapter 2

Materials and Methods

Antibodies and reagents

mTurquoise-Rab8 T22N and -Rab11 S25N were detected using mouse anti-GFP antibody from Santa Cruz Biotechnologies (Santa Cruz, CA). Mouse anti-Rac1 and mouse anti-actin were purchased from BD Biosciences (San Jose, CA). Rabbit anti-ARF6 was obtained from Sigma (St. Louis, MO). Rabbit anti-ARF1 was a gift from Sylvain Bourgoin (Laval University, Quebec Canada). Mouse anti-HA, rabbit anti-HA.11, mouse anti-GAPDH, and mouse anti-myc (9E10) were obtained from Covance (Princeton, NJ). DyLight™ 594-conjugated donkey anti-mouse and DyLight™ 649-conjugated donkey anti-rabbit were purchased from Jackson Immuno Research Laboratories (West Grove, PA). Sepharose CL-4B beads were purchased from Sigma (St. Louis, MO). Glutathione agarose was obtained from Thermo Scientific (Rockford, IL). Western blots were incubated with HRP-labeled secondary antibodies purchased from Jackson Immuno Research Laboratories (West Grove, PA) and subsequently with SuperSignal West Femto Maximum Sensitivity Substrate from Thermo Scientific (Rockford, IL). A c-Digit imaging system from LI-COR Biosciences (Lincoln, NE) was used to image blots. SecinH3 was purchased from EMD Millipore (Billerica, MA). Human fibronectin matrix was obtained from Corning (Lowell, MA).
Cell lines

MDCK cells were obtained from Clontech (Mountain View, CA). Cells were maintained in DMEM containing 10% FBS and a 1% mixture of penicillin, streptomycin, and fungizone. Cell media was purchased from Mediatech, Inc. (Manassas, VA). FBS was obtained from Gemini Bio-products (West Sacramento, CA). Cells were cultured at 37°C and 5% CO₂.

Plasmid constructs

Human EHD1, Rab5a, Rab8, and Rab11 were a generous gift from Dr. James Goldenring (Vanderbilt University, Nashville, TN). The Rac1 FRET biosensor, pTriEx4-Rac1-2G, was obtained from Addgene (plasmid #66110) where it was deposited by Dr. Olivier Pertz (University of Bern, Switzerland)[197].

Primers

GRASP Site 1 mutation FWD primer:
5’-GAAGCCGCTCGCCGCCGCCGCCGCCGCCCTAGCCTCCCGCGCTGCGGTCCGCCCGCTCCGCCCG-3’

GRASP Site 1 mutation RVS primer:
5’-CGGGCGGAGCGCCGCCAGCGCGCGAGGCTCGGCGGGCGCGCGCGCGCGCG-3’

The amino acid sequence PSGPP (amino acids #37-41) was mutated to ASGAA

GRASP Site 2 mutation FWD primer:
5’-CCGGCTGCCGCCGCCGCTGCGCGCCGAGCCGCCGCCGCCGCGAGGAGAGGAGCTGTA-3’
GRASP Site 2 mutation RVS primer:

5’-TACAGCTCCTCCCCGCGGCTCCGGCCGAGGCGGAGGCCGAGCCC-3’

The amino acid sequence PPGAP (amino acids #46-50) was mutated to AAGAA

**siRNA mediated knockdown**

The siRNA targeting human ARF1 (target sequence: 5’-

CAAUCCUGUACAUUGAA-3’), human ARF6 (target sequence: 5’-

CCUGAGUACAAGAAACAU-3’), dog GRASP (target sequence 5’-

GCTTTAGATCCAGACTTA-3’) and scrambled non-targeting control were obtained from Dharmacon (Lafayette, CO). 1 x 10⁶ MDCK cells were transfected with siRNA (200 nM) using the Neon® transfection system (Thermo Scientific, Rockford, IL). Transfections were performed using the manufacturer’s recommended protocol for MDCK cells (1650 V, 20 ms, 1 pulse). For HGF time course and Dock180 recruitment experiments, 2 x 10⁵ cells were plated on square fibronectin-coated coverslips (20 ug/mL). For Rac1-GTP pull-down assays, 5 x 10⁵ cells were plated in 60 mm dishes.

**Live cell imaging**

MDCK cells were plated in 35 mm glass bottom dishes. Cells were imaged in FluoroBrite™ DMEM with 1% FBS and 10 mM HEPES purchased from Thermo Scientific (Rockford, IL). During imaging, cells were kept in a humidified chamber at 37°C and 5% CO₂. The temperature of the objective was maintained at 37°C by an objective warmer.
Immunofluorescence and image analysis

Cells were plated on flame-sterilized fibronectin-coated (20 ug/mL) glass coverslips. ProLong® Gold Antifade mountant from Life Technologies (Rockford, IL) was used to fix cells. Cells were imaged with an Olympus IX83 inverted microscope (Tokyo, Japan). Images were analyzed and processed by the imaging software Slidebook 6.0 from Intelligent Imaging Innovations (Denver, CO) or Fiji [198].

Localization of GRASP/Tamalin and Dock180 with endosome markers

First, a Laplacian filter was applied to the channel containing the marker protein (EHD1, Rab8, Rab11, Rab5a, or Rab7). Masks were created over the filtered marker channel and the whole cell area. Slidebook 6.0 calculated the fluorescent sum intensity for GRASP (WT or DM) or Dock180 in both masks. For each protein, the fluorescent sum intensity in the marker channel was normalized to the fluorescent sum intensity of the whole cell.

Localization of GRASP/Tamalin and Dock180 at the plasma membrane

Slidebook 6.0 (Fig. 3.3) and Fiji (Fig. 3.4, 3.5, 4.4) were used to determine the levels of GRASP and Dock180 at the cell periphery.

Slidebook 6.0: First, the Dock180 channel of each image was masked to get a mask of the whole cell area. The fluorescent sum intensity of GRASP and Dock180 in the whole cell area was calculated. Next, the interior of the whole cell mask was erased so that the remaining portion of the mask only covered the cell periphery. This time, the fluorescent sum intensity of GRASP and Dock180 in the peripheral mask area was
calculated. For each given cell, the peripheral fluorescent sum intensity of GRASP or Dock180 was then normalized to the fluorescent sum intensity of the whole cell.

**Fiji:** The threshold function of the software was used to create a mask covering the whole cell area of each cell. Minimizing the mask of the whole cell by five pixels then created an interior mask. The fluorescent sum intensity of each protein was calculated for the whole cell mask and the interior cell mask. The fluorescent sum intensity of GRASP and Dock180 at the periphery was calculated by subtracting the fluorescent sum intensity of either protein in the interior mask from the fluorescent sum intensity of that protein in the whole cell mask. Again, the fluorescent sum intensity of GRASP or Dock180 at the periphery was normalized to the fluorescent sum intensity of the whole cell.

*Calculation of FRET signal*

Slidebook 6.0 (Fig. 3.8, 3.9, 4.1) and Fiji (Fig. 3.7) were used to calculate and quantify FRET signal.

**Slidebook 6.0:** Images were taken using either CFP donor/YFP acceptor or YFP donor/mCherry acceptor defined channels. Standardized exposure settings were used for each experiment. Bleed-through coefficients were calculated using control images expressing either donor or acceptor fluorescent proteins alone. The corrected FRET channel was calculated for each image and whole cell masks were created for each cell using the Dock180 channel. The whole cell masks were used to quantify the fluorescent sum intensity of the corrected FRET channel and the whole cell area. The FRET channel fluorescent sum intensity was then normalized to the whole cell area. Images shown are
colored so that warm values correlate with strong FRET signal and cold values correlate with weak FRET signal.

**Fiji:** Fiji imaging software was used to analyze the Rac1 FRET probe images. Images were taken using the CFP donor/YFP acceptor defined channels. Dividing the FRET signal channel by the YFP signal channel created a ratio image for each cell. A whole cell mask was made over the ratio image and minimizing the whole cell mask by ten pixels created an interior mask. Fiji calculated the FRET fluorescent sum intensity in the whole cell mask and the interior mask. FRET fluorescent sum intensity at the periphery was calculated by subtracting the interior fluorescent sum intensity from the whole cell fluorescent sum intensity. Fluorescent sum intensity at the periphery was normalized to the fluorescent sum intensity of the whole cell.

All mask raw values were uploaded to Microsoft® Excel® for Mac 2011. Two sample T-tests were performed in Microsoft® Excel®. All means show are ± standard error.

**HGF stimulation**

For experiments involving knockdown, cells were transfected with desired siRNA by Neon transfection as described above. Following Neon transfection, cells recovered overnight in antibiotic-free media supplemented with 10% FBS. Cells were switched to new media (10% FBS, 1% antibiotics) and transfected with the desired constructs using Lipofectamine 3000 purchased from Invitrogen (Carlsbad, CA). Cells were allowed to express for 10-12 hours and then switched to serum-free media (containing 1% FBS).
overnight. The following morning, cells were incubated in the presence or absence of 10 ng/mL HGF (Rac FRET probe experiments were incubated with 20 ng/mL HGF). After zero, six, eight, or 12 hours, cells were rinsed with PBS, fixed, and mounted using ProLong Gold Antifade mountant (Life Technologies). HGF stimulation experiments not involving knockdown followed the above protocol beginning with Lipofectamine 3000 transfection.

**Bimolecular fluorescence complementation assay**

MDCK cells were transfected with VN-tagged Cytohesin-2 or VC-tagged GRASP constructed using Lipofectamine 3000 and plated on fibronectin-coated coverslips. After approximately 24 hours, cells were incubated with 10 ng/mL HGF for six hours. Cells were then fixed and blocked with immunofluorescence blocking buffer (10% normal goat serum and 0.02% Saponin in PBS) for 10 minutes. Following blocking, cells were incubated with a 1:50 dilution of primary antibody in a humidified chamber for one hour and 30 minutes. Cells were washed with PBS and then incubated with a 1:400 dilution of secondary antibody for 30 minutes. Cells were again washed with PBS. After a final rinse with water, coverslips were mounted unto slides and imaged by deconvolution microscopy.
**Rac1 activation assay**

*Dominant negative rab studies*

MDCK cells were transfected with either mTurquoise-Rab8 S22N or –Rab11 T25N using Lipofectamine 3000 (Invitrogen). After 10-12 hours of expression, cells were switched to serum-free media overnight. The next morning, cells were incubated in the presence or absence of 20 ng/mL HGF. After six hours, cells were lysed for pull-down.

*ARF6 and ARF1 knockdown studies*

MDCK cells were transfected with siRNA against ARF1 or ARF6 using the Neon transfection system. Following transfections, cells were incubated in antibiotic-free media for 18 hours to recover. Cells were switched to fresh media (10% FBS, 1% antibiotics) for 10-12 hours and then to serum-free media overnight. The next morning, cells were incubated in the presence or absence of 20 ng/mL HGF and lysed after six hours.

*GRASP double mutant studies*

MDCK cells were transfected with YPET-GRASP double mutant using Lipofectamine 3000 (Invitrogen). Cells were allowed to express for 10-12 hours and then switched to serum-free media over night. The next morning, cells were incubated in the presence or absence of 20 ng/nL HGF and lysed after six hours.

Cells were lysed in 50 mM Tris, pH 7.5, 100 mM NaCl, 2 mM MgCl₂, 1% NP-40, 10% glycerol, 1 mM DTT and 1 mg/mL each pepstatin, leupeptin, and antipain. Lysate was clarified by CL-4B Sepharose beads and a 10 minute spin at 14,000 x g. A portion
(9%) of the clarified lysate was saved to check protein expression. The remaining clarified lysate (500 ul) was incubated for 45 minutes with GST-PBD bound to glutathione-Sepharose beads. Beads were washed three times with lysis buffer and eluted into 30 ul SDS-PAGE sample buffer. Samples were heated for 10 min at 65°C before analysis by Western blot. Levels of endogenous Rac1 were detected using Rac1-specific antibodies. Amount of active Rac1 was normalized to levels of total Rac1 in the whole cell lysate. In order to calculate fold HGF-stimulated Rac1 activation, the normalized levels of Rac1 in HGF-stimulated cells was divided by the normalized levels of Rac1 in untreated control cells.

**GRASP/Cytohesin-2 binding assay**

The coding sequence of either wild type GRASP or the GRASP double mutant was inserted into the pGEX2T plasmid. This produces a fusion of GRASP to GST. BL21 E. coli were transformed with the indicated plasmids and grown to OD$_{600}$ = 0.4 before induction with IPTG. Cells were grown at room temperature for 24 hours before lysis in 0.01% Triton X-100 in PBS for 10 minutes. Lysate was clarified by centrifugation for 20 minutes at 13,000 x g and then rotated with glutathione sepharose beads for two hours at 4°C. Beads were washed three times with PBS and stored at -80°C.

A 15 cm plate of MDCK cells was infected with adenovirus encoding myc-tagged Cytohesin-2. After six hours, cells were lysed in 50 mM Tris, pH 7.5, 150 mM NaCl, 10 mM NaF, 1 mM NaVO$_4$, 10 mM sodium pyrophosphate, 1% Triton X-100, 0.1 mM PMSF as well as 1 mg/mL each of pepstatin, leupeptin, and antipain. Lysate was clarified for 10 min at 12,000 x g with sepharose CL-4B beads. A portion (3%) of the
cleared lysate was saved to confirm protein expression. The remaining lysate was split in half and incubated with either GST-GRASP WT or GST-GRASP DM glutathione beads on a rotating shaker for 45 min at 4°C. Following incubation, beads were washed three times with lysis buffer and then eluted into 30 ul SDS-PAGE sample buffer. Samples were heated for 10 min at 65°C before analysis by Western blot.

**Reverse transcription PCR**

Total RNA was isolated from MDCK cells using the RNeasy mini kit (Qiagen, Valencia, CA). Custom primers were used to amplify GRASP (GRASP FWD: 5’-GGAGGAGAACCAGACCTTCG-3’; GRASP RVS: 5’-GCTGCTCTTTGCACCATTAGG-3’). ReadyMade primers used to amplify GAPDH were purchased from Integrated DNA Technologies (Coralville, IA). RT-PCR was performed with 50 ng of template RNA for GAPDH and 100 ng of template RNA for GRASP using the Azura Genomics One-Step Ultra RT-PCR kit (Raynham, MA). RT-PCR samples were run on an agarose gel and band intensities were measured using Fiji.
Chapter 3

**ARF6 and ARF1 Regulate Recycling of GRASP/Tamalin and the Rac1-GEF**
**Dock180 during HGF-Stimulated Rac1 Activation**

* The following chapter was published as a manuscript in *Small GTPases*.


**Introduction**

HGF is a signaling factor that causes cells to dissociate and scatter [199]. In order for migration to occur, HGF activates migratory signaling cascades that are regulated by small GTPases, such as ARF6 and Rac1 [147,200]. Small GTPases are regulated by guanine nucleotide exchange factors and the ARF-GEF Cytohesin-2, in particular, is known be involved in changes in cell shape and migration [132,151,201]. Inhibition of cytohesin GEF activity impairs HGF-stimulated wound healing, while overexpression of Cytohesin-2 mimics HGF-stimulated cell scattering [161,178]. The Rac1-GEF Dock180 co-localizes with Cytohesin-2 at the cell periphery and expression of Cytohesin-2 stimulates Dock180-induced Rac1 activation [194]. Inhibition of Cytohesin-2 GEF activity or expression of a dominant negative Dock180 mutant impairs HGF-stimulated Rac1 activation and migration [161]. These results suggest that both the ARF-GEF Cytohesin-2 and the Rac1-GEF Dock180 are also involved in the HGF-signaling pathway.

Our lab previously explored whether Cytohesin-2 and Dock180 form a complex in the cell and found that they interact indirectly through the scaffolding protein GRASP. The leucine-rich region of GRASP binds the coiled-coil domain of Cytohesin-2 while the proline-rich region of GRASP interacts with the SH3 domain of Dock180. Thus, GRASP
serves as a bridge between the two GEFs. Expression of a Cytohesin-2 mutant lacking its coiled coil domain fails to promote Rac1 activation, suggesting that formation of the complex is required for cytohesin-induced Rac1 activation [161]. Interestingly, expression of a Cytohesin-2 mutant lacking its GEF activity, but still containing its coiled-coil domain, is also unable to stimulate Rac1 activation [178]. These results suggest that cytohesin-induced Rac1 activation requires both the scaffolding function and the GEF activity of Cytohesin-2. It appears that activation of ARF6 is required for cytohesin-induced Rac1 activation. However, it’s not clear how crosstalk between ARF6 and Rac1 occurs. We hypothesized that ARF6 regulates trafficking of GRASP and Dock180 to the plasma membrane. This trafficking positions Dock180 to activate the plasma membrane localized Rac1. We examined localization of GRASP and Dock180 before and after HGF stimulation and found that both ARF6 and ARF1 regulate trafficking of GRASP and Dock180 from recycling endosomes to the plasma membrane. We also explored the dynamics of GRASP and Dock180 association following HGF-stimulation. The following chapter is taken from the results section of a manuscript published in Small GTPases. Figure legend numbering has been changed to match the dissertation numbering.

**Results**

**GRASP/Tamalin and Dock180 localize to recycling endosomes**

ARF6 is a key regulator of membrane traffic and is particularly involved during trafficking between recycling endosomes and the plasma membrane. Therefore, recycling endosomes are a promising cellular location for GRASP/Tamalin and Dock180 if ARF6
Figure 3.1 GRASP and Dock180 localize to recycling endosomes. A-C) MDCK cells were plated on fibronectin-coated coverslips and transfected with mTurquoise- Rab8 (recycling endosome marker), Rab11 (recycling endosome), or Rab5a (early endosome) with either YPET-GRASP or mCherry-Dock180 using Lipofectamine 3000. After 10-12 hours of expression, cells were switched to serum-free media overnight. The next day, cells were fixed and imaged by deconvolution microscopy as described in the Materials and Methods. In merge images, GRASP is pseudocolored yellow, Dock180 is pseudocolored red, and endosome marker is pseudocolored blue. Scale bars: 10 um. D) Dock180 and GRASP are more often found with the recycling endosome markers than with the early endosome marker Rab5a. Fluorescent sum intensity of Dock180 or GRASP in marker masks normalized to whole cells fluorescent sum intensity was calculated using Slidebook 6.0 in 79-80 cells. Data are means ± standard error. * p < 5 x 10^{-25}, T Test.

is indeed involved in their recycling. Recycling endosomes are a subpopulation of endosomes located around the microtubule-organizing center and are composed of a dynamic tubular network. They connect the endocytic pathway to the exocytic pathway and their dynamic nature suggests that they are high trafficking regions [202,203]. We hypothesized that if ARF6 regulates trafficking of GRASP and Dock180, then GRASP and Dock180 should be located on recycling endosomes. The membrane fission protein Eps-15 homology domain-containing protein 1 (EHD1) and the small GTPases Rab8, and Rab11 work in concert with ARF6 during trafficking and are all known to co-localize
with ARF6 at recycling endosomes [203-205]. We recently demonstrated that Cytohesin-2 localizes to EHD-1 positive endosomes [206]. EHD1, Rab8, and Rab11, therefore, can all be used as markers for recycling endosomes.

To test our hypothesis that GRASP and Dock180 localize to recycling endosomes, we expressed fluorescent-tagged GRASP and Dock180 constructs with either the recycling endosome markers Rab8 or Rab11 in MDCK cells. The early endosome marker Rab5a was used as a negative control. A proportion of the total GRASP or Dock180 population accumulated in a perinuclear region with the recycling endosome markers (Fig. 3.1A, B). Localization with Rab5a (early endosome) was not as pronounced (Fig. 3.1C). We found that the fraction of GRASP and Dock180 that localized with Rab8 or Rab11 was significantly greater than the fraction of these proteins that localized with the early endosome marker Rab5a, suggesting that GRASP/Tamalin and Dock180 localize to recycling endosomes in resting cells (Fig. 3.1D).

We then transfected MDCK cells with both GRASP and Dock180 and one of the recycling endosome markers EHD1, Rab8, or Rab11. The early endosome marker Rab5a was again used as a negative control. Both GRASP and Dock180 accumulate with the recycling endosome markers in an internal perinuclear region (Fig. 3.2A-C). Similar to before, GRASP and Dock180 were less likely to localize to the same regions as Rab5a (Fig. 3.2D). GRASP and Dock180 were again more often found with the recycling endosome markers EHD1, Rab8, and Rab11 than they were with the early endosome marker Rab5 (Fig. 3.2E, F). We also treated cells with HGF to observe dynamics of localization following cell stimulation. After stimulation, GRASP and Dock180 appeared
Figure 3.2 GRASP and Dock180 localization with recycling endosomes decreases upon HGF stimulation. A-D) MDCK cells were transfected with YPET-GRASP, mCherry-Dock180 and either mCerulean-C1-EHD1 (recycling endosome marker), mTurquoise 2-Rab8 (recycling endosome), mTurquoise 2-Rab11 (recycling endosome), or mTurquoise 2-Rab5a (early endosome) using Lipofectamine 3000. After 10-12 hours of expression, cells were switched to serum-free media overnight. The next day, cells were incubated with or without 10 ng/ml and fixed after 6 hours. Cells were imaged, and analyzed by deconvolution microscopy as described in the Materials and Methods. In merged images, GRASP is pseudocolored yellow, Dock180 is pseudocolored red, and endosome marker is pseudocolored blue. Scale bars: 10 μm. E,F) Sum Intensity of Dock180 (E) and GRASP (F) in marker masks normalized to whole cells masks was calculated using Slidebook 6.0 in 64-71 cells. Data are means ± standard error. * p < 5 x 10^{-5}, ** p < 5 x 10^{-13}, T Test.

more diffuse and no longer as tightly accumulated with EHD1, Rab8, or Rab11 (Fig. 3.2A-C). HGF treatment led to a decrease in localization of GRASP and Dock180 with the recycling endosome markers. However, localization of GRASP and Dock180 with Rab5a was unchanged (Fig 3.2E, F). These results support our hypothesis that HGF
promotes the movement of GRASP and Dock180 from recycling endosomes. We conclude that GRASP/Tamalin and Dock180 localize to recycling endosomes in resting cells and that HGF treatment promotes the movement of GRASP/Tamalin and Dock180 off of these structures.

*HGF stimulates cytohesin-dependent recycling of GRASP/Tamalin and Dock180 to the cell periphery*

Overexpression of Cytohesin-2 stimulates Rac1 activation and cell migration [178]. However, Cytohesin-2 is ARF specific and is not able to activate Rac1 directly, making it unclear how the ARF-GEF promotes Rac1 activation [157]. Deletion of the coiled-coil domain of Cytohesin-2 and elimination of its GEF activity both impair cytohesin-induced Rac1 activation [161,178]. This suggests that both formation of the GRASP and Dock180 complex and activation of ARF6 are required for cytohesin-induced Rac1 activation. ARF6 oversees the endocytosis and recycling of membrane adhesion proteins and Cytohesin-2 has been implicated in the regulation of integrin recycling [162,207,208]. We hypothesized that cytohesin dependent ARF activation regulates trafficking of GRASP and Dock180 to the plasma membrane. Transport to the plasma membrane would position Dock180 to activate the membrane localized Rac1 [209].

We tested if levels of GRASP and Dock180 increase at the periphery following stimulation of cells with HGF. We found that levels of both GRASP and Dock180 increased at the periphery over time following treatment with 10 ng/mL HGF (Fig. 3.3B, D, E). On the other hand, GRASP and Dock180 remained internal with some slight
Figure 3.3 HGF treatment stimulates recycling of GRASP and Dock180 to the plasma membrane. MDCK cells were co-transfected with YPET-GRASP and mCherry-Dock180 constructs using Lipofectamine 3000 and allowed to express for 10-12 hours on fibronectin-coated coverslips. Cells were switched to serum-free medium (1% FBS) overnight and treated as indicated the next morning. Cells were fixed 0, 6, 8, and 12 hours after treatment and imaged by deconvolution microscopy. Scale bars: 10 um. A) GRASP and Dock180 remain internal in control cells treated with serum-free media. B) Stimulation of cells with HGF (10 ng/mL) leads to movement of GRASP and Dock180 to the periphery. C) Concurrent treatment with the cytohesin inhibitor SecinH3 (15 μM) impairs HGF-stimulated (10 ng/mL) movement of GRASP and Dock180 to the periphery. D, E) Quantification of levels of Dock180 (D) and GRASP (E) at the periphery as calculated by Slidebook 6.0 in 65-72 cells. Data are means ± standard error of the peripheral sum intensity normalized to the whole sum intensity. Asterisk indicates statistical significance compared to zero hour of set. * = p < 5 x 10^{-3}, ** = p < 5 x 10^{-6}, T test.
perinuclear accumulation in control cells treated with serum-free media (Fig. 3.3A, D, E). SecinH3 is a triazolo compound that binds to the Sec7 catalytic domain of cytohesins and inhibits their GEF activity [166]. We have previously shown that treatment of MDCK cells with SecinH3 blocks HGF-stimulated migration and HGF-stimulated Rac activation [161]. Concurrent treatment of cells with SecinH3 and HGF inhibited HGF-stimulated movement of GRASP and Dock180 to the periphery (Fig. 3.3D, E). The appearance of cells treated with both SecinH3 and HGF was very similar to control cells (Fig. 3.3C). These results suggest that HGF stimulation promotes trafficking of GRASP and Dock180 to the periphery and this movement is dependent on cytohesin GEF activity.

Expression of Rab8 T22N or Rab11 S25N impairs HGF-stimulated recycling of GRASP/Tamalin and Dock180

The small GTPases Rab8 and Rab11 coordinate with ARF6 during trafficking from recycling endosomes to the plasma membrane [210,211]. Expression of the dominant negative forms of these proteins (Rab8 T22N and Rab11 S25N) traps cargo at recycling endosomes [212,213]. We hypothesized that inhibiting trafficking by expressing Rab8 T22N or Rab11 S25N should block movement of GRASP and Dock180 to the cell periphery. GRASP and Dock180 accumulated in a perinuclear location along with the dominant negative Rabs (Fig. 3.4A, B). We found that expression of Rab8 T22N and Rab11 S25N impaired HGF-stimulated movement of GRASP/Tamalin and Dock180 to the periphery (Fig. 3.4C, D). These results suggest that functional ARF6-associated trafficking pathways are required for HGF-stimulated recycling of GRASP/Tamalin and Dock180 to the periphery.
Figure 3.4 Rab8 T22N and Rab11 S25N impair HGF-stimulated recycling of GRASP and Dock180.

MDCK cells were transfected with YPET-GRASP, mCherry-Dock180 and either mTurquoise- Rab8 T22N or Rab11 S25N using Lipofectamine 3000. Cells were allowed to express for 10-12 hours and then switched to serum-free media (1% FBS) overnight on fibronectin-coated coverslips. The next morning, cells were treated with HGF (10 ng/mL) and fixed after the indicated time points. Cells were imaged by deconvolution microscopy. **A, B**) Expression of Rab8 T22N (A) or Rab11 S25N (B) blocks HGF-stimulated movement of Dock180 and GRASP to the periphery. In the merged images, Rabs are pseudocolored blue, GRASP is yellow, and Dock180 is red. Scale bars: 10 um. **C, D**) Fiji was used to calculate levels of Dock180 (C) and GRASP (D) at the periphery in 65-71 cells as described in the Materials and Methods. Data are means ± standard error of the peripheral sum intensity normalized to the whole sum intensity. Asterisk indicates statistically significant compared to zero hour of set.

* = $p < 5 \times 10^{-3}$, ** = $p < 10^{-5}$, T test.
**Knockdown of ARF6 or ARF1 blocks HGF-stimulated recycling of GRASP/Tamalin and Dock180**

The ARF protein family regulates a wide range of membrane trafficking routes in eukaryotic cells. The ARF family is composed of six isoforms (ARF1-6) separated into three classes (Class I-III) based on their structural similarities. ARF1-3 belong to Class I, ARF4,5 belong to Class II, and ARF6 is the sole member of Class III. The ARFs vary based on the cellular organelles to and from which they regulate trafficking. ARF6 is the most widely studied ARF and is primarily involved in regulating endocytosis and exocytosis at the plasma membrane [214]. ARF1, on the other hand, is believed to regulate cargo traffic at the Golgi and some endosomes [215]. While Cytohesin-2 is able to catalyze nucleotide exchange on both ARF1 and ARF6 in vitro, previous work suggested that ARF6 is its primary substrate in vivo [178]. However, ARF1 has been found to regulate recruitment of paxillin to focal adhesions and there is growing evidence that ARF1 may also be present at the plasma membrane [174,216].

ARFs have been known to act in pairs and it is possible that both ARF1 and ARF6 may regulate recycling of GRASP and Dock180 [140,174,217]. In order to explore which ARF isoform may be involved in recycling of GRASP and Dock180, we knocked down either ARF6 or ARF1 and transfected cells with exogenous GRASP and Dock180 constructs (Append. A Fig. 3.1). We then observed levels of GRASP and Dock180 at the periphery following stimulation with HGF. In control cells, prior to stimulation, GRASP and Dock180 were slightly accumulated in a perinuclear region. Following treatment with 10 ng/mL HGF, GRASP and Dock180 could be observed at the periphery, on tubules, and in bright puncta (Fig. 3.5A). On the other hand, after ARF1 or ARF6 knock
Figure 3.5 ARF1 and ARF6 knock-down impairs HGF-stimulated recycling of GRASP and Dock180. 

A-C) MDCK cells were transfected with control siRNA or siRNA against either ARF6 (B) or ARF1 (C) by Neon transfection and plated on fibronectin-coated coverslips as described in the Materials and Methods. The following day, cells were transfected with GRASP and Dock180 by Lipofectamine 3000 and allowed to express for 10-12 hours. Cells were switched to serum-free media overnight and treated with HGF (10 ng/mL) the next morning. Cells were fixed at the indicated time points and imaged by deconvolution microscopy. Scale bars: 10 um. 

D,E) Fiji imaging software was used to calculate levels of Dock180 (D) and GRASP (E) at the cell periphery in 62-78 cells as described in the Materials and Methods. Data are means ± standard error of the peripheral sum intensity normalized to the whole sum intensity. Asterisk indicates statistically significant compared to zero hour of set. *= p < 5 x 10^{-5}, t-test.
down, Dock180 appeared slightly cytosolic with some accumulation in a perinuclear region and this did not change following HGF stimulation. GRASP was often observed as bright puncta with some accumulation in perinuclear regions as the time course progressed (Fig. 3.5B, C). In addition, we found that knockdown of either ARF6 or ARF1 impaired trafficking of GRASP and Dock180 to the periphery (Fig 3.5D, E). Based on these results, we conclude that both ARF6 and ARF1 regulate HGF-stimulated recycling of GRASP/Tamalin and Dock180 to the plasma membrane.

**Inhibition of ARF6 recycling pathways impair HGF-stimulated Rac1 activation**

Both the spatial and temporal activation of Rac1 are key to its proper function in the cell. Rac1 proteins are c-terminally isoprenylated after translation at a cysteine that belongs to a conserved CAAX sequence. This isoprenoid moiety anchors Rac1 to membranes [218]. Rho-GDI’s bind to Rho proteins such as Rac1 and block the hydrophobic isoprenoid moiety, keeping Rac1 soluble, cytosolic, and inactive [219]. Following cell stimulation, it is believed that Rac1 is recruited to the plasma membrane. Recruitment could occur by hydrophilic interaction of Rac1’s polybasic domain with the negative lipid heads present in the plasma membrane [220]. At the membrane, Rac1 can then be activated by Rac1-GEF’s, such as Dock180, and subsequently bind effectors to initiate downstream effects. Our results above suggest that inhibiting ARF6 trafficking pathways traps Dock180 at an internal location within the cell. Based on this, we hypothesize that inhibition of ARF6 recycling pathways should also impair activation of Rac1, as Dock180 is no longer able to localize to Rac1. To impair trafficking, we again transfected MDCK with either Rab8 T22N or Rab11 S25N constructs. The levels of
Figure 3.6 Inhibition of ARF-associated recycling pathways impairs HGF-stimulated Rac1 activation. A, B) Expression of Rab8 T22N or Rab11 S25N impairs HGF-stimulated Rac1 activation. MDCK cells were transfected with mTurquoise-Rab8 T22N, Rab11 S25N, or empty vector using Lipofectamine 3000. Cells were allowed to express for 10 hours and switched to serum-free media overnight. Cells were incubated in the presence or absence of HGF (20 ng/mL) and lysed after six hours. GST-PBD pull-downs were performed to isolate Rac1-GTP. Starting lysate and pull-down samples were run on a Western and blotted for Rac and GFP (Rabs). Representative gels are shown. Experiments were quantified by the LI-COR imaging system for Rab8 T22N (A, n = 8) and Rab11 S25N (B, n=7). Data shown are means ± standard error. * = p < 0.05, paired T test. C,D) ARF1 and ARF6 knockdowns impair HGF-mediated Rac activation. MDCK cells were transfected with control siRNA or siRNA against ARF1 or ARF6 by Neon transfection. After 30 hours of expression, cells were switched to serum-free media overnight. The next day, cells were incubated in the absence or presence of 20 ng/mL HGF. After six hours, cells were lysed and Rac1-GTP was isolated by GST-PBD pull-down as described above. ARF6 (C) n = 7, ARF1 (D) n = 9. Data shown are means ± standard error. * = p < 0.05, paired T test.
Rac1-GTP before and after stimulation with HGF were quantified using an active Rac1 GST-PBD pull-down assay. When Rab8 T22N or Rab11 S25N was expressed, HGF-stimulated Rac1 activation was significantly impaired (Fig. 3.6A, B). These results suggest that ARF6-associated recycling pathways are required for HGF-stimulated Rac1 activation.

We next explored the effect that knockdown of either ARF1 or ARF6 had on HGF-mediated Rac1 activation. As already described in the paragraph directly above, we know that inhibition of trafficking pathways associated with ARF6 impair HGF-stimulated Rac activation. We hypothesized that directly knocking down ARF1 or ARF6 should also impair Rac1 activation. We found that knockdown of either ARF1 or ARF6 impaired HGF-stimulated Rac1 activation (Fig. 3.6C, D). From these results, we conclude that functional ARF1 and ARF6 trafficking pathways are necessary for efficient activation of Rac following HGF stimulation. The results suggest that recycling of Dock180 is required for HGF-stimulated Rac1 activation; presumably to trans-locate Dock180 from recycling endosomes to the plasma membrane where Rac1 is located and waiting to be activated.

*Rac1 is activated at the plasma membrane*

Our model is dependent on the assumption that Rac1 is activated at the periphery by Dock180 and not at an interior location. Our findings that inhibition of ARF6 trafficking pathways impairs movement of Dock180 as well as HGF-stimulated Rac1 activation support this assumption. However, we wanted to further confirm the localization of Rac1 activation. A second generation Rac1 FRET biosensor (pTriEx4-
Rac1-2G) was recently created to detect localization of active Rac1 in vivo [197]. We used this probe to test the hypothesis that active Rac1 would increase at the periphery of the cell following HGF stimulation. We found that treatment with HGF led to a significant increase in FRET signal at the periphery of the cell (Fig. 3.7A,C). This increase in FRET signal correlates with an increase in levels of active Rac1 at the periphery. The same experiment was then repeated live, which produced a more robust FRET signal. Cells were placed in a temperature and CO₂ controlled chamber on the

![Figure 3.7](image)

**Figure 3.7 Rac1 is active at the periphery.** A,B) HGF stimulation promotes activation of Rac at the periphery. MDCK cells were transfected with Rac FRET probe using Lipofectamine 3000 and allowed to express for 10-12 hours. Following overnight serum-starvation, cells were incubated in the presence or absence of 20 ng/mL HGF and imaged after zero and six hours. Cells were either fixed (A) or imaged live (B) by deconvolution microscopy as described in the Materials and Methods. Scale bars: 10 um. C,D) Quantification of active Rac at the periphery in fixed (C, n = 64-74) or live (D, n = 74-110) cells using Fiji imaging software as described in the Materials and Methods. Data shown are means ± standard error. * p < 5 x 10⁻⁶, T test.
microscope stand. Again, levels of active Rac1 increased at the periphery following HGF stimulation (Fig. 3.7B,D). These results support our model that Rac1 is activated at the periphery rather than at an internal location.

**HGF stimulates association of GRASP/Tamalin and Dock180**

Binding of GRASP to Dock180 is necessary for cytohesin-induced Rac1 activation; however, the dynamics of GRASP and Dock180 association are not currently understood [161]. Fluorescence resonance energy transfer (FRET) is an imaging technique that detects energy transfer between donor and acceptor fluorophores. This information can be used to identify fluorescently labeled proteins of interest that are within 10-50 Å of each other. Thus, a high FRET signal indicates that the labeled protein pairs are physically bound together while a low FRET signal indicates no interaction [221].

We used FRET imaging techniques to study the dynamics of GRASP and Dock180 binding. YPET-GRASP was used as the donor fluorophore and mCherry-Dock180 as the acceptor fluorophore. We found that treatment with HGF led to an increase in association of GRASP and Dock180 as indicated by a significant increase in FRET signal following stimulation with 10 ng/mL HGF (Fig 3.8B, D). Association of GRASP and Dock180 did not change in control cells treated with serum-free media (Fig. 3.8A, D). Interestingly, GRASP and Dock180 binding remained unchanged in cells treated concurrently with HGF and the cytohesin GEF inhibitor SecinH3 (Fig. 3.8C, D). These results suggest that HGF stimulation promotes binding of GRASP and Dock180 and this association is cytohesin GEF dependent.
HGF stimulation promotes binding of GRASP and Dock180. A-B) HGF treatment stimulates association of GRASP and Dock180. MDCK cells were plated on fibronectin-coated coverslips and transfected with the FRET pair YPET-GRASP and mCherry-Dock180 using Lipofectamine 3000. Cells were allowed to express for 10-12 hours before switched to serum-free media overnight. The next morning, cells were incubated in the presence or absence of 10 ng/mL HGF. Cell were fixed at the stated time points and imaged by deconvolution microscopy. Images were processed as described in Materials and Methods. C) Concurrent inhibition of cytohesin GEF activity and stimulation with HGF impairs association of GRASP and Dock180. Following transfection and starvation as described above, cells were treated with both HGF (10ng/mL) and cytohesin inhibitor SecinH3 (15 uM) prior to fixation at the indicated time points. Scale bar: 10 um. D) Slidebook 6.0 was used to calculate the FRET sum intensity and whole cell area in 65 cells as described in the Materials and Methods. Data are means ± standard error of the FRET sum intensity for the whole cell normalized to the cell area. Asterisk indicates statistically significant compared to zero hour
of set. * = p < 5 \times 10^{-4}, T test. Corrected FRET intensity values in scale bar are in arbitrary units. E) Association of Dock180 and Elmo is not dynamic. MDCK cells were plated on fibronectin-coated coverslips and transfected with mTurquoise 2-ELMO and YPET-Dock180 by Lipofectamine 3000. Cells were allowed to express for 10-12 hours before serum-starved overnight. Cells and treated with 10 ng/mL of HGF the following morning and fixed at the indicated time points. Cells were imaged by deconvolution microscopy as described in the Materials and Methods. Scale bar: 10 um. F) Slidebook 6.0 was used to calculate FRET sum intensity in 68 cells. Data are means ± standard error, T test. Corrected FRET intensity values in scale bar are in arbitrary units.

**Inhibition of ARF6 trafficking pathways traps GRASP/Tamalin and Dock180 together**

We next explored the effect inhibiting recycling has on association of GRASP and Dock180. It may be that inhibition of trafficking traps GRASP and Dock180 in their bound association. On the other hand, inhibiting trafficking might prevent GRASP and Dock180 from coming together and binding. MDCK cells were transfected with the GRASP and Dock180 FRET constructs along with either Rab8 T22N or Rab11 S25N. Following serum-starvation, cells were stimulated with 10 ng/mL HGF. We found that expression of Rab8 T22N or Rab11 S25N led to a significant increase in GRASP and Dock180 association in resting cells prior to HGF stimulation compared to control cells. (Fig. 3.9A-C, H; Append. A Fig. 3.3). In addition, treatment with HGF failed to stimulate further interaction between GRASP and Dock180 (Fig. 3.9H). We hypothesized that the increase in binding we saw in resting cells expressing Rab8 T22N or Rab11 S25N is due to the fact that trafficking was inhibited overnight and that this extended inhibition of trafficking accumulates GRASP/Tamalin and Dock180 at recycling endosomes allowing them to interact in an unregulated manner. This is in contrast to the experiments where SecinH3 was used to acutely inhibit ARF activation and trafficking. We were curious if we could reproduce the high level of GRASP and Dock180 interaction by overnight treatment with the cytohesin GEF inhibitor, SecinH3, before treatment with HGF the next
Figure 3.9 Inhibition of ARF trafficking pathways traps GRASP and Dock180 together. A-C) Expression of Rab8 T22N and Rab11 S25N traps GRASP and Dock180 in resting cells. MDCK cells were plated on fibronectin-coated coverslips and transfected with YPET-GRASP, mCherry-Dock180, and either mTurquoise 2-Rab8 T22N, Rab11 S25N or empty vector by Lipofectamine 3000. Cells were allowed to express for 10-12 hours, serum-starved overnight, and treated with HGF (10 ng/mL) the following morning. Cell were fixed and imaged by deconvolution microscopy as described in the Materials and Methods. D) Extended inhibition of cytohesin GEF activity promotes GRASP and Dock180 binding. Following transfection and 10-12 hours of expression as described previously, cells were switched to serum-free media and treated with SecinH3 (15 uM). The next morning, cells were treated with HGF (10 ng/mL) and fixed at the designated time points. Scale bar: 10 um. E-G) Knockdown of ARF1 and ARF6 promotes GRASP and Dock180 interaction. MDCK cells were Neon transfected with control siRNA or siRNA against either ARF1 or ARF6 and plated on fibronectin-coated coverslips. The next day, cells were transfected with YPET-GRASP and mCherry-Dock180 by Lipofectamine 3000, allowed to express for 10-12 hours, and switched to serum-free media overnight. Cells were treated with HGF (10 ng/mL) the next morning and fixed at the indicated time points. Scale bar: 10 um. H) Quantification of corrected FRET signal by Slidebook 6.0 in 67-70 cells expressing either Rab8 T22N, Rab11 S22N or treated with SecinH3. Data are means ± standard error, * = p < 5 x 10^{-4}, ** p < 5 x 10^{-7}, *** p < 5 x 10^{-20}, T test. Corrected FRET intensity values in scale bar are in arbitrary units. I) Whole cell FRET sum intensity was calculated using Slidebook 6.0 in 62-78 cells. Data are means ± standard error. Asterisk indicates statistically significant compared to zero hour of set. * p < 5 x 10^{-9}, T test. Corrected FRET intensity values in scale bar are in arbitrary units.
morning. When we did so, we again saw a significant increase in GRASP and Dock180 association in resting cells prior to treatment with HGF (Fig. 3.9D, H; Append. A Fig. 3.3).

Next, we explored whether knockdown of ARF1 or ARF6 would have similar effects on GRASP and Dock180 association. MDCK cells were transfected with either control siRNA or siRNA against ARF1 or ARF6. Cells were transfected with the GRASP and Dock180 FRET constructs and treated with HGF as described in previous experiments. Again, knockdown of ARF6 or ARF1 led to a significant increase in GRASP and Dock180 binding in resting cells before HGF treatment (Fig. 3.9E-G, I; Append. A Fig. 3.3). These results all continue to suggest that inhibiting ARF6 trafficking pathways traps GRASP/Tamalin and Dock180 in their bound conformation within the cell.

Association of Dock180 and ELMO is unaffected by HGF treatment

The ELMO protein family is a group of scaffolding proteins lacking any known catalytic activity. Both ELMO1 and ELMO2 contain proline-rich regions, which appear to physically interact with the SH3 domain of Dock180 [191]. However, the interaction between the two proteins is complex and yet to be fully mapped out [190]. Some studies suggest that the interaction between Dock180 and ELMO enhances the Rac1-GEF activity of Dock180 by increasing the affinity of Dock180 to the nucleotide-free Rac1 [191]. Other studies have hypothesized that ELMO may assist in recruiting Dock180 to the plasma membrane where Rac1 is located; possibly through an ARF6 trafficking pathway [194].
We used FRET to explore the dynamics of ELMO and Dock180 interactions. We hypothesized that a hand off of Dock180 from GRASP to ELMO might occur in cells following HGF stimulation. We co-expressed the FRET pair mTurquoise-ELMO with YPET-Dock180 in MDCK cells. However, we did not observe any difference in FRET signal in resting cells compared to cells treated with 10 ng/mL HGF (Fig. 3.8E; Append. A Fig. 3.2). All cells exhibited a strong FRET signal indicative of ELMO and Dock180 interaction regardless of the treatment conditions (Fig. 3.8F). These results suggest that Dock180 and ELMO always associate in resting cells and this interaction does not change following stimulation with HGF. Thus, there does not appear to be any sort of hand off of Dock180 from GRASP to ELMO.

Conclusions

We found that the scaffolding protein GRASP and the Rac1-GEF Dock180 localize to recycling endosomes in resting cells. Treatment with HGF stimulates trafficking of GRASP and Dock180 to the plasma membrane. The small GTPases ARF1 and ARF6 regulate this trafficking of GRASP and Dock180. Localization to the plasma membrane following stimulation positions Dock180 to activate the membrane localized Rac1. Inhibition of GRASP and Dock180 trafficking impairs HGF-stimulated Rac1 activation. We also found that treatment with HGF stimulates association of GRASP and Dock180. Inhibiting ARF6-associated trafficking pathways traps GRASP and Dock180 together in the cell and HGF treatment no longer stimulates additional association (Fig. 3.10).
Figure 3.10 Trafficking of GRASP and Dock180 during ARF6 to Rac1 cross talk. GRASP and Dock180 localize to recycling endosomes in resting cells. HGF stimulation promotes association of GRASP and Dock180 and trafficking to the plasma membrane. Movement to the plasma membrane positions Dock180 to activate the membrane-localized Rac1. Inhibition of ARF6-associated trafficking pathways blocks HGF-stimulated movement of GRASP and Dock180 to the periphery and Rac1 activation. Inhibition of trafficking also traps GRASP and Dock180 together in the cell.
Chapter 4

Creation and Characterization of a GRASP Mutant Unable to Bind Dock180

Introduction

GRASP/Tamalin is a scaffolding protein found in many multi-protein complexes due to the presence of several protein-protein interacting domains. These domains include a PSD-95/discs-large/ZO-1 (PDZ) domain, a carboxyl-terminal PDZ binding motif, a proline-rich region, and a leucine-zipper region [196,222]. GRASP is highly expressed in neuronal tissue where it interacts with a number of neuronal proteins including group 1 metabotropic glutamate receptors, SAP90/PSD-95-associated protein, S-SCAM, PSD-95, Mint2/X11betz/X11L, and cytohesins [222,223]. While predominantly expressed in neuronal tissue, GRASP can also be found at low levels in heart, embryo, kidney, lung, and ovary tissue [196].

Previously, our lab found that knockdown of GRASP impairs cytohesin-induced Rac1 activation and cell scattering [195]. It is not unrealistic then to hypothesize that GRASP may also be involved in the HGF signaling pathway. Indeed, GRASP knockdown impairs HGF-induced migration, wound healing, and Rac1 activation [224]. These results suggest that GRASP mediates protein-protein interactions that promote ARF-Rac1 crosstalk during HGF-signaling. To further elucidate these protein-protein interactions, our lab showed that the leucine-rich region of GRASP interacts with the coiled-coil domain of Cytohesin-2 and the proline-rich region of GRASP binds the SH3 domain of Dock180 [161].
We sought to create a mutant form of GRASP that is not able to bind Dock180. We believe this mutant form of GRASP will be a more useful tool than expression of GRASP truncation mutants or simple GRASP knockdown. Compared to GRASP knockdown, which would result in complete loss of function of GRASP, the GRASP mutant would still be able to interact with its other binding partners and we would only be removing its ability to associate with Dock180. For instance, the GRASP mutant will still interact with Cytohesin-2 and thus would not affect the processes that depend on that association. Thus, we can use this tool to link loss of the Dock180 interaction with Cytohesin-2 associated processes. For example, we could explore the affect that the GRASP mutant has on activation and localization of ARF6. Is ARF6 localization and activation linked to the interaction between GRASP and Dock180 or is the process independent? Will ARF6 be activated and move to the plasma membrane regardless of GRASP and Dock180’s binding ability? We will also be able to explore the affect that the GRASP mutant has on Cytohesin-2 localization. Will Cytohesin-2 still localize to recycling endosomes when the GRASP mutant is expressed or is formation of the entire Dock180/GRASP/Cytohesin-2 complex required for proper localization of all proteins involved? In future experiments, this GRASP mutant could also be used as a dominant negative tool or as a control.

The proline-rich region of GRASP contains two potential SH3 binding sites characterized by a –X-P-P-X-P motif. Mutation of either site alone is not sufficient to impair binding of GRASP to Dock180. We found that mutation of both SH3 binding sites impaired GRASP’s ability to interact with Dock180. This GRASP mutant is mis-localized and its expression impairs HGF-stimulated trafficking of GRASP and Dock180.
In addition, it appears that the interaction between GRASP and Dock180 recruits Dock180 to recycling endosomes. Finally, our results indicate that the interaction between GRASP and Dock180 is required for efficient HGF-stimulated Rac1 activation.

**Results**

*Mutation of both SH3 binding sites prevents binding of GRASP to Dock180*

GRASP has two potential SH3 binding sites in its proline-rich region which we designated Site 1 (PSGPP) and Site 2 (PPGAP). We began by creating two single site mutants that had either the prolines in the first site or in the second site mutated to alanines (Fig 4.1A). We will refer to the first site mutant as GRASP Site 1 single mutant and the second site mutant as GRASP Site 2 single mutant. We also created a GRASP mutant in which both sites were mutated. We will refer to this GRASP mutant as the GRASP double mutant.

We have previously shown that HGF stimulation promotes association of wild type GRASP and Dock180. This is indicated by a significant increase in FRET signal following treatment with HGF. We co-expressed mCherry-Dock180 and the various YPET-tagged GRASP constructs as FRET pairs in MDCK cells. An increase in GRASP and Dock180 association, as indicated by an increase in FRET signal, can be observed at six hours following HGF stimulation when mCherry-Dock180 is expressed with YPET-GRASP WT (Fig. 4.1B, F; Appen. B Fig. 4.1). Mutation of either Site 1 or Site 2 does not appear to prevent GRASP from binding Dock180 as a FRET signal was observed following treatment with HGF when mCherry-Dock180 and YPET-GRASP Site 1 single mutant or YPET-GRASP Site 2 single mutant were expressed as FRET pairs (Fig. 4.1C, D, F). Interestingly, we observed a significant increase in FRET signal in resting cells
Figure 4.1 Mutation of both SH3 binding domains in GRASP prevents binding of GRASP to Dock180. A) Domain organization of wild type GRASP. The amino acid sequences for the two potential SH3 binding sites are underlined and indicated as Site 1 or Site 2. The red prolines were mutated to alanines. B-E) The GRASP double mutant does not bind Dock180. MDCK cells were plated on fibronectin-coated coverslips and transfected with mCherry-Dock180 and either YPET-GRASP WT, -GRASP Site 1 single mutant, -GRASP Site 2 single mutant, or -GRASP double mutant using Lipofectamine 3000. After 10-12 hours of expression, cells were switched to serum-free media (1% FBS) overnight. The next morning, cells were incubated in the presence or absence of 10 ng/mL HGF. Cells were fixed at the indicated time points and imaged by deconvolution microscopy. Images were processed as described in the Materials and Methods. Corrected FRET intensity values in scale bar are in arbitrary units. Scale bar: 10 um. F) Slidebook 6.0 was used to calculate the FRET sum intensity and whole cell area in 65-84 cells. Data are means ± standard error of the FRET sum intensity for the whole cell normalized to the cell area. Asterisk indicates statistically significant compared to zero hour of set. * = p < 0.05, ** = p < 5 x 10^-4, T Test.

expressing the GRASP Site 1 single mutant compared to cells expressing wild type GRASP (Fig. 4.1C, F). In addition, treatment with HGF did not result in any further increases in FRET signal, suggesting that the GRASP mutant and Dock180 exhibit maximal binding at resting conditions. These results suggest that the GRASP Site 1 single mutant has an enhanced interaction with Dock180. We hypothesize that the first
SH3 binding domain may have an additional function and perhaps is involved in another interaction that can inhibit GRASP-Dock180 binding in resting cells. An alternative hypothesis is that GRASP exists in an auto-inhibited confirmation through the Site 1 SH3 domain. Thus, mutation of the first site relieves this auto-inhibition. There is evidence that GRASP can adopt in auto-inhibited confirmation. Previous studies have found that at low concentrations and lacking another protein to interact with, GRASP forms a dimeric auto-inhibited complex in which its C-terminal EESQL sequence acts as an internal ligand and interacts with its PDZ domain. GRASP’s auto-inhibition impairs it from acting as a scaffold until it encounters an extrinsic ligand and the inhibition is relieved. This creates a reserve of GRASP that is readily available upon stimulation. During mGluR trafficking, the C-terminal region of mGluR displaces the internal ligand, which relieves GRASP’s auto-inhibition. GRASP is then able to bind the synaptic scaffolding molecule S-SCAM and regulate trafficking of mGluRs [223,225,226].

The Site 2 GRASP mutant behaved similarly to wild type GRASP in terms of its interactions with Dock180. The fact that the Site 2 mutant can still interact with Dock180 implies that the first SH3 binding site is still sufficient for Dock180 binding. Interestingly, we observed that the FRET intensity of the Site 2 mutant with Dock180 is significantly less than the FRET intensity of wild type GRASP with Dock180 after six hours (Fig. 4.1D, F). This may be because the first biding site is able to interact with another factor that competes with Dock180 for binding. Thus, we don’t see the same level of association as we do with the wild type GRASP, which has two binding sites available. When we expressed the GRASP double mutant and Dock180 as FRET pairs, we did not detect a FRET signal in resting cells or upon HGF treatment (Fig. 4.1 E, F).
This suggests that mutation of both SH3 binding domains prevents GRASP from binding Dock180.

To further confirm the interaction (or lack of interaction) between Dock180 and the GRASP mutants, we created wild type and mutant GRASP constructs fused to GST and attached to glutathione beads. We can use these GST-GRASP constructs as “bait” to pull-down Dock180. We anticipate that wild type GRASP, the GRASP Site 1 single mutant, and the GRASP Site 2 single mutant will all be able to pull-down Dock180 from cell lysate but the GRASP double mutant will not. An additional method that we can employ to study the interaction between Dock180 and GRASP is a Bimolecular fluorescence complementation assay (BiFC). Dock180 fused to the N terminal fragment of the Venus fluorescent protein and wild type/mutant GRASP fused to the C terminal fragment will be expressed in cells. If the two proteins bind, the VN and VC fragments will be able to interact and fluoresce (Kodama and Hu, 2010) [227]. We predict that we will observe YFP fluorescence when VN-Dock180 is expressed with VC -GRASP wild type, -GRASP Site 1 single mutant, or –GRASP Site 2 single mutant, but not when it is expressed with VC-GRASP double mutant.

*The GRASP double mutant retains its ability to bind Cytohesin-2*

A mutant form of GRASP that is unable to bind Dock180 should still maintain its ability to interact with its other binding partners. This is in contrast to knockdown of GRASP, which would eliminate all of GRASP’s interactions in the cell. Expression of the GRASP mutant lacking its Dock180 binding abilities allows us to ask questions that knockdown of GRASP would not answer. For example, does GRASP recruit
Cytohesin-2 to recycling endosomes and is this recruitment independent of the GRASP-Dock180 interaction? Is ARF6 activation and localization to the plasma membrane upon its activation separate from GRASP’s ability to bind Dock180 or are these processes linked? Before we answer these questions, we wanted to first confirm that the GRASP double mutant is still able to interact with Cytohesin-2, regardless of its ability to bind Dock180. The C-terminal leucine-rich region of GRASP interacts with the coiled-coil domain of Cytohesin while the two SH3 binding domains in GRASP’s N-terminal
proline-rich region interact with the SH3 domain of Dock180 [161]. We hypothesized that because GRASP’s Cytohesin-2 and Dock180 interacting domains are distinct, the GRASP double mutant should still interact with Cytohesin-2.

To test our hypothesis, we created GST-fused GRASP wild type or GRASP double mutant constructs that could be expressed in E. Coli, purified, and attached to glutathione beads. MDCK cells were infected with adenovirus encoding myc-tagged Cytohesin-2 and lysed after six hours. Cell lysate was incubated with either wild type GRASP or GRASP double mutant attached to glutathione beads. We found that both wild type and double mutant GRASP were able to pull-down Cytohesin-2 from the cell lysate (Fig. 4.2A). These results indicate that the GRASP double mutant is still able to interact with Cytohesin-2, regardless of its Dock180 binding abilities.

Next, we confirmed the ability of the GRASP double mutant to bind Cytohesin-2 in intact cells. To do so, we used a Bimolecular fluorescence complementation assay (BiFC). The N terminal fragment of the yellow fluorescent protein Venus was fused to Cytohesin-2 while the C terminal fragment of Venus was fused to either wild type GRASP or the GRASP double mutant. MDCK cells were transfected with equimolar concentrations of VN-Cytohesin-2 and either VC-GRASP WT or VC-GRASP DM. Cells were fixed and antibody stained as described in Materials and Methods. Production of YFP fluorescence indicates that the proteins are able to bind while lack of YFP fluorescence indicates that the proteins do not interact. Expression of VN-Cytohesin-2 with either VC-GRASP WT or VC-GRASP DM results in production of YFP fluorescence (Fig. 4.2B). These results continue to show that the GRASP double mutant
retains its ability to interact with Cytohesin-2. Thus, in the future, we can use the GRASP double mutant to answer the questions described above.

*The GRASP double mutant is mis-localized*

Previously, we showed that GRASP and Dock180 localize to recycling endosomes in resting cells [228]. This conclusion was based on the observation that GRASP and Dock180 are more often localized with the recycling endosome markers EHD1, Rab8, and Rab11 than they are with the early endosome marker Rab5a. We wondered if mutation of the two SH3 binding sites would affect the localization of GRASP. To explore this question, we transfected MDCK cells with the GRASP double mutant and either Rab8 (recycling endosome marker), Rab11 (recycling endosome), Rab5a (early endosome), or Rab7 (lysosome). Interestingly, we observed a decrease in localization of the GRASP double mutant with Rab8 compared to the localization of wild type GRASP with Rab8 (Fig. 4.3A, E). There was also a slight decrease in localization of the GRASP double mutant with the lysosome marker Rab7 (Fig. 4.3D, E). We also observed an increase in localization of the GRASP double mutant with the early endosome marker Rab5a compared to wild type GRASP and Rab5a (Fig. 4.3B, E). However, localization of the GRASP double mutant with the recycling endosome marker Rab11 was not significantly different from localization of wild type GRASP with Rab11 (Fig. 4.3C, E). It may seem surprising that localization of the GRASP double mutant with the recycling endosome marker Rab8 changed while its localization with another recycling endosome marker, Rab11, remained the same. However, this is not actually that unusual. It has been shown in multiple studies that recycling endosomes are
Figure 4.3 The GRASP double mutant is mis-localized. A-D) MDCK cells were plated on fibronectin-coated coverslips and transfected using Lipofectamine 3000 with mTurquoise 2 -Rab8 (recycling endosomes), -Rab5a (early endosomes), -Rab11 (recycling endosomes), or -Rab7 (late endosomes) and YPET-GRASP WT or YPET-GRASP double mutant. Cells were allowed to express for 10-12 hours and then switched to serum-free media (1% FBS) overnight. The next morning, cells were fixed and imaged by deconvolution microscopy as described in the Materials and Methods. In merge images, GRASP constructs are pseudocolored yellow and Rab markers are pseudocolored blue. Scale bars: 10 um. E) Slidebook 6.0 was used to calculate fluorescent sum intensity of GRASP constructs in markers masks and in whole cell masks. Fluorescent sum intensity of GRASP in markers masks was normalized to whole cell fluorescent sum intensity in 73-79 cells. Data are means ± standard error. * = p < 5 x 10^{-12}, T Test.

heterogeneous both between separate structures and within subdomains of the same recycling endosome. Therefore, proteins of interest may localize with one recycling endosome marker and not with another [123,229,230]. It is common practice then to use
more than one recycling endosome marker to detect localization to these structures. It
would be interesting to visualize Rab8, Rab11, and the GRASP double mutant in cells at
the same time. This would allow us to observe concurrently the localization of the
GRASP double mutant with Rab11 as well as its lack of localization with Rab8 in a
single cell. Based on our results, we conclude that the GRASP double mutant exhibits a
shift in localization away from Rab8-associated recycling endosomes toward Rab5a-
labelled early endosomes. However, its localization with Rab11-associated recycling
endosomes remains unchanged.

*Expression of the GRASP double mutant impairs HGF-stimulated trafficking of GRASP
and Dock180*

Stimulation of cells with HGF leads to ARF6 and ARF1-regulated trafficking of
GRASP and Dock180 from recycling endosomes to the plasma membrane [228]. In
addition, GRASP has been found to regulate the ARF6-dependent recycling of the major
histocompatibility complex-I and ER stress induced cell surface trafficking of core-
glycosylated CFTR [231,232]. We hypothesized that trafficking of GRASP and Dock180
is dependent on their complex formation. If our hypothesis is correct, then expression of
the GRASP mutant should impair HGF-stimulated recycling of GRASP and Dock180.

MDCK cells were co-transfected with GRASP double mutant and Dock180
constructs and levels of GRASP and Dock180 at the periphery following HGF
stimulation were calculated. In wild type cells, GRASP and Dock180 could be observed
at the periphery of cells following treatment with HGF (Fig. 4.4A). Calculation of the
Figure 4.4 The interaction between GRASP and Dock180 is necessary for efficient HGF-stimulated trafficking of GRASP and Dock180. A, B) MDCK cells were plated on fibronectin-coated coverslips and transfected with YPET-GRASP WT (A) or -GRASP DM (B) and mCherry-Dock180 using Lipofectamine 3000. After 10-12 hours of expression, cells were switched to serum-free media (1% FBS) overnight. The next morning, cells were incubated in the presence of 10 ng/mL HGF. Cells were fixed after the indicated time points and imaged by deconvolution microscopy. Scale bars: 10 um. C, D) Quantification of fluorescent sum intensity of Dock180 (C) and GRASP (D) at the periphery in 60-73 cells as calculated by Fiji imaging software and described in the Materials and Methods. Data are means ± standard error of the peripheral sum intensity normalized to the whole sum intensity. Asterisk indicates statistical significance compared to zero hour of set. * = p < 5 x 10^{-2}, ** = p < 5 x 10^{-3}, *** = p < 5 x 10^{-6}. T Test.
fluorescent sum intensity of GRASP and Dock180 confirmed that HGF stimulation led to a significant increase in levels of GRASP and Dock180 at the periphery (Fig. 4.4C, D). On the other hand, the GRASP double mutant and Dock180 appeared to remain internal throughout the time course in cells expressing the GRASP double mutant and the presence of GRASP and Dock180 at the periphery was not as pronounced as that in wild type GRASP expressing cells. In addition, some perinuclear accumulation could also be observed (Fig. 4.4B). Quantification of fluorescent sum intensity indicated that levels of GRASP and Dock180 at the periphery following HGF stimulation were significantly less than levels of GRASP and Dock180 in cells expressing wild type GRASP (Fig. 4.4C, D). These results suggest that the interaction between GRASP and Dock180 is necessary for efficient HGF-stimulated trafficking of the two proteins.

An integral role of GRASP in the trafficking of Dock180 is not surprising as GRASP is known to regulate a variety of trafficking pathways. In neurons, GRASP regulates AMPA receptor recycling by coupling the early endosome-associated Rab4 with Rab11 recycling endosomes through an interaction with the SNARE protein Syntaxin 13 [233]. Another group found that GRASP forms a multi-protein complex with Cytohesin-2 and mGluR. Overexpression of GRASP leads to an increase in cell-surface mGluR while expression of a GRASP unable to bind mGluR reduced cell-surface mGluR levels. These results are reminiscent of what we observed in our own study [222]. In addition, overexpression of GRASP and its subsequent accumulation at early recycling endosomes blocks ARF6-dependent trafficking of major histocompatibility complex-I (MHC-1) [231].
GRASP recruits Dock180 to recycling endosomes

We have just shown that the interaction between GRASP and Dock180 is necessary for efficient trafficking of the two proteins. However, we do not know why this interaction is required. Interestingly, GRASP contains four YXXL motifs. These motifs are known to interact with the medium chains of the clathrin-associated AP-2 protein complex. Thus, GRASP may be able to link cytohesins to trafficking machinery in order to regulate intracellular recycling of proteins such as Dock180 [222,234]. Another possibility is that GRASP may recruit Dock180 to recycling endosomes. Results from another study led the authors to speculate that GRASP may be responsible for targeting of Cytohesin-3 to endosomal structures [231]. We hypothesize that the role of GRASP in trafficking of Dock180 is to recruit Dock180 to recycling endosomes.

To explore our hypothesis, we transfected MDCK cells with either control siRNA or siRNA targeting endogenous GRASP (Append. B Fig. 4.2). The next day, cells were transfected with Dock180 and either Rab11 (recycling endosome marker) or Rab5a (early endosome marker). In control knockdown cells, Dock180 is more often localized with the recycling endosome marker Rab11 than it is with the early endosome marker Rab5a (Fig. 4.5A, C, E). However, knockdown of GRASP led to a shift in localization of Dock180 from recycling endosomes to early endosomes (Fig. 4.5B, D, E). This suggests that GRASP recruits Dock180 to recycling endosomes and loss of GRASP results in localization of Dock180 to early endosomes. We would like to confirm this conclusion by rescuing cells with either wild type GRASP or the GRASP double mutant. If GRASP does recruit Dock180 to recycling endosomes through its interaction with Dock180’s
SH3 domain, then rescue with wild type GRASP following GRASP knockdown should shift localization of Dock180 back to recycling endosomes. However, expression of the GRASP double mutant following GRASP knockdown should have no affect on Dock180 localization and Dock180 would remain localized to early endosomes.

Figure 4.5 GRASP recruits Dock180 to recycling endosomes. A-D) MDCK cells were transfected with control siRNA or siRNA against GRASP by Neon transfection and plated on fibronectin-coated coverslips as described in the Materials and Methods. The following day, cells were transfected with Dock180 and either Rab11 (recycling endosome marker) or Rab5a (early endosome) by Lipofectamine 3000 and allowed to express for 24 hours. Cells were fixed and imaged by deconvolution microscopy. Scale bars: 10 um. In merged images, endosome markers are pseudocolored blue and Dock180 is pseudocolored red. E) Slidebook imaging software was used to calculate the fluorescent sum intensity Dock180 in the endosome marker mask and the whole cell mask in 75-84 cells as described in the Materials and Methods. Data are means ± standard error. * p < 5 x 10⁻⁷.
Expression of the GRASP double mutant impairs HGF-stimulated Rac1 activation

We have previously shown that inhibiting ARF6-associated trafficking pathways impairs HGF-stimulated Rac1 activation and trafficking of GRASP and Dock180 to the periphery [228]. In addition, knockdown of GRASP impairs HGF-stimulated Rac1 activation [224]. It is possible that inhibiting ARF6 recycling pathways impairs HGF-stimulated Rac1 activation because Dock180 is no longer trans-located to the plasma membrane where Rac1 is waiting to be activated. We have just shown that expression of the GRASP double mutant impairs HGF-stimulated trafficking of GRASP and Dock180 to the plasma membrane. We hypothesized that expression of the GRASP double mutant should also then impair HGF-stimulated Rac1 activation.

To explore our hypothesis, we transfected MDCK cells with empty vector or GRASP double mutant construct. Levels of active Rac1 before and after HGF stimulation were quantified using an active Rac1 GST-PBD pull-down assay. We found that expression of the GRASP double mutant significantly impaired HGF-stimulated Rac1 activation compared to control cells transfected with empty vector (Fig. 4.6). Interestingly, expression of the GRASP double mutant appeared to lead to a slight increase in Rac1 activation in untreated resting cells. However, results were variable and a comparison of control cells versus GRASP double mutant expressing cells at resting conditions had a p-value of 0.048. We hypothesize that perhaps expression of the GRASP double mutant leads to mis-regulation of Dock180, as the two proteins are no longer able to interact. The results may have been variable and not significant due to the presence of endogenous wild type GRASP.
The ambiguity of the results in untreated cells expressing the GRASP double mutant make it difficult to draw clear conclusions from the overall data. In order to obtain a stronger picture of what might be occurring, we would like to knockdown endogenous GRASP and rescue with either wild type GRASP or the GRASP double mutant. We would then incubate cells with or without HGF and perform a Rac1-GTP pull-down assay after six hours. We believe this would allow us to determine if the Rac1 activation that we observe in untreated GRASP double mutant expressing cells is significant and we hope this experiment would also confirm that expression of the GRASP double mutant impairs HGF-stimulated Rac1 activation.

Figure 4.6 The interaction between GRASP and Dock180 is required for efficient HGF-stimulated Rac1 activation. A) Expression of the GRASP double mutant impairs HGF-stimulated Rac1 activation. MDCK cells were transfected with YPET-GRASP DM or empty vector using Lipofectamine 3000. Cells were allowed to express for 10-12 hours before switching to serum-free media (1% FBS) overnight. The next morning, cells were incubated in the presence or absence of 20 ng/mL HGF. After 6 hours, cells were lysed and GST-PBD pull-downs were performed to calculate active Rac1. Starting lysate and pull-down samples were run on a Western and blotted for Rac1 and GFP (YPET-GRASP DM). Experiments were quantified by LI-COR imaging systems. Data shown are means ± standard error. * = p < 0.05, paired T Test, n = 12. Representative gel is shown.
Conclusions

In this study, our goal was to characterize the interaction between GRASP and Dock180 during HGF signaling while maintaining GRASP’s association with its other binding partners. To do so, we created a mutant form of GRASP that is unable to bind Dock180. We found that the proline-rich region of GRASP contains two potential SH3 binding sites and mutation of both of these sites prevents GRASP’s ability to interact with Dock180. GRASP mutants containing a single mutated SH3 binding site retain their ability to bind Dock180. Interestingly, the double mutant form of GRASP (containing two mutated SH3 binding sites) appears to shift its localization from recycling endosomes (where wild type GRASP is typically found) to early endosomes. We also show that GRASP’s interaction with Dock180 is necessary for efficient trafficking of the two proteins. This is not surprising, as GRASP is known to be involved in the trafficking of other various protein receptors. We believe that the role of GRASP in trafficking of Dock180 is to recruit Dock180 to recycling endosomes. In support of this hypothesis, we showed that knockdown of GRASP leads to a decrease in localization of Dock180 with recycling endosomes and an increase in Dock180’s localization with early endosomes. Finally, we found that the interaction between GRASP and Dock180 is required for HGF function, such as Rac1 activation. Future experiments will aim to further delineate the role of GRASP in the HGF signaling pathway. For example, does GRASP recruit Cytohesin-2 to recycling endosomes as well? Is ARF6 activation and localization to the plasma membrane dependent on GRASP and Dock180 association or are these steps sequential?
Figure 4.8 The interaction between GRASP and Dock180 is required for efficient HGF function. HGF stimulation promotes binding of GRASP and Dock180. This interaction is required for HGF-stimulated trafficking of GRASP and Dock180 and Rac1 activation.
Chapter 5
Discussion

The big picture: From HGF to migration

This final chapter will outline a comprehensive model from initial HGF binding to polarized cell migration using information presented in Chapters 1, 3, and 4. The goal of this chapter is to illustrate how my work has helped elucidate the role of active ARF6 in HGF signaling, Rac1 activation, and polarized migration. In addition, I discuss what is still unknown regarding Dock180/GRASP association and trafficking and present our current hypotheses. At the end of this chapter, the reader will understand how my results fit into the broader context of the HGF signaling pathway.

Signaling from HGF to active ARF6

Hepatocyte growth factor (HGF) is a potent signaling factor that promotes dissociation and scattering of epithelial cells [199]. HGF is essential for the maturation of several epithelial organs during development [235,236]. In addition, HGF and its receptor c-Met promote tissue recovery, such as following lung, spinal cord, or muscle damage [84,237,238]. In order to stimulate cell movement, the signals downstream of HGF lead to changes in cell shape and polarity and these changes are often regulated by small GTPases [200,239]. One of the small GTPases known to regulate migration downstream of HGF is ARF6. Treatment with HGF leads to an increase in levels of intracellular active ARF6 and the presence of ARF6 is necessary for HGF-stimulated cell migration [147].
There are multiple potential routes from initial HGF stimulation to ARF6 activation and each of these potential routes will be discussed below (Fig. 5.1). To begin, binding of HGF promotes c-MET homodimerization and auto-phosphorylation of two tyrosine residues found within the tyrosine kinase domain [68]. Two more tyrosine residues in the carboxy-terminal tail are phosphorylated next and act as docking sites for effector proteins [240]. A number of signaling molecules bind c-MET and activate changes in migration, invasion, and proliferation [241].

**Figure 5.1 Signaling from HGF to active ARF6.** Activation of ARF6 following HGF stimulation is regulated by a number of different pathways. 1) Activation of the MAPK/ERK signaling pathway leads to an increase in Cytohesin-3 expression. 2) Active AKT phosphorylates cytohesins to relieve their auto-inhibition and promote membrane recruitment. 3) R-Ras recruits RLIP and Cytohesin-2 to recycling endosomes.

**MAPK/ERK signaling pathway**

One of the signaling molecules recruited by c-MET phosphorylation is the adaptor protein Growth factor receptor-bound protein 2 (GRB2) [242]. The SH2 and SH3 domains of GRB2 interact with the Rat sarcoma viral oncogene homolog (RAS)-GEF
Son of Sevenless (SOS), which subsequently leads to an increase in levels of active Ras [69,243]. Ras-GTP binds to the serine-threonine kinase Rapidly accelerated fibrosarcoma (RAF) and indirectly causes its activation through recruitment of RAF to the plasma membrane [244]. RAF kinase activates MAPK/ERK Kinase 1 (MEK1) by phosphorylation of two serine residues in its activation loop [245,246]. MEK1 then phosphorylates tyrosine and threonine residues on the serine/threonine kinase Mitogen activated protein-kinase (MAPK also known as ERK), which leads to its activation [247,248]. Activated ERK trans-locates to the nucleus where it alters gene expression by phosphorylation and activation of a number of transcription factors [249].

*Regulation of cytohesin expression*

Unpublished data from our lab suggests that treatment with HGF leads to an increase in transcription of the 3G splice variant of the ARF GEF Cytohesin-3/GRP1. Levels of Cytohesin-2 transcripts remain relatively stable, but are two-three fold higher than levels of Cytohesin-3/GRP1 transcript [250]. An increase in expression of cytohesins could drive activation of ARF6. It is possible that changes in Cytohesin-3 expression are promoted by transcription factors activated through the HGF-stimulated MAPK/ERK signaling pathway. One possible transcription factor that could regulate Cytohesin-3 expression is Twist. The basic helix loop transcription factor Twist is known to act downstream of HGF and knockdown of Twist impairs HGF-stimulated wound healing [250]. Phosphorylation of Twist at a serine by p38, JNK, or ERK prevents its degradation and promotes its stability [251]. Following HGF stimulation, Twist regulates a decrease in E-cadherin expression that allows cells to separate from their neighbors and
adopt a migratory phenotype [252,253]. A previous study observed that Twist promoted Cytohesin-3 expression [254]. Another transcription factor that could regulate Cytohesin-3 expression is the latent cytoplasmic transcription factor STAT-3. STAT-3 is phosphorylated and activated between two to six hours following HGF stimulation. Delayed activation of STAT-3 is stimulated by the production of interleukin-6 via a PI 3-kinase mediated pathway [255]. This time frame aligns with the increase we observe in Cytohesin-3 expression. A final transcription factor that could promote Cytohesin-3 expression is ETS1. Following HGF signaling, ERK phosphorylates a threonine on ETS1 and this induces its transcriptional activity [256]. Expression of a dominant negative form of ETS1 inhibits scattering of MDCK cells [257,258]. This phenotype links ETS1 to cytohesins as overexpression of Cytohesin-2 promotes cell scattering. Further studies are needed to explore transcription factor regulation of Cytohesin-3 expression following HGF stimulation.

PI3K/AKT signaling pathway

The p85 subunit of PI3K binds the phosphorylated c-Met receptor directly or indirectly through the adaptor protein GRB2 associated binding protein 1 (GAB1) [259,260]. At the plasma membrane, PI3K phosphorylates PtdIns(3,4)P₂ to form PtdIns(3,4,5)P₃ [261]. The PH domain of AKT (also known as Protein kinase B, PKB) binds PtdIns(3,4,5)P₃ and recruits AKT to the plasma membrane following the PI3K-dependent production of PtdIns(3,4,5)P₃ [262,263]. PtdIns(3,4,5)P₃ production also recruits the serine/threonine kinase 3’-phosphoinositide dependent kinase 1 (PDK1). PDK1 activates AKT by phosphorylation of a threonine residue in AKT’s activation loop.
Active AKT phosphorylates a threonine residue in the PH domain of cytohesins. When the threonine residue is un-phosphorylated, the coiled-coil domain of cytohesins is able to interact with the c-terminal region and this prevents membrane association. Phosphorylation of the threonine residue by AKT impairs this binding and promotes membrane recruitment [173]. Thus, activation of the PI3K/AKT pathway could serve to recruit cytohesins to recycling endosomes where they can then activate ARF6.

**R-Ras/RLIP signaling pathway**

The small GTPase R-Ras is involved in a number of cellular processes such as focal adhesion formation, vascular differentiation, and actin organization [266-268]. R-Ras shares only 55% sequence similarity with the other Ras isoforms, H-, K-, and N-Ras, with the most divergence occurring in the C-terminal hypervariable domain [269,270]. Unlike the other Ras isoforms, the Ras-GEF SOS does not activate R-Ras and R-Ras does not appear to be involved in the MAPK/ERK signaling pathway described above [271,272]. However, MDCK cells expressing constitutively active R-Ras scatter and form branching tubules in 3D collagen matrices [273]. These phenotypes are similar to those seen in cells treated with HGF or expressing Cytohesin-2. Active R-Ras can be found at the plasma membrane and recycling endosomes [274-276].

RLIP76 is a ubiquitously expressed protein that acts as a multifunctional GTPase-activating protein [277]. The D64 residue on the effector loop of R-Ras interacts with RLIP76 through RLIP76’s 292-392 residues [278]. RLIP76 interacts only with active R-Ras and is an R-Ras specific effector [278,279]. Interestingly, RLIP76 also interacts with Cytohesin-2 through serine residues in its N-terminal region [278,280]. A recent study found that Cytohesin-2, RLIP76, and R-Ras form a multi-protein complex on recycling
endosomes. Cytohesin-2 is typically believed to be a cytosolic protein with some perinuclear accumulation [174,194]. RLIP76 is also found in both the cytosol and on membranes [281,282]. The authors of the study hypothesize that active R-Ras recruits Cytohesin-2 and RLIP76 from the cytosol to recycling endosomes. They believe that recruitment of this complex then drives ARF6 activation [280].

**ARF6 to Rac1 crosstalk**

Previous work in our lab suggests that activation of ARF6 is required for Rac1 activation. For example, expression of the ARF-GEF Cytohesin-2 promotes Dock180-induced Rac1 activation and migration [194]. Cytohesin-2 also forms a multi-protein complex with the scaffolding protein GRASP and the Rac1-GEF Dock180. Formation of this complex is required for cytohesin-induced Rac1 activation [161]. Interestingly, the GEF activity of Cytohesin-2 must also be functional in order to promote cytohesin-induced Rac1 activation [194]. It appears that both the scaffolding function and the GEF activity of Cytohesin-2 are required for cytohesin-induced Rac1 activation. Cytohesin-2 is not able to activate Rac1 directly, however, there does appear to be some form of crosstalk occurring between active ARF6 and Rac1 (Fig. 5.3A). Therefore, the question driving my dissertation project was: **How does active ARF6 promote Rac1 activation?** Given what is known about the typical functions of ARF6, we hypothesized that ARF6 is either regulating the trafficking of Dock180 to a location where Dock180 can then activate Rac1 or ARF6 is directly recruiting Dock180 to the plasma membrane through modification of membrane lipid composition. Based on previous results from our lab (E.g. Cytohesin-2 promotes trafficking of integrins and R-Ras from recycling endosomes
to the plasma membrane [206]), we believed that active ARF6 was required for trafficking of Dock180 and we developed the hypothesis described below.

**Hypothesis**: *Active ARF6 regulates trafficking of GRASP and Dock180 from recycling endosomes to the plasma membrane.*

ARF6 is known to regulate trafficking of membrane adhesion proteins through recruitment of coat proteins, modification of membrane-lipid composition, and remodeling of actin organization [132]. This ARF6-regulated trafficking typically occurs between the recycling endosome and the plasma membrane [125,145,146]. In addition, Cytohesin-2 localizes to recycling endosomes and is known to promote trafficking of integrin and R-Ras from recycling to the plasma membrane [162,206,208]. Thus, GRASP and Dock180 may also localize to recycling endosomes. Active ARF6 then regulates trafficking of GRASP and Dock180 from recycling endosomes to the plasma membrane. At the plasma membrane, Dock180 can then activate Rac1 (Fig. 5.2).

![Diagram](attachment:image.png)

**Figure 5.2 Potential role for active ARF6 in cytohesin-induced Rac1 activation.** Active ARF6 regulates trafficking of GRASP and Dock180 from recycling endosomes to plasma membrane. Trafficking of Dock180 positions the Rac1-GEF to activate a plasma membrane localized Rac1.
**ARF1 and ARF6 regulate trafficking of GRASP and Dock180 from recycling endosomes to the plasma membrane**

**Summary of findings**

We tested our hypothesis stated above by exploring the following three questions:

1. Where do GRASP and Dock180 localize in the cell?
2. How does Cytohesin-2 GEF activity promote Rac1 activation?
3. What is the importance of the GRASP-Dock180 interaction in HGF signaling?

We began by exploring the localization of GRASP and Dock180 with various endosome markers and determined that the two proteins localize to recycling endosomes in resting cells. Interestingly, treatment with HGF led to a decrease in localization of GRASP and Dock180 with recycling endosome markers, suggesting that GRASP and Dock180 are moving off of these structures upon stimulation. To further explore GRASP and Dock180 recycling, we measured levels of GRASP and Dock180 at the periphery following stimulation with HGF. We found that levels of GRASP and Dock180 increased at the cell periphery following treatment with HGF and this increase was cytohesin GEF dependent.

Inhibition of ARF6-associated trafficking pathways through expression of dominant negative Rab8 and Rab11 impaired GRASP and Dock180 recycling. These results suggest that active ARF6 regulates this movement. To confirm this, we directly knocked down ARF6 as well as ARF1. Interestingly, knockdown of both ARF6 and ARF1 impaired HGF-stimulated trafficking of GRASP and Dock180.

If the purpose of trafficking Dock180 is to transport it to the plasma membrane where it can activate Rac1, then impairing trafficking of Dock180 should also impair Rac1 activation. In line with this, we found that expression of dominant negative Rab8
and Rab11 impaired HGF-stimulated Rac1 activation. Similarly, knockdown of both ARF1 and ARF6 also impaired HGF-stimulated Rac1 activation. It appears then that conditions that impair HGF-stimulated trafficking of GRASP and Dock180 also impair HGF-stimulated Rac1 activation (Fig. 5.3B).

**ARF6 and ARF1: Trafficking partners**

Interestingly, we found that both ARF1 and ARF6 regulate trafficking of GRASP and Dock180 to the plasma membrane during HGF-stimulated Rac1 activation. ARF1 and ARF6 are the best studied of the ARF isoforms and also the most divergent [141]. The GTP-bound conformations of ARF1 and ARF6 are almost identical and the two proteins interact with many of the same effectors. However, the GDP-conformations are quite dis-similar and are believed to bind different GEFs [148,283]. ARF1 is primarily cytosolic in its inactive form. In its GTP-bound active form, ARF1 regulates the recruitment of COPI coat proteins and vesicle budding during intra-Golgi transport [151,284]. ARF6, on the other hand, is typically found associated with membranes in both its GDP- and GTP-bound forms [285-287]. ARF6 localizes to the plasma membrane and is involved in actin remodeling, endocytosis, and cytokinesis [288-290]. Previously, it was believed that ARF1 acted only at the Golgi, but recent evidence suggests a role for ARF1 at the plasma membrane as well. For example, insulin stimulation recruits ARF1 to the plasma membrane in the presence of Cytohesin-2 expression [291]. In addition, ARF1 localizes to endosomes at the apical poles in kidney tubule epithelial cells and regulates recruitment of paxillin during focal adhesion assembly. [216,292]. It is also not unusual to find ARF proteins working in pairs. A previous study found that Golgi structure and
Figure 5.3 Elucidating ARF6 and Rac1 crosstalk. A) Previous work indicated that both the scaffolding function and GEF activity of Cytohesin-2 is required for cytohesin-induced Rac1 activation. This suggested a role for active ARF6 in cytohesin-induced Rac1 activation. However, it was not clear how crosstalk between the two small GTPases occurred. B) We show that ARF1 and ARF6 regulate trafficking of GRASP and Dock180 from recycling endosomes to the plasma membrane. This situates Dock180 to activate a plasma membrane localized Rac1.

transport was unaffected upon knockdown of single ARF proteins. However, knockdown of pairs of ARF proteins led to defects in secretory and endocytic trafficking [140].

Cytohesins have two auto-inhibitory mechanisms used to regulate their activity. The first mechanism involves phosphorylation of the PH domain at a threonine residue,
which promotes membrane association [173]. The second makes use of the linker region between the PH and Sec7 domain. This linker acts as a pseudosubstrate and inhibits docking of GTPases. Inhibition is released by binding of active ARF6 to cytohesins [172]. In our study, it is possible that active ARF6 first binds to Cytohesin-2 to relieve auto-inhibition. Upon release of inhibition, Cytohesin-2 may then go on to activate ARF1. It is also known that active ARF6 is able to bind to the PH domain of Cytohesin-2 and act as an adaptor to recruit the ARF-GEF to the plasma membrane. This interaction appears to be conserved throughout the cytohesin family as active ARF6 was also able to bind Cytohesin-3. In that study, Cytohesin-2 activated ARF1 at the plasma membrane and actually preferred ARF1 as a substrate to ARF6 in vitro [174]. It is possible that a similar mechanism occurs in our proposed pathway.

HGF stimulation promotes GRASP and Dock180 association

Summary of findings

As previously described, the proline-rich region of GRASP interacts with the SH3 domain of Dock180 to form a multi-protein complex. While we knew that formation of this complex is necessary for HGF-stimulated Rac1 activation, we did not know the dynamics of GRASP and Dock180 association. We were curious if GRASP and Dock180 always remain in complex or if their interaction changes upon HGF stimulation. To explore their association, we expressed GRASP and Dock180 as FRET pairs. While there does appear to be some interaction between GRASP and Dock180 in resting cells, treatment with HGF led to a significant increase in their association. We wondered if inhibition of ARF6-associated trafficking pathways would impair this association.
Instead, expression of dominant negative Rab8 and Rab11 and knockdown of ARF1 and ARF6 led to a significant increase in GRASP and Dock180 interaction in resting cells. These results suggest that inhibiting trafficking pathways traps GRASP and Dock180 together in the cell (Fig. 5.3B).

It is not clear how HGF treatment promotes the binding of GRASP and Dock180. Promotion of GRASP and Dock180’s interaction may simply depend on their localization. Perhaps in order for GRASP and Dock180 to interact, they need to be directed to recycling endosomes. Upon both proteins reaching recycling endosomes, they are then in close enough proximity to interact without any need for conformational or auto-inhibition relief. We have shown that localization of GRASP and Dock180 to recycling endosomes decreases six hours after HGF stimulation (Fig. 3.2). However, we have not explored whether localization of GRASP and Dock180 with the recycling endosome markers increases soon after HGF treatment (such as after two or three hours), before decreasing around the six-hour mark. This would indicate that HGF treatment promotes recruitment of GRASP and Dock180 to the recycling endosomes and would support the hypothesis that an increase in GRASP and Dock180 recruitment leads to an increase in their association.

Dock180 contains a DHR-1 domain that consists of surface loops that form a basic pocket lined with three lysine residues that specifically bind PtdIns(3,4,5)P$_3$ [293,294]. Expression of PI 3-kinases results in the accumulation of PtdIns(3,4,5)P$_3$ on membranes and translocation of Dock180 to these PtdIns(3,4,5)P$_3$-enriched regions [294,295]. Point mutations in the basic pocket or elimination of the DHR-1 region prevent Dock180-induced cell elongation or directional migration. These mutants,
however, retain their ability to act as Rac1 GEFs [293,295]. ARF6-GTP activates PIP-5K which catalyzes the production of PtdIns(4,5)P$_2$ [296,297]. PtdIns(4,5)P$_2$ serves as a substrate for PI 3-kinase in its production of PtdIns(3,4,5)P$_3$ [298]. Thus, active ARF6 may use this pathway to direct a localized increase in levels of PtdIns(3,4,5)P$_3$ at recycling endosomes following HGF stimulation. An increase in PtdIns(3,4,5)P$_3$ would recruit Dock180 to recycling endosomes and position Dock180 for trafficking. It would be interesting to express a Dock180 mutant unable to bind PtdIns(3,4,5)P$_3$ and explore association of GRASSP and Dock180 following HGF stimulation. If Dock180 needs to be on recycling endosomes in order to interact with GRASSP, then the Dock180 mutant lacking its membrane recruitment abilities should not bind GRASSP.

*Connections between trafficking and association*

Interestingly, we found that inhibiting ARF6-associated trafficking pathways leads to an increase in GRASSP and Dock180 binding in resting cells prior to stimulation with HGF (Fig. 3.9). The cells are not being stimulated, so what is promoting the interaction between GRASSP and Dock180? If the hypothesis stated directly above is correct (recruitment to recycling endosomes places GRASSP and Dock180 in position to interact), it could provide us with a potential answer to this question. Expression of dominant negative Rab8 and Rab11 impairs trafficking and leads to the accumulation of proteins in tubular structures that resemble recycling endosomes [212,213,299,300]. Similarly, knockdown of ARF1 and ARF4 results in extensive tubulation of Rab11 positive endosomes. Knockdown of ARF6 or expression of dominant negative ARF6 leads to internal accumulation of E-cadherin and transferrin, respectively [301-303].
When we impaired trafficking, we saw similar accumulation of GRASP and Dock180 at internal puncta and perinuclear regions of the cell (Fig. 3.4 and Fig. 3.5). If GRASP and Dock180 binding is promoted by their localization to the same area of the cell, it then seems logical that inhibiting trafficking, and subsequent accumulation of GRASP and Dock180 to the same cellular region, would lead to an increase in GRASP and Dock180 association.

The GRASP-Dock180 interaction is required for efficient HGF function

Summary of findings

As described above, HGF stimulation promotes GRASP and Dock180 association. We were curious if this interaction between the two proteins is required for HGF function. To answer this question, we began by creating a mutant form of GRASP that is not able to bind Dock180. In contrast to simply knocking down GRASP, this mutant would retain its ability to interact with its other binding partners, such as Cytohesin-2. The proline-rich region of GRASP contains two potential SH3 binding domains that we labeled as either Site 1 or Site 2. We found that GRASP mutants in which only a single site is mutated are still able to interact with Dock180. However, mutation of both sites prevents the ability of GRASP to bind Dock180. We refer to this GRASP mutant as our GRASP double mutant. Interestingly, we found that expression of the GRASP double mutant impairs HGF-stimulated trafficking of GRASP and Dock180. We hypothesized that GRASP recruits Dock180 to recycling endosomes. If GRASP is unable to localize Dock180 to recycling endosomes (due to their inability to bind), then Dock180 will not be in position for its ARF-regulated trafficking to the plasma
membrane. In support of our hypothesis, we show that knockdown of GRASP results in a shift in Dock180 localization from recycling endosomes to early endosomes. Finally, we found that the interaction between GRASP and Dock180 is required for efficient HGF function, as expression of the GRASP double mutant appears to impair HGF-stimulated Rac1 activation.

**GRASP and Dock180: Always together or occasionally apart?**

While we know that HGF stimulation promotes GRASP and Dock180 binding and the interaction between the two proteins is necessary for efficient HGF function, the dynamics of GRASP and Dock180 association following HGF treatment are not clear. It would be interesting to explore whether GRASP and Dock180 translocate to the plasma membrane bound together, if they translocate separately, or if they come apart once they reach the plasma membrane. There are a few different methods that could be employed to examine these questions. First, a Bimolecular fluorescence complementation assay (BiFC) using a split Venus system could be used to test whether or not GRASP and Dock180 travel to the plasma membrane separately or together. GRASP tagged with the C-terminal sequence of Venus and Dock180 tagged with the N-terminal sequence would be expressed in cells. Once GRASP and Dock180 associate, the complementary Venus fragments would reassemble into a functional fluorescent protein and the two fragments would be irreversibly bound. This means that GRASP and Dock180 would also be bound in complex together. If GRASP and Dock180 need to come apart for translocation to the plasma membrane, expressing the two proteins as split Venus pairs would result in them becoming trapped inside the cell. HGF stimulation would no longer promote their
movement to the plasma membrane. Another method that could be used to study the
dynamics of GRASP and Dock180 would be to express the two proteins as FRET pairs
and observe them over time following HGF stimulation in a single live cell. The presence
or loss of FRET signal at the plasma membrane would help elucidate the localization of
GRAP and Dock180 interaction.

**Trafficking and polarity direct cell migration**

Following trafficking of Dock180 to the plasma membrane, Dock180 is now in
position to activate a plasma membrane localized Rac1. This model fits with and
reinforces the role of polarity in cell migration. A key feature of migration is the
establishment of polarity. Without a leading and a trailing edge, a cell would never gain
substantial ground in a given direction. Specific spatial and temporal intracellular
trafficking pathways establish polarity during cell migration [12,304]. Trafficking
machinery asymmetrically recruits effectors to the leading edge where they activate
enzymes such as PLC, PKC isoforms, and PI 3-Kinases. Activation of these enzymes
leads to accumulation of PtdIns(3,4,5)P$_3$ at the leading edge [28,305]. PTEN maintains
the concentration gradient of PtdIns(3,4,5)P$_3$ at the front of the cell by hydrolyzing any
PtdIns(3,4,5)P$_3$ found elsewhere [306]. Accumulation of PtdIns(3,4,5)P$_3$ at the leading
edge leads to the proper recruitment and activation of signaling factors such as Rac1 and
CDC42. Rac1 and CDC42 promote migration through localized actin polymerization and
subsequent formation of lamellipodia and filopodia. CDC42 interacts with and activates
the Wiskott-Aldrich syndrome proteins (WASp) while Rac1 indirectly activates the
Scar/WAVE family [307]. Active WASp, Scar, and WAVE proteins regulate the actin polymerizing Arp2/3 complex [308,309].

The directed trafficking of Dock180 to the leading edge would help ensure that Rac1 activation is limited to the front of the cell. Another group found that membrane from the recycling endosome is transported to and incorporated into the leading edge during migration. The additional membrane from the recycling endosome helps the lamellipodia expand. Perhaps in addition to providing more surface area, trafficking of the recycling endosome membrane may also asymmetrically transport Dock180 to the plasma membrane to activate Rac1 (Fig. 5.4) [310].

Figure 5.4 Polarity and trafficking pathways promote directed migration. During migration, the cell establishes a front/back polarity and recycling is directed to the leading edge. Delivery of Rac1 activation machinery, such as Dock180, to the leading edge results in further protrusions at the leading edge and promotes persistence of directed migration. N = nucleus, ERC = early recycling endosome.
Final Conclusions

To summarize, we have detailed a comprehensive model of the role of active ARF6 in HGF signaling, Rac1 activation, and directed cell migration. HGF stimulation leads to an increase in active ARF6, possibly through a MAPK/ERK, PI3-K, or R-Ras signaling pathway. Crosstalk between active ARF6 and Rac1 occurs through the ARF1 and ARF6 regulated trafficking of GRASP and Dock180 to the plasma membrane. Polarized trafficking of Dock180 to the leading edge would promote persistence of Rac1 regulated lamellipodia and reinforce the direction of migration (Fig. 5.5).

Delineating the mechanisms of epithelial cell migration is crucial to the understanding of how cells use migration to promote tissue healing and how cancerous cells migrate when they should remain where they are. Therefore, it is important to continue exploring the small GTPase signaling pathways that regulate these processes. In addition, it can be difficult to target small GTPases themselves without causing other adverse side effects within the cell. However, the targeting of scaffolding proteins or other proteins that connect small GTPase signaling could provide a promising alternative strategy and lead to the development of novel therapeutics to halt metastasis or promote wound healing.
Figure 5.5 Comprehensive model from HGF stimulation to migration. ARF6 is activated by way of a PI3-K, R-Ras, or MAPK/ERK pathway following HGF stimulation. Active ARF6 and ARF1 regulate trafficking of Dock180 to the plasma membrane. Polarized trafficking of Dock180 to the leading edge would promote Rac1 activation at the leading edge and directed migration.
Appendix A: Supplemental Material for Chapter 3

Appendix A Figure 3.1 Efficient knockdown of ARF1 and ARF6. Representative gels of ARF6 (A) and ARF1 (B) knockdown. Dharmacon control siRNA was used as a control. Lysate from samples was blotted with antibodies against ARF1, ARF6, and actin. Images were analyzed using the Li-COR imaging system.

Appendix A Figure 3.2 Dock180 and ELMO images for FRET figure. Individual YPET-Dock180 and mCherry-ELMO channels for FRET images in Figure 3.8.
Appendix A Figure 3.3 Dock180 and GRASP images for FRET figure.

Individual YPET-GRASP and mCherry-Dock180 channels for FRET images in Figure 3.9. Figure letters correspond to figure letters in Figure 3.9 (Example: Suppl. Fig. 3A corresponds to Fig. 3.9A). Scale bar = 10 μm.
Appendix B: Supplemental Materials for Chapter 4

Appendix B Figure 4.1 Dock180 and GRASP images for FRET figure. Individual YPET-GRASP (WT, Site 1, Site 2, or DM) and mCherry-Dock180 channels for FRET images in Figure 4.1. Figure letters correspond to figure letters in Figure 4.1. Scale bar = 10 um.

Appendix B Figure 4.2 Knockdown of GRASP for Dock180 recruitment assay. Efficient GRASP knockdown for Dock180 recruitment to recycling endosomes experiment. Representative gel of GRASP knockdown. One step RT-PCR was performed using GRASP (100 ng starting RNA) and GAPDH primers (50 ng starting RNA). Gel band intensity was quantified using Fiji. An average 91% knockdown of GRASP was achieved.


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