# Regulation of cancer cell migration by GGA3 and cytohesin-1

by

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# Abstract

Cells interact with their environment to ensure proper signaling, tissue patterning and normal function. This interaction can be regulated by cell surface molecules called receptor tyrosine kinases (RTKs) as well as by integrin receptors that respond to extracellular growth factors and matrix, respectively. Upon activation by ligand engagement, both RTKs and integrins assemble a complex of downstream signaling molecules. While this process initiates at the plasma membrane, work over the past 3 decades has clearly demonstrated a role for spatially restricted signaling in cell migration. In this thesis I show that the small GTPase, Arf6, acts as a unique switch whereby its effector, Golgi-localized, γ-ear-containing, Arf-binding protein 3 (GGA3), regulates integrin trafficking and its guanine nucleotide exchange factor (GEF), cytohesin-1, regulates RTK signaling. Both of these processes promote cancer cell migration.

Proper trafficking of integrin receptors through the endolysosomal network is required for cancer cell migration. In this thesis, I show that the endosomal adaptor, GGA3, forms a complex with β1 integrin and the endosomal sorting nexin, SNX17. Amongst GGA family members, GGA3 specifically regulates β1 integrin stability and cell migration. This identified a new role for the endosomal sorting adaptor, GGA3 in cancer cell migration.

In the 3rd Chapter of my thesis I identify a role for the Arf6 GEF, cytohesin-1 in Met RTK-dependent cell migration. Using CRISPR/Cas9 gene editing technology we find that Cytohesin-1 regulates Met RTK-dependent cell migration through the actin cytoskeleton and membrane ruffling. I find that a splice variant of cytohesin-1 that lacks a 3-nucleotide microexon is specifically required for Met RTK-dependent cell migration. Microexon splicing is a novel phosphoinositide switch whereby the shorter cytohesin-1 isoform binds $PI(3,4,5)P_3$ *in vivo* and the longer isoform binds $PI(4,5)P_2$. For the first time, this work defines a functional role for microexon alternative splicing in cell migration.

Through GEFs and effectors, Arf6 is at the interface between membrane trafficking and the actin cytoskeleton. Together these studies identify a role for the Arf6 effector, GGA3, in integrin trafficking and the Arf6 exchange factor, cytohesin-1, in RTK signalling. Both integrin trafficking and RTK signaling promote cancer cell migration, highlighting the processes that must be coordinated during cancer cell migration and emphasize the need for further studies examining the role of the membrane in cell migration during normal development or cancer progression.

# Abrégé

Les cellules interagissent avec leur environnement afin d'assurer leur fonction, maintenir une bonne communication cellulaire par voie de signalisation et une organisation adéquate des tissues. Ces interactions sont régulées en partie par des molécules présentes au niveau de la membrane cellulaire telles que les récepteurs tyrosine kinases (RTK) et les récepteurs intégrines. Alors que les RTK sont activés par les facteurs de croissance, les intégrines sont sensibles aux molécules de matrice extracellulaire (MEC). Suite à leur activation par interaction avec leurs ligands, ces récepteurs recrutent et activent une complexe moléculaire de signalisation intracellulaire. Alors que ce processus est initié au niveau de la membrane cellulaire, ces trois dernières décennies de recherche ont clairement démontré l'importance des voies de signalisation restreintes à des compartiments sub-cellulaires pour réguler la migration cellulaire. Dans cette thèse, je montre que la GTPase Arf6 agit comme un véritable interrupteur « marche/arrêt » de la migration cellulaire contrôle le trafic intracellulaire des intégrines de par sa protéine effectrice, GGA3. De plus, j'ai pu mettre en évidence la régulation de la signalisation en aval des RTKs par la cytohésine-1, un facteur d'échange nucléotidique (GEF) pour Arf6. Ces deux processus favorisent la migration des cellules cancéreuses.

Le trafic cellulaire physiologique des intégrines à travers le réseau endo-lysosomal est nécessaire à la migration des cellules tumorales. Les travaux présentés ici montrent que l'adapteur endosomal GGA3 forme un complexe avec l'intégrine β1 et la nexine de tri endosomal, SNX17. Comparé aux autres membres de la famille de protéine GGA, GGA3 régule spécifiquement la stabilité de l'intégrine β 1 et la migration cellulaire. Mon travail identifie ici un un nouveau rôle pour l'adaptateur de tri endosomal GGA3 dans la migration des cellules tumorales.

Dans le troisième chapitre de ma thèse, j'identifie le rôle du GEF de Arf6, cytohésine-1, dans la migration cellulaire dépendante du RTK Met. À l'aide de la technologie CRISPR/Cas9, nous avons montés que Cytohésine-1 régule la migration cellulaire dépendante de Met à travers son action sur le cytosquelette d'actine. Plus spécifiquement, j'ai découvert qu'un variant d'épissage de Cytohésine 1 auquel il manque un micro exon de 3 nucléotides est nécessaire à la migration cellulaire dépendente de Met. Cet épissage de microexon constitue un nouveau mécanisme de réponse aux phosphoinositides, puisque l'isoforme courte se lie à $PI(3,4,5)P_3$ in

vivo, alors que l'isoforme longue se lie à $PI(4,5)P_2$. Pour la première fois, ces travaux ont révélé un rôle pour l'épissage alternatif de microexons dans la migration cellulaire.

Arf6 se place à l'interface entre le trafic membranaire et le cytosquelette d'actine à travers ses GEFs et effecteurs. Dans leur ensemble, mes travaux identifient un rôle pour l'effecteur de Arf6 GGA3 dans le trafic cellulaire des intégrines ainsi qu'un rôle du GEF cytohésine-1 dans la signalisation cellulaire en aval des RTKs. Ces deux processus favorisent la migration des cellules tumorales, soulignant ainsi l'importance de leur coordination pour la migration cellulaire. Mes travaux mettent également en lumière le besoin de mieux comprendre le rôle de la membrane dans la migration cellulaire au cours du développement embryonnaire ou de la progression tumorale.

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# Preface

This thesis is written in manuscript-based format. It contains one published manuscript and one submitted manuscript incorporated into Chapters 2 and 3 respectively.

**First author publications arising from this work:**

1. "Regulation of Cell Migration and β1 Integrin Trafficking by the Endosomal Adaptor GGA3."
   Ratcliffe CDH*, Sahgal P*, Parachoniak CA, Ivaska J, Park M. Traffic. 2016 Jun;17(6):670-88.

2. "Distinct functional roles for Cytohesin-1 microexon splice variants in Met receptor stimulated cell migration."
   Ratcliffe CDH, Siddiqui N, Sonenberg N, Park M. Manuscript in preparation.

**Contributions of Authors:**

Chapter 2: P Sahgal performed and analyzed all experiments related to figures 2.1 D, 2.2 B, 2.4 D, E, F and Figure 2.9 A, B, C, D, E, F, G. CA Parachoniak made the initial observation that β1 integrin levels are reduced and traffic to a perinuclear compartment in GGA3 silenced cells. I performed and analyzed data related to figures 2.1 A, B, C; 2.2 A, C, D, E; 2.2 A, C, D, E; 2.3 A, B, C, D; 2.4 A, B, C; 2.5 A, B, C, D, E, F; 2.5 A, B, C, D, E, F; 2.6 A, B, C, D, E; 2.7 A, B, C, D; 2.8 plus supplemental figures 2.1 A, B; 2.2 A, B, 2.3 A, B; 2.4 A, B. I wrote the original draft of the manuscript. P Sahgal, J Ivaska, M Park and I edited the manuscript.

Chapter 3: Nadeem Siddiqui performed and analyzed experiments related to figures 3.3 A, B, C, D and supplemental Figure 3.3. I performed and analyzed all other experiments relating to figures 3.1 A, B, C, D; 3.2 A, B, C, D, E; 3.4 A, B, C, D, E, F, G, H, I, J, K; 3.5 A, B, C, D, E; supplemental figures 3.1 A, B; 3.2 A, B, C, D; 3.3. I wrote the original draft of the manuscript. N Siddiqui, M Park and I edited the manuscript.

Chapter 4: Data arising from unpublished works are presented in Chapter 4. I designed, performed and analyzed experiments related to Figures 4.1, 4.2, 4.3 and 4.4.

**Additional Publications:**

1. Lai AZ, Durrant M, Zuo D, **Ratcliffe CDH** and Park M. Met kinase-dependent loss of the E3 ligase Cbl in gastric cancer. J Biol Chem. 2012. 287(11):8048-59. 2014
2. Rajadurai CV, Harvylov S*, Coelho PP*, **Ratcliffe CDH***, Zaoui K*, Monast A, Chughtai N, Gertler FB, Siegel P, Park M. 5'-Inositol Phosphatase SHIP2 Recruits Mena to Stabilize Invadopodia for Cancer Cell Invasion. J Cell Biol. 2016 Sep 12;214(6):719-34.
3. Knight JF, Sung VYC, Kuzmin E, Couzens AL, de Verteuil DA, **Ratcliffe CDH**, Coelho P, Johnson RM, Gruosso T, Smith HW, Lee W, Saleh SM, Zuo D, Zhao H, Guiot MC, David RR, Gregg JP, Moraes C, Gingras AC, Park M. Kibra (WWC1) is a metastasis suppressor gene affected by chromosome 5q loss in triple negative breast cancer. *Cell Reports*. 2018 Mar 20;22(12):3191-3205.
4. Bell ES, Coelho PP, Rajadurai CV, Peschard P, Vaillancourt R, **Ratcliffe CDH**, Zuo D, Park, M. Autophagy regulates Met RTK-mediated cell signaling, motility and invasion. *Under revision*

*denotes equal contribution

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# List of Abbreviations

| | |
|---|---|
| Arf | ADP-ribosylation factor |
| B2AR | β2-adrenergic receptor |
| BORC | BLOC-1-related complex |
| CCP | Clathrin coated pit |
| CIE | Clathrin-independent endocytosis |
| CME | Clathrin-mediated endocytosis |
| DOR | δ–opioid receptor |
| DUB | De-ubiquitinase |
| ECM | Extracellular matrix |
| EEA1 | Early endosomal antigen 1 |
| EGF | Epidermal growth factor |
| EGFR | Epidermal growth factor receptor |
| ESCRT | Endosomal sorting complex required for transport |
| FAK | Focal adhesion kinase |
| FEME | Fast endophilin-mediated endocytosis |
| FERM | Band4.1/ezrin/radixin/moesin |
| Gab1 | Grb2 associated binder 1 |
| GAE | Gamma adaptin ear |
| GAP | GTPase-activation protein |
| GAT | GGA and Tom1 |
| GEF | Guanine nucleotide exchange factor |
| GGA3 | Golgi-localized, γ-ear-containing, Arf-binding protein |
| GPCR | G-protein-coupled receptors |
| Hrs | Hepatocyte growth factor-regulated tyrosine kinase substrate |
| ILV | Intralumenal vesicle |
| LAMP1 | Lysosomal associated membrane protein 1 |
| MIDAS | Metal ion-dependent adhesion site |
| MMP | Matrix metalloproteinase |
| PAK | p21-activated kinases |
| PDGF | Platelet derived growth factor |
| PH | Pleckstrin homology |
| PIP | Phosphatidylinositol phosphate |
| PSI | Plexin-semaphorin-integrin |

| | |
|---|---|
| PTB | Phosphotyrosine-binding |
| PTEN | Phosphatase and tensin homolog deleted on chromosome 10 |
| PX | Phox-homology |
| RCP | Rab coupling protein |
| ROCK | Rho-associated protein kinase |
| RTK | Receptor Tyrosine Kinase |
| SNX | Sorting Nexin |
| Sos | Son of sevenless |
| VHS | VPS-27, Hrs and STAM |
| WT | Wild type |

# CHAPTER 1

# 1 LITERATURE REVIEW

Cell migration is a normal physiological process. Whether it is in the developing embryo where myogenic precursor cells migrate from the somites to the limb bud or in adults in response to a wound, cells moving relative to their surroundings require spatial and temporal cues. Intracellular signalling pathways that respond to extracellular cues are tightly regulated. However, these normal physiological processes can be dysegulated. For example, aberrant growth factor signalling to receptor tyrosine kinases (RTKs) triggers activation of molecular cascades that promote cancer cell migration. Work over the past 3 decades has clearly demonstrated a role for spatially restricted signalling during cancer cell migration. By controlling the localization and abundance of signalling receptors, cancer cells respond to their extracellular microenvironment. A thorough understanding of the molecular mechanisms that regulate cancer cell migration will contribute to our understanding of the 90% of deaths caused by metastatic cancer.

Cell migration is regulated at the interface between cell membranes and the actin cytoskeleton. This regulation is mediated by members of the evolutionarily conserved Ras superfamily of small GTPases (Wennerberg, 2005). The Ras proteins are the founding members of this superfamily and pioneering studies established mutant H-Ras and K-Ras as potent oncogenes (Cox and Der, 2014). Roles for members of this superfamily have expanded to include cell signalling, membrane trafficking and regulation of the cytoskeleton (Donaldson and Jackson, 2011; Heasman and Ridley, 2008; Simanshu et al., 2017; Stenmark, 2009). The small GTPase ADP-ribosylation factor 6 (Arf6) associates with the plasma membrane and internal structures called endosomes. Activation of Arf6 promotes rearrangement of the actin cytoskeleton and can enhance cell migration. Herein, I describe novel roles for activators and effectors of Arf6 in cancer cell migration.

## 1.1 Cell migration

In 1970 Michael Abercrombie published a series of papers on “The locomotion of fibroblasts in culture” where he filmed embryonic chick heart fibroblasts migrating on cover slips (Abercrombie et al., 1970c; 1970a; 1970b). These studies provided the first quantitative insights into cell migration. A physical link between the microenvironment and the cytosol was identified

in 1986 when cloning of cDNA encoding the β subunit of the fibronectin receptor revealed a transmembrane domain (Tamkun et al., 1986). In addition to binding ECM, this receptor formed a complex with the cytosolic protein talin (Horwitz et al., 1986). Talin was known to localize specifically to sites of cell adhesion (Burridge, 1983). Many more integrin receptors have since been identified and work over the past 30 years has established that focal adhesions are dynamic structures that couple extracellular matrix to the actin cytoskeleton (De Franceschi et al., 2015). Therefore, the β1 integrin receptor became the founding member of a family of transmembrane receptors that regulate cell migration in addition to vascular biology and embryonic development.

The most common form of cell migration studied today is cancer cell migration. Approximately 90% of deaths due to cancer are associated with metastasis (Lambert et al., 2017). Metastasis is a multistage process where cancer cells from a primary tumour colonize a distant site in the body. This requires survival and migration in different microenvironments. One of the first steps in metastasis is breaking of the basement membrane and invasion of the stroma surrounding the tumour. Migration of cancer cells closely mimics mechanisms of cell migration that a variety of cells use under normal physiological conditions. Thus, several modes of cancer cell migration have been defined (Paul et al., 2016). Invariably, the actin cytoskeleton plays a role in each of these modes, however its arrangement differs depending on the mode. Different modes favour different extracellular microenvironments. For example, cancer cells migrating in dense collagen matrices must degrade the matrix in order to invade via a matrix metalloproteinase (MMP) dependent mechanism (Wolf et al., 2013). However, cells may use a bleb-based contractility mechanism of cell migration to squeeze through larger collagen pores. A molecular understanding of cancer cell migration may not only provide new therapeutic opportunities but also help us understand outcomes of clinical trials that aim to treat metastatic cancer.

### 1.1.1 Molecular mechanisms of cancer cell migration

Cancer cell migration can be modelled as a stepwise process and therefore requires spatial coordination of the cytoskeleton, adhesion proteins and signalling pathways. First, a cell must polarize and extend its plasma membrane. This is followed by adhesion, traction and retraction. A cell migrates through continuous cycling of these steps. Actin polymerization and dynamics are regulated by the Rho family of small GTPases and these play important roles in cell migration. Rac1, Cdc42 and RhoA are the best characterized members of this subfamily of small GTPases

and each mediate specific functions during cancer cell migration. Membrane trafficking also plays an essential role in cancer cell migration through active delivery of receptors or signalling complexes to the appropriate subcellular localization. Appropriate activation of small GTPases in specific subcellular localizations allows for polarization of the cell and forward migration.

### 1.1.2 Membrane protrusion

The polarized front of the cell is termed the "leading edge". The leading edge may be in the form of broad lamellipodium, narrow filopodium or spherical blebs. The lamellipodium was first described by Abercrombie in 1970 and is arguably the best characterized form of leading edge. Lamellipodia are large flat structures that are enriched in branched-actin filaments that drive membrane protrusion. Growth factor signalling activates the small GTPase Rac1 which in turn drives actin polymerization and lamellipodia formation (Ridley et al., 1992). Activation of Rac1 is required for this process since a dominant negative Rac1 mutant (S17N), inhibits platelet derived growth factor (PDGF) dependent membrane ruffling. A dominant active Rac1 (G12V) is also sufficient to promote membrane ruffling. Active Rac1 promotes ruffling by recruiting effectors such as N-WASP, p21-activated kinases (PAK) and the branched actin nucleation complex known as Arp2/3 (Bishop and Hall, 2000). Activation of the Arp2/3 complex promotes nucleation of branched actin filaments and reorganization of the actin cytoskeleton. Anterograde flow of F-actin at the leading engages focal adhesion complexes thereby coupling actin polymerization to membrane protrusion.

### 1.1.3 Adhesion

Attachment of cells to the extracellular matrix is mediated by the integrin family of receptors. Integrin receptors recruit adaptors such as talin, focal adhesion kinase and paxillin. These in turn mediate integrin signalling and couple to the actin cytoskeleton through actin binding proteins such as vinculin. Focal complexes are formed as a cell extends the leading edge forward (Zaidel-Bar et al., 2006). These are highly dynamic structures where integral membrane proteins cluster and recruit adaptor proteins. Recent mass spectrometry approaches have facilitated the identification of a core adhesome and advances in super-resolution microscopy have spatially mapped a subset of components within the adhesion complex (Horton et al., 2015; Kanchanawong

et al., 2010). Focal complexes serve as an anchor point, or a molecular clutch, for branched actin polymerization at the leading edge (Case and Waterman, 2015). Focal complexes can couple retrograde flow of polymerized actin with the extracellular matrix, allowing for mechanotransduction to the plasma membrane and membrane protrusion. Tension promotes focal complex reinforcement and maturation, while relaxation induces disassembly (Balaban et al., 2001; Choquet et al., 1997; Galbraith et al., 2002; Riveline et al., 2001). Larger focal complexes also interact with actin stress fibers. These are bundles of 10-30 actin filaments that often cross the length of the cell and terminate at focal complexes (Tojkander et al., 2012). In contrast to branched actin at the leading edge, actin stress fibers are contractile structures.

#### 1.1.4 Contraction and disassembly

In order to migrate, the cell must disassemble adhesions and retract its trailing edge. The actin cytoskeleton plays an important role in this process as well. RhoA is a small GTPase that promotes actin stress fiber formation and contractility (Ridley and Hall, 1992). RhoA promotes stress fiber contractility by recruiting Rho-associated protein kinase (ROCK) and phosphorylation of Myosin II (Kimura et al., 1996). Phosphorylated Myosin II then promotes contraction of actin stress fibers and retraction at the rear of the cell. Retraction also requires disassembly of focal adhesions at the rear of the cell. Focal adhesion disassembly is regulated by paxillin phosphorylation and calpain cleavage of focal adhesion kinase (Chan et al., 2010; Zaidel-Bar et al., 2006).

## 1.2 Integrin receptors

Hundreds of proteins are potentially involved in focal adhesion regulation and consequently cancer cell migration (Horton et al., 2015). However, a central role for integrin receptors as regulators of focal adhesion signalling and mechanotransduction has been clearly established. Integrin receptors are type I transmembrane cell surface receptors that link the extracellular matrix to intracellular signalling pathway and the actin cytoskeleton. The receptors function as heterodimeric pairs consisting of an $\alpha$ chain and $\beta$ chain. In humans, there are 18 $\alpha$ subunits and 8 $\beta$ subunits that form 24 known heterodimeric pairs that recognize different components of the extracellular matrix, including collagen, fibronectin and vitronectin. Integrin

receptors play a critical role in cancer progression and regulate cell survival, proliferation as well as promote cell migration and invasion. Integrin receptors have been identified as possible therapeutic targets.

Shortly after their discovery, it was appreciated that integrin receptors traffic through subcellular compartments within the endolysosomal network and this has role in cell migration (Bretscher, 1989). Over the past nearly 30 years, the consequences of dysregulated integrin trafficking on cancer cell migration have been defined (Caswell et al., 2009; De Franceschi et al., 2015; Pellinen, 2006). However, key questions remain in determining the specificity and pathways of integrin traffic.

### 1.2.1 Integrin receptor structure and ligand binding

Integrin heterodimers are classified based on ligand recognition. The four subgroups are RGD-receptors, collagen receptors, laminin-receptors and leukocyte-specific receptors (Hynes, 2002). While the β subunit may be shared by many heterodimers, it is the α subunit that determines ligand specificity. The α subunit is made up of a seven-bladed β-propeller, a thigh domain and two calf domains (Figure 1.4) (Campbell and Humphries, 2011). Half the α subunits, including the collagen binding α1, α2, α10 and α11, also have an α-I domain inserted between blades 2 and 3 of the β propeller (Larson, 1989). The I domain is required for ligand binding of α1β1 and α2β1 heterodimers and contains a metal ion-dependent adhesion site (MIDAS) motif that coordinates a $Mg^{2+}$ ion and recognizes a GFOGER (O = hydroxyproline) motif in a collagen fibril. A crystal structure of the α2-I domain bound to a collagen peptide revealed the molecular basis for coordination of the divalent cation by the MIDAS motif and its role in bridging the interaction between α2 integrin and collagen (Emsley et al., 2000). Mutation of key residues within the MIDAS motif (D151, S153, T221 and D254) abrogates collagen binding arguing that coordination of $Mg^{2+}$ is an essential step in ligand recognition. However, the MIDAS motif also contributes to ligand binding through side chains that interact with the collagen strand through a salt bridge, hydrophobic interactions and hydrogen bonds. The requirement for a divalent cation is a recurring theme in all known integrin:ligand interactions. However, ligand specifity is determined by the surrounding residues.

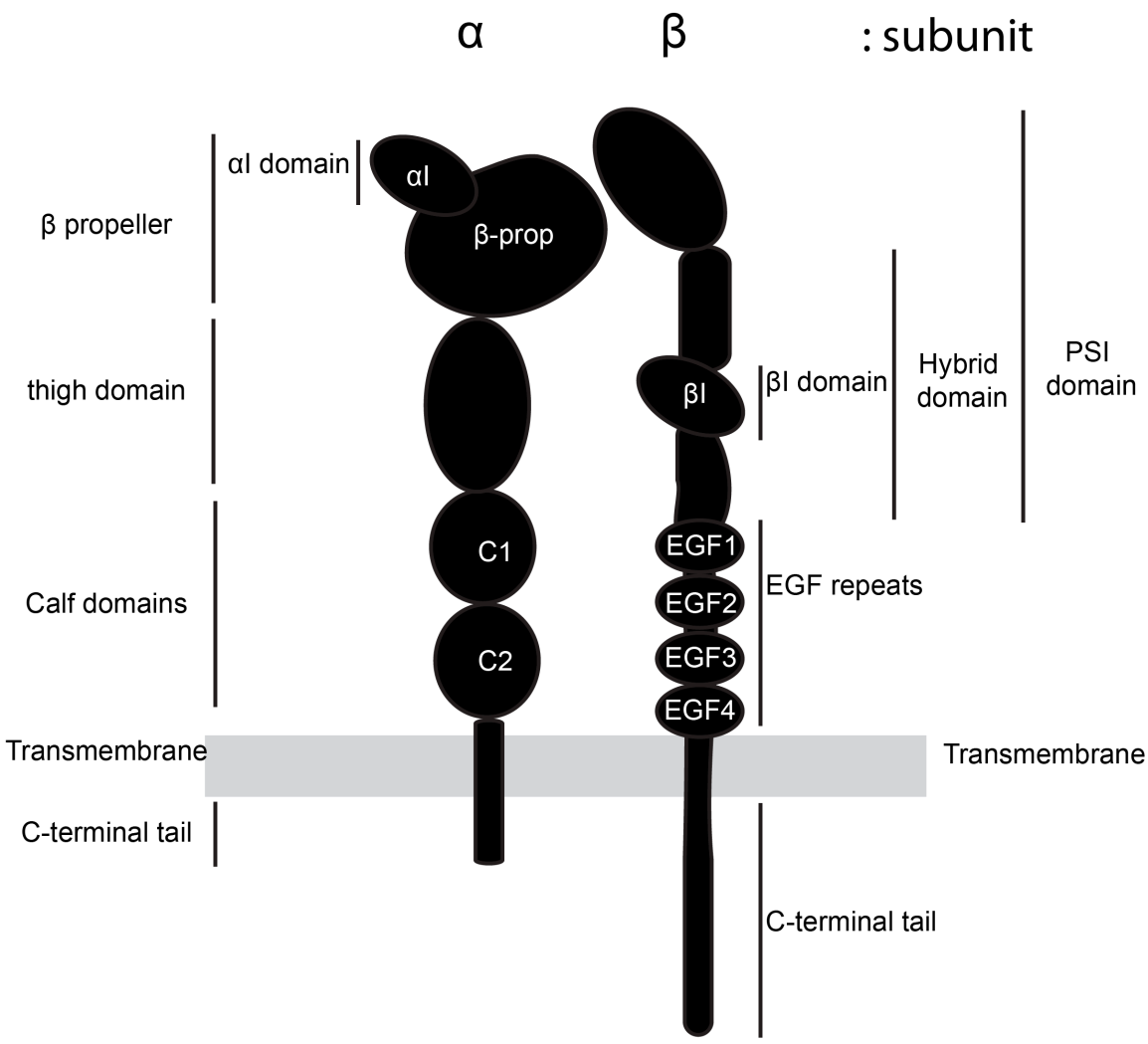


**Figure 1.1 Structural overview of integrin receptors.** Integrin receptors are type I transmembrane receptors that form heterodimers comprising one α subunit and one β subunit. Shown are the structural domains of each integrin subunit.

The β subunit is made up of a β-I domain, a hybrid domain, plexin-semaphorin-integrin (PSI) domain followed by four EGF-repeats, a transmembrane region and a cytoplasmic tail (Figure 1.4) (Campbell and Humphries, 2011). Heterodimerization is mediated by an interaction between the hybrid domain and the β-propeller of the α subunit. The cytoplasmic tail of β1 integrin contains binding sites that are specifically recognized by molecules that link integrins to the actin cytoskeleton (e.g. talin, vinculin), signalling molecules (focal adhesion kinase (FAK)) or trafficking proteins (SNX17).

### 1.2.2 Integrin activation and signalling

Despite the hundreds of proteins that are part of the adhesome, integrin tails are relatively short (40 to 60 amino acids) and consequently have limited protein recognition motifs. The β1 cytoplasmic tail has two NPXY motifs that are binding sites for phosphotyrosine-binding (PTB) domain containing proteins or FERM domain containing proteins. Integrin signalling is unique in the sense that it proceeds bi-directionally (Hynes, 2002). Ligand binding stabilizes an active conformation and promotes recruitment of cytoplasmic signalling molecules to the receptor (Takagi et al., 2002). This is termed "outside-in" signalling. "Inside-out" signalling occurs upon binding of talin or kindlin to the cytosolic tail of the β subunit. Talin binds to the membrane proximal NPXY motif while kindlin recognizes to the distal NPXY motif (Anthis et al., 2009; Harburger et al., 2009). Talin binding releases autoinhibitory interactions between the α and β subunit leading to stabilization of a high affinity state of the integrin heterodimer (Ye et al., 2011). This induces a conformational switch and increases the affinity of the receptor for extracellular ligands. Therefore, bidirectional signalling of integrin receptors provides a functional and spatially restricted link for the cell to sense the extracellular environment as well as respond to it. Talin has multiple F-actin binding sites, as well as vinculin binding sites (Klapholz and Brown, 2017). Vinculin can also interact with F-actin (Hüttelmaier et al., 1997; Janssen et al., 2006; Menkel, 1994). Therefore, talin and vinculin can act as structural adaptors between integrins and the actin cytoskeleton (Humphries et al., 2007). Paxillin is another protein adaptor that localizes to focal adhesions and regulates focal adhesion turnover (Turner et al., 1990; Zaidel-Bar et al., 2006). FAK is phosphorylated upon integrin engagement and recruited to focal adhesion. FAK is a tyrosine kinase that has been implicated in cell migration downstream from integrins and

growth factor receptors (Chen and Chen, 2006; Guan et al., 1991; Schaller et al., 1992). FAK may also signal from an endosomal compartment to prevent anoikis (Alanko et al., 2015).

### 1.2.3 Integrin receptors in cancer

Integrin receptors are important for tumour initiation, growth and metastasis in models of cancer. Deletion of β1 integrin in genetically engineered mouse models of breast cancer and pancreatic cancer impairs processes associated with tumour progression (Huck et al., 2010; Kren et al., 2007; White et al., 2004). β1 integrin is required for tumour initiation in a mouse model of breast cancer driven by the polyomavirus middle T oncogene. Mammary epithelial ablation of β1 integrin in a PyVmT driven mouse model of breast cancer, resulted in fewer hyperplastic nodules during tumour initiation (White et al., 2004). β1 integrin deletion in PyVmT cancer cell lines results in lower FAK phosphorylation (White et al., 2004). However, a requirement for β1 integrin in tumour progression is context dependent. In contrast to the aforementioned model, β1 integrin is not required for tumour initiation in a breast cancer model driven by activated ErbB2 (Huck et al., 2010). In this model, where β1 integrin has been deleted in the mammary epithelium, there are fewer metastases but only a modest effect on tumour initiation. There is also reduced FAK and Src phosphorylation in β1 integrin knockout tumours. Our group has identified a gene signature associated with invasion in human basal B breast cancer cell lines and Met receptor tyrosine kinase dependent mouse tumours that includes α5 integrin (Knight et al., 2013). However, the functional impact of α5 integrin in this model has not been tested. β1 integrin signalling is also important for resistance to BRAF inhibition in an orthotopic mouse model of melanoma (Hirata et al., 2015). In this model, β1 integrin and FAK signalling are upregulated upon BRAF inhibition and sustain ERK/MAPK activity for tumour growth. β1 integrin signalling is promoted by tumour associated fibroblasts that deposit extracellular matrix to provide a niche for cancer cell proliferation. Combined inhibition of BRAF and FAK reduces tumour growth in this model of melanoma as well as a patient derived xenograft model (Hirata et al., 2015). Together these data support a role for integrin receptors in cancer and demonstrate that signalling cues derived from the tumour microenvironment can protect a tumour from therapeutic intervention. However, the role that β1 integrin trafficking plays *in vivo* is unknown.

Given their role in cancer cell migration, integrins are attractive therapeutic targets. However, despite promising data from preclinical models, targeting integrins in a clinical setting

has yielded disappointing results. Abituzumab and cilengitide are two therapeutics that target αVβ3. Abituzumab was tested in combination in K-RAS wild-type metastatic colorectal cancer, whereas cilengitide combined with radiotherapy was tested in glioblastoma (Élez et al., 2014; Stupp et al., 2014). Neither treatment provided clinical benefit to patients. One possible explanation for this is compensatory feedback loops that exist between other integrin receptors or growth factor signalling. Clearly therefore our current understanding of integrin biology is not sufficient to predict clinical response.

### 1.2.4 Endolysosomal trafficking of integrins

Subcellular localization of integrin receptors can regulate integrin activation, signalling and function. While integrins interact with the extracellular matrix at the plasma membrane, they are internalized through a process called endocytosis, and enter the early endosome. Recycling of integrin receptors from endosomes back to the plasma membrane is the predominant pathway for internalized receptors and occurs on the order of 5-15 minutes (Bretscher, 1989). However, integrin receptors may also be degraded via the late endosome with a half-life of 8-12 hours (Lobert et al., 2010).

Active, ligand bound, integrin receptors have a higher propensity to internalize than their inactive counterparts (Arjonen et al., 2012). Once internalized the inactive and active integrin receptor diverge. Whereas the active receptor colocalizes with the late endosomal marker, Rab7, inactive β1 integrin receptors recycle back to the plasma membrane. Blocking receptor recycling leads to an accumulation of intracellular inactive β1 integrin. Several endocytic adaptors have been identified that regulate endocytosis of specific integrin subunits, as well as integrin receptors more broadly. α2 integrin and several other α subunits are recognized by the endocytic adaptor AP-2. The small GTPase Rab21 also associates with the α subunit tail to regulate integrin endocytosis and is required for cytokinesis (Pellinen et al., 2008). Rab21 associates with a conserved GFFKR sequence that is a single amino upstream from the AP-2 binding motif, suggesting that steric considerations would preclude simultaneous binding of Rab21 and AP-2 to α integrin tails and these pathways might function in parallel. The endocytic adaptor, Numb, also regulates β1 integrin endocytosis in migrating cells. Numb localizes to clathrin coated pits (CCPs) at the leading edge of migrating cells in close proximity to focal adhesions (Nishimura and Kaibuchi, 2007). Numb competes for binding of integrin tails with talin suggesting that Numb predominantly regulates

integrin subunits that are not linked to signalling machinery or the actin cytoskeleton. The clathrin adaptor Dab2 can directly interact with the same NPXY motif in the β1 integrin tail to promote clathrin dependent endocytosis suggesting that there is functional redundancy in integrin endocytosis (Teckchandani et al., 2012; 2009). Given the diversity of mechanisms that regulate integrin endocytosis it is surprising that a given mechanism is required. Therefore, it will be important to understand whether endocytic complexes assemble in distinct CCPs or whether they assemble stochastically on many integrin receptors that have been sorted into a single clathrin coated pit.

Due to the long half-life of integrin degradation relative to recycling, the molecular mechanisms of degradation of integrins and the functional consequences thereof were not the focus of initial studies. However, it has become clear that integrin ubiquitination and turnover is important for cell migration (Lobert and Stenmark, 2014; Lobert et al., 2010). In addition to promoting internalization, ligand binding also promotes α5β1 ubiquitination (Lobert et al., 2010). Mutation of 4 lysine residues on the α5 subunit reduces its ubiquitination, enhances its stability and impairs cell migration. Ubiquitinated integrin receptors are recognized by the endosomal sorting complex required for transport (ESCRT) machinery on late endosomes and targeted for degradation. Concurrent depletion of ESCRT subunits, Hrs and Tsg101 inhibits incorporation of both α5 and β1 subunits into multivesicular bodies and leads to accumulation of these subunits. Interestingly, silencing of ESCRT targeting proteins HD-PTP and UBAP1, also stabilizes α5 (Kharitidi et al., 2015). However, in contrast to the α5 ubiquitin deficient mutant, silencing HD-PTP and UBAP1 increases cell migration (Kharitidi et al., 2015). ESCRT targeting proteins subunit are not specific to integrin receptors and therefore any effects of depleting these components on cell migration may be due to other effects. However, mutational analysis also has its limitations given the small size of integrin cytoplasmic tails. Any mutations in the integrin tails may affect effector binding sites or structural determinants of integrin biology. Therefore, to predict whether a given perturbation will affect cell migration, greater understanding of the interplay between degradation and recycling is required.

The interaction between fibronectin and α5β1 is destabilized by the mildly acidic environment of the early endosome (Kharitidi et al., 2015). This leads to differential trafficking of ligand and receptor and concomitant deubiquitination of the receptor. Deubiquitination is regulated by a family of DUBs that remove ubiquitin moieties. α5β1 is recognized by the DUB, USP9x

(Kharitidi et al., 2015). Silencing Usp9X increases α5β1 ubiquitnation, decreases its stability and decreases cell migration. In pancreatic cancer, low levels of USP9x correlate with poor survival (Pérez-Mancera et al., 2012). These data are consistent with deubiquitinated α5β1 recycling back to the plasma membrane.

Integrin receptor recycling occurs through both Rab4 and Rab11 recycling endosomes. Recycling of β1 integrin heterodimers requires Arf6 activation and is increased by Arf6 activity (Morgan et al., 2013; Powelka et al., 2004). Consistent with this data, silencing of Rab35, a negative regulator of Arf6, promotes β1 integrin recycling and cell migration (Allaire et al., 2013). Phosphorylation of the transmembrane receptor Syndecan-4 by c-Src decreases Arf6 activation, reduces α5 recycling and stabilizes focal adhesion (Morgan et al., 2013). Therefore, control of Arf6 activity is an important switch to regulate integrin recycling and cell migration. SNX17 is a molecular link between integrins and the endolysosomal network. SNX17 directly binds to the distal NPXY motif in the β1 tail through its FERM domain (Böttcher et al., 2012; Steinberg et al., 2012). This interaction stabilizes β1 integrin and SNX17 silencing specifically reduces β1 integrin levels. However, prior to this thesis, there was no known link between SNX17 and Arf6.

The 8p11-12 amplicon is found in 10-25% of breast cancer and is associated with the luminal B breast cancer subtype and poor outcome (Zhang et al., 2009). The adaptor Rab Coupling Protein (RCP) is a candidate driver of the 8p-11 amplicon is an effector of Rab11 family members (RAB11A, RAB11B and RAB25) and forms a complex with α5β1 to promote cancer cell migration (Caswell et al., 2008). RCP promotes cancer cell migration by increasing endosomal recycling of α5β1 integrin. However, RCP dependent cargo is not limited to integrins. EGFR, Met and EphA2 have all be shown to be regulated by RCP under specific contexts such as inhibition of αVβ3 integrin or p53 mutation (Caswell et al., 2008; Gundry et al.; Muller et al., 2013; 2009). RCP dependent recycling is promoted by treating cells with the αVβ3 agonist, cilengitide, as well as osteopontin or a cyclic peptide (cRGDfV). Treatment of cancer cells with these agonists promotes activation of RhoA at the invasive front of cancer cell migrating in 3D microenvironments and consequently increases invasion (Paul et al., 2015). Therefore, this body of work supports a model whereby dysregulated endolysosomal trafficking promotes cancer cell migration and metastasis.

## 1.3 Endocytosis and membrane trafficking

### 1.3.1 Compartments of the endolysosomal network

The cell is made up of many membrane-bound organelles that have distinct chemical properties and compartmentalize biological processes. These structures are dynamic and serve as platforms for signalling receptors or transporting cargo. The endolysosomal network consists of the plasma membrane, endosomes and lysosomes (Figure 1.1). There is extensive crosstalk between the endolysosomal network and other organelles such as the trans-Golgi or the endoplasmic reticulum. Early discoveries on the dynamics of endolysosomal network were made in the late 1800s when Ilya Mechnikov observed a change in colour (blue to red) of litmus particles that had been internalized by cells. This indicated that cells contained an acidic compartment. Roth and Porter expanded on this work and their early studies of endocytosis focused on the uptake of yolk proteins by mosquito oocytes (Roth, 1964). Here they identified sequential compartments of internalized cargo. Over subsequent decades it was found that many ligands and their receptors could be internalized into subcellular compartments. These compartments could be isolated, characterized and each has distinct biochemical features. The identity of membrane bound organelles can be defined morphologically, chemically or molecularly (Huotari and Helenius, 2011). The Rab proteins have emerged as molecular determinants of the membrane identity of these organelles. Rabs can be recognized by downstream effectors to regulate organelle identity, their tethering and fusion as well as position within the cell (Stenmark, 2009). A subset of the Rab family serve as molecular markers to define early endosomes (Rab5), late endosomes (Rab7 or 9) and recycling endosomes (Rab4 or 11). The early endosome is characterized by a radius of between 50 and 100nm, a pH between 6.1 and 6.8, and the presence of the Rab5 small GTPase. Early endosomes also progressively acquire the phospholipid, phosphatidylinositol 3-phosphate (PI3P) (Christoforidis et al., 1999). Lysosomes are larger with a radius between 200 and 500nm; a pH of 4.8 to 6.0; and the Rab7 small GTPase. Using these characteristics as fiducial markers, I have studied the pathways and molecular requirements for receptor trafficking through the endolysosomal network. Upon internalization and entry into early endosomes, receptors may undergo distinct fates. They can be recycled to the plasma membrane or trans-Golgi network via tubulo-vesicular structures called recycling endosomes; or targeted for lysosomal degradation via late endosomes or autophagosomes. Mutations in trafficking proteins are associated with diseases

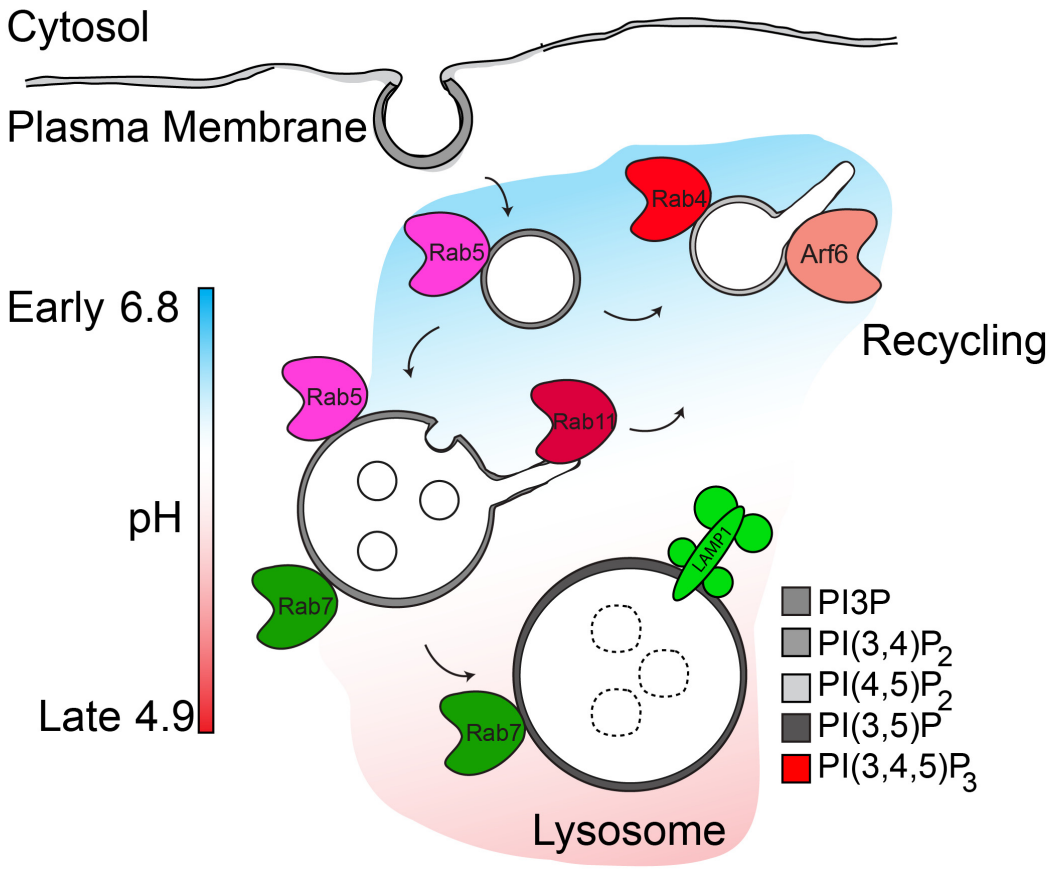


**Figure 1.2 Overview of the endolysosomal network.** The endolysosomal network starts at the plasma membrane. Clathrin coated pits, or clathrin independent routes internalize cargo and solutes. Upon internalization, vesicles acquire early endosome markers such as Rab5 and EEA1. From the early endosome, cargo may be recycled by to the plasma membrane to targeted for degradation via the lysosome. Each organelle of the endolysosomal network is characterized by chemical, morphological or molecular features. These may be, but are not limited to, size, pH, presence of different phosphoinoisitides or presence of specific Rab small GTPases.

and dysregulated trafficking has been identified as a mechanism that promotes cancer cell migration.

#### 1.3.1.1 Plasma membrane and endocytosis

For at least 35 years, models for endocytosis and trafficking through the endolysosomal network have started at the plasma membrane (Helenius et al., 1983). The plasma membrane is a bilayer that separates the cytosol and the extracellular space; it is made up of lipid, carbohydrate

and proteins. Phospholipids are the most abundant lipid constituent of the plasma membrane, with the remaining fraction predominantly comprised of cholesterol. Phospholipids are made up of two hydrophobic fatty acid chains and a hydrophilic head group. While the length and saturation of the acyl chains may vary, phospholipids are classified based on their head groups. The major phospholipids are phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine and sphingomyelin. Phosphatidylinositol is a minor constituent of the plasma membrane; however, this lipid family plays an important role in signal transduction and regulation of cancer cell migration. The plasma membrane is a dynamic structure. Endocytosis and recycling are two processes that regulate the molecular composition of the plasma membrane.

Endocytosis occurs through both clathrin mediated endocytosis (CME) and clathrin-independent endocytosis (CIE) (Traub, 2009). Clathrin mediated endocytosis is arguably the best studied pathway for receptor internalization and is used by integrins and RTKs alike. CME can be divided into multiple steps: initiation, growth, maturation, scission and uncoating. Initiation occurs when a clathrin triskelion and two AP-2 molecules coordinately recognize $PI(4,5)P_2$ at the plasma membrane (Cocucci et al., 2012). This provides the initial scaffold for additional AP-2 and clathrin triskelion to be recruited and promote membrane curvature. However, not all clathrin coated pits will bud from the plasma membrane. Different populations of clathrin coated pits appear at the plasma membrane and can be grouped according to their life-time (Aguet et al., 2013). Shortlived clathrin structures (<20 secs) are pits that have initiated but failed to mature and bud off the membrane. Long-lived clathrin structures (~40 secs half-life) are productive clathrin coated pits that endocytose. The membrane is progressively deformed, a clathrin lattice forms, and dynamin accumulates at the neck of the newly formed bud (Aguet et al., 2013; Avinoam et al., 2015). Accessory proteins that sense and promote membrane curvature, such as FCHo1/2, amphiphysin and SNX9, can also be recruited. The final step of clathrin mediated endocytosis is driven by GTP hydrolysis by dynamin and scission of the neck of the clathrin coated bud(Antonny et al., 2016; Cocucci et al., 2014). Upon internalization, clathrin rapidly dissociates from vesicles in a Hsc70 dependent reaction and any cargo taken up by the clathrin coated pit has now entered the endolysosomal network.

CIE can be further subdivided into dynamin-dependent and independent endocytosis, caveolin-dependent, the CLIC/GEEC pathway, macropinocytosis and the recently described fast endophilin-mediated endocytosis (FEME) (Boucrot et al., 2015; Mayor et al., 2014; Renard et al.,

2015). These pathways have emerged as regulators of cargo that do not engage AP-2 or clathrin. The CLIC/GEEC pathway forms from large tubules that are insensitive to inhibition of clathrin or dynamin. Cholesterol, Arf1, GRAF1 and Cdc42 are important regulators of the CLIC/GEEC pathway. Macropinocytosis is another morphologically distinct clathrin independent mechanism of endocytosis (Bohdanowicz and Grinstein, 2013). During macropinocytosis, the plasma membrane is remodelled and actin polymerization drives formation of cup-like structures that engulf solutes or antigens. Macropinocytosis is also dependent on Cdc42 (Schlam et al., 2015). The FEME pathway regulates endocytosis of cargo at the leading edge of migrating cells including the β1-adrenergic receptor, Met RTK and Shiga toxin (Boucrot et al., 2015; Renard et al., 2015). This pathway is independent of clathrin, caveolin-1 and GRAF1 but is dependent on endophilin, $PI(3,4)P_2$ and the actin cytoskeleton.

Many mechanisms of endocytosis are defined based on their morphology or independence from regulators of other types of endocytosis. It is unclear whether some of these mechanisms represent truly independent forms of endocytosis or a particular requirement for a given component in special situations. There exist many pathways for endocytosis, however once internalized, cargo is invariably delivered to the early endosome.

#### 1.3.1.2 Early endosomes

The early endosome is a hub for cargo entering the endolysosomal network. In early endosomes, cargo is sorted, and receptors may continue to signal. The best characterized Rab is the early endosomal marker Rab5. Over the past 30 years Rab5 has emerged as a central regulator of the endolysosomal network and its absence results in defects or loss of the endolysosomal network in yeast and mouse liver (Singer-Krüger, 1994; Zeigerer et al., 2012). Rab5 regulates endosome homeostasis by recruiting effectors that were identified by biochemical elution experiments (Christoforidis et al., 1999). These effector proteins include the endosomal tether, early endosomal antigen 1 (EEA1); the Class III PI3K, Vps34; Rabaptin-5; and the Rab5 exchange factor, Rabex-5 (Horiuchi et al., 1997; Stenmark et al., 1995). Vps34 is a class III PI3K that generates PI(3)P on early endosomes. EEA1 is a coiled-coil protein that tethers early endosomes and promotes homotypic docking and fusion(Murray et al., 2016). From this set of interactors, it is clear that Rab5, through its effectors, regulates the size and number of endosomes and orchestrates early endosome identity.

Rab5 also regulates early endosome maturation. The earliest clue for endosome maturation came from observations by Metchnikov where he observed a gradual colour change of litmus particles turning from blue to red upon internalization in macrophages. He noted colour heterogeneity of the particles within the same cell. This provided the first evidence for chemically distinct compartments within a single cell. Today this acidic compartment is known as the lysosome. However, key insights into the molecular basis of endosome maturation came in 2005 through biochemical and imaging experiments (Rink et al., 2005). It was observed that Rab5 endosomes could acquire Rab7 and an intermediate compartment was formed. However, this was shortlived and Rab5 would be lost and the same membrane bound organelle would remain Rab7 positive. The molecular switch for Rab conversion was later identified (Poteryaev et al., 2010). Activation of Rab5 on endosomes requires Rabex-5. Rabex-5 is also a Rab5 effector and therefore a positive feedback loop exists which promotes Rab5 activation on early endosomes. Buildup of the product of Vps34, PI3P, promotes recruitment of the Rab7 GEF Mon1/Ccz1. Mon1/Ccz1 not only activates Rab7 and recruits it to the endosome, but Mon1/Ccz1 can also displace Rabex-5. Therefore, feedback inhibition from a Rab7 GEF reduces Rab5 activation. Buildup of PI(3)P also promotes recruitment of the Rab5 GTPase Activating Protein (GAP), TBC-2 (Law et al., 2017). With these data, a molecular switch model has emerged for the maturation of endosomes from Rab5 to Rab7. However, whether similar switches exist between the over 60 other Rab family members remains to be determined.

Overexpression of Rab5 is observed in a subset of human cancers and can promote cancer cell migration and invasion *in vivo* (Frittoli et al., 2014). Indeed, overexpression of dominant negative RAB5A (S34N) reduced metastatic outgrowths of MDA-MB-231 cells injected into the mammary fat pad of immunocompromised mice. Overexpression of Rab5 in MCF10A cells also prevents kinetic arrest of cell monolayers and promotes collective motility (Malinverno et al., 2017). These results highlight a role for endolysosomal machinery in cell migration.

#### 1.3.1.3 Late endosomes and lysosomes

The late endosome and lysosome are commonly defined by the presence of Rab7 and Lysosomal Associated Membrane Protein (LAMP1) respectively. The lysosome has a pH ranging from pH 4.5 to 5.0 and is rich in acid hydrolases that mediate protein degradation and turnover of cellular components. This was the acidic compartment observed by Metchnikoff in 1893 and the

electron dense compartment observed by Alex Novikoff and Christian de Duve in 1956 (Novikoff, 1956; Roth, 2005). While the lysosome has been described as a purely degradative compartment, recent efforts have established that it also acts as a signalling organelle, as well as a carrier for extracellular matrix (ECM) remodeling matrix metalloproteases (Perera and Zoncu, 2016). The lysosome acts as a hub for nutrient sensing. Under conditions of nutrient availability, the mTORC1 complex localizes to the lysosomal cytosolic surface and promotes mRNA translation (Saxton and Sabatini, 2017). Under conditions of stress mTORC1 is cytosolic and inactive. Inactivation of mTORC1 releases transcription factors, such as TFEB, that promote lysosome biogenesis (Napolitano and Ballabio, 2016). In this way, the lysosome can act as a signalling hub to promote cellular anabolism or catabolism. Several lines of evidence have supported a function for lysosomes in cancer cell invasion. Lysosomes contribute to cancer cell invasion by transporting, Na+/H+ transporters and matrix metalloproteases that acidify and degrade extracellular matrix (Steffan et al., 2009; Steffen et al., 2008). Lysosomes also contribute to osteoclast function and bone resorption, highlighting the ability of cancer cells to subvert normal physiological processes (Lacombe et al., 2013).

Lysosomes are often depicted as a perinuclear and terminal compartment of the endolysosomal network. However, live cell imaging of lysosomal proteins clearly reveals dynamic compartments that move from the perinuclear region to the cell periphery. This movement is microtubule based and regulated by the BLOC-1-related complex (BORC) complex (Pu et al., 2015). The BORC complex is associated with the cytosolic face of lysosomes and recruits the small GTPase Arl8 which, in turn, couples lysosomes to the plus-end of the microtubule, thus promoting lysosome movement to the cell periphery. Work from the Ferguson and Grinstein laboratories have implicated lysosome position with function. Peripheral LAMP1 positive lysosomes have a higher pH (6.0) compared to perinuclear lysosomes (4.8) and impaired proteolytic activity (Johnson et al., 2016). This data is consistent with an enrichment of cathepsin B staining in lysosomes of neuronal cell bodies compared to distal components and accumulation of BACE1 at sites of amyloid placques (Gowrishankar et al., 2015). It has also recently been found that BORC interacts with Ragulator upon amino acid starvation. Ragulator negatively regulates BORC resulting in a perinuclear clustering of lysosomes (Pu et al., 2017). Clearly therefore, energy sensing and metabolism may play an important role in dynamics within the endolysosomal network. The extent to which this is true beyond lysosomes remains unknown.

#### 1.3.1.4 Recycling Endosomes

A cell internalizes and recycles the equivalent surface area of the entire plasma membrane over the course of one hour (Steinman, 1983). Given the relatively rapid dynamics, early studies suggested that recycling was the default pathway for most internalized receptors and degradation required specific targeting. While this may be true for some receptors, work over the past 20 years has established that receptor recycling is also regulated by the chemical, physical and molecular characteristics of the endolysosomal network. Once internalized, membrane bound receptors such the transferrin receptor, integrin receptors, G-protein-coupled receptors (GPCRs) and multiple RTKs are sorted and recycled back to the plasma membrane. This process can prolong stability, signalling and specifically localize receptors to plasma membrane subdomains. In many case this is a highly regulated process and disruption of recycling promotes lysosomal degradation of the receptor.

Recycling endosomes initially form as tubules extending from vesicles. These tubules sort membrane bound cargo from luminal fluid. Multiple recycling routes have been described, however the degree of overlap between these compartments is not clear (Goldenring, 2015). The best characterized routes for cargo are the Rab4 positive "fast-recycling" route and a Rab11 "slow recycling" route. Specific cargo may enter one route or another or both. Rab4 regulates endosome tubulation by regulating recruitment of adaptor proteins, such as AP-1, AP-3 and Golgi-localized, γ-ear containing, Arf-binding protein 3 (GGA3), to early endosomes (D'Souza et al., 2014). Once formed, recycling endosomes will be transported along microtubules or propelled by the actin cytoskeleton to the cell periphery where these endosomes will fuse with the plasma membrane and deliver their cargo.

Recycling endosomes are highly dynamic structures. Rapid imaging (<30msec per frame) of GFP-clathrin light chain reveals that in addition to the plasma membrane, there is a fraction of "gyrating" clathrin that moves at approximately 3.7 μm/sec but with low directional persistence (Zhao and Keen, 2008). "Gyrating" clathrin structures do not associate with dextran or epidermal growth factor (EGF) and are therefore distinct compartments from internalized cargo targeted for lysosomal degradation. However, they do colocalize with markers of recycling endosomes, contain transferrin and mediate recycling of β1 integrin and Met RTK (Luo et al., 2013; Majeed et al., 2014; Parachoniak et al., 2011). Consistent with a role for "gyrating" clathrin in receptor recycling, silencing of clathrin light chain reduces cancer cell migration (Majeed et al., 2014).

These studies highlight the dynamic nature of the endolysosomal network and the importance of coordination for cell function. It is not only the presence of individual compartments that is important but also how they interact with one another. However, there are exceptions to any strict definition of the system. Compartments can mature or fuse with one another and therefore intermediates are present at any given time. However, this framework provides us with a set of experimentally testable tools to investigate the contribution of these components to cell biology. While markers of recycling endosomes and cargo that transit via recycling endosomes have been identified, the molecular determinants of sorting remained largely unexplored at the onset of this thesis.

### 1.3.2 Cargo sorting in the endolysosomal network

The protein composition of the endolysosomal network is heterogeneous. In order to spatially and temporally coordinate receptor localization throughout the network, specific linear peptide motifs may be recognized by the endolysosomal machinery and hence sorted into specific compartments (Figure 1.2). Endosomal adaptor proteins specifically recognize sorting motifs and provide a molecular link between cargo and the endolysosomal network. Subsets of adaptor proteins act at distinct subcellular localizations, recognize different sorting motifs and can therefore coordinate selective trafficking of receptors.

#### 1.3.2.1 Receptor mediated endocytosis

Upon ligand binding, many receptors at the plasma membrane become activated and recruit downstream signalling molecules. Furthermore, a number of molecular adaptors that recognize cargo for entry into the endolysosomal network have been identified. As more receptors are characterized it has become clear that ligand bound, active receptors, are internalized at a higher rate than their inactive counterparts (Arjonen et al., 2012; Di Fiore and Zastrow, 2014; Goh et al., 2010; Harding, 1983; Li et al., 2007b; Vieira et al., 1996). Receptor activation promotes recruitment to endocytic pits and assembly of large macromolecular complexes. Endocytosis of protein cargo requires specific recognition and incorporation either CCPs or clathrin independent carriers. The best characterized pathway for cargo entry is CME. During CME, the clathrin adaptor AP-2 specifically recognizes short peptide motifs or “sorting signals” on cargo proteins. This creates a molecular link between cargo and the clathrin coat. Known sorting motifs include

tyrosine-based motifs (NPXY or YXXØ – where Ø is a large hydrophobic amino acid) and dileucine based motifs ([DE]XXXL[LI]) (Bonifacino and Traub, 2003). Cargo molecules, including many integrin receptors, contain an evolutionarily conserved YXXØ motif that is directly recognized by the μ2 subunit of the AP-2 complex (De Franceschi et al., 2016). Mutation of the YXXØ motif reduces binding to AP-2, colocalization of α2 integrin with AP-2 in cells and endocytosis of the receptor. Notably, introduction of this sequence into the αV subunit, which does not contain a YXXØ motif, promotes endocytosis. These data demonstrate that sorting motifs are both necessary and sufficient for recognition by trafficking cargo. In addition to integrins, endocytic sorting motifs are present in many receptors including transferrin receptor, CD4 and epidermal growth factor receptor (EGFR) (Aiken et al., 1994; Collawn et al., 1990; Goh et al., 2010).

Cargo proteins influence the dynamics of clathrin coated pits and fine tune the rate of receptor internalization. Global levels of CCP initiation and density are not affected by cargo activation or overexpression, however cargo capture does appear to be a key step in CCP maturation (Ehrlich et al., 2004; Loerke et al., 2009; Puthenveedu and Zastrow, 2006; Santini et al., 1998). This prevents internalization of "empty" CCPs. However, the precise function that cargo has in CCP maturation remains unclear. Several proposals have been put forward. Membrane bound receptors may phosphorylate endocytic machinery. Clathrin heavy chain is phosphorylated by c-Src tyrosine kinase upon activation of EGFR (Wilde et al., 1999). In turn, this promotes clathrin recruitment to the cell periphery and depletion of c-Src reduces the rate of EGFR endocytosis but does not eliminate it. EGFR also negatively regulates Synaptojanin recruitment to CCPs (Delos Santos et al., 2017). Synaptojanin negatively regulates CCP formation and by inhibiting Synaptojanin recruitment, EGFR may prevent abortive CCPs. Recently, using coincident sensors of clathrin and different phosphoinositides, PI4P was shown to accumulate slowly over time in CCPs (He et al., 2017). Combined knockout and silencing of the 5' phosphatases Synaptojanin and OCRL, respectively, increases $PI(4,5)P_2$ levels in CCPs, suggesting that recruitment of lipid phosphatases and kinases to CCPs may promote their maturation.

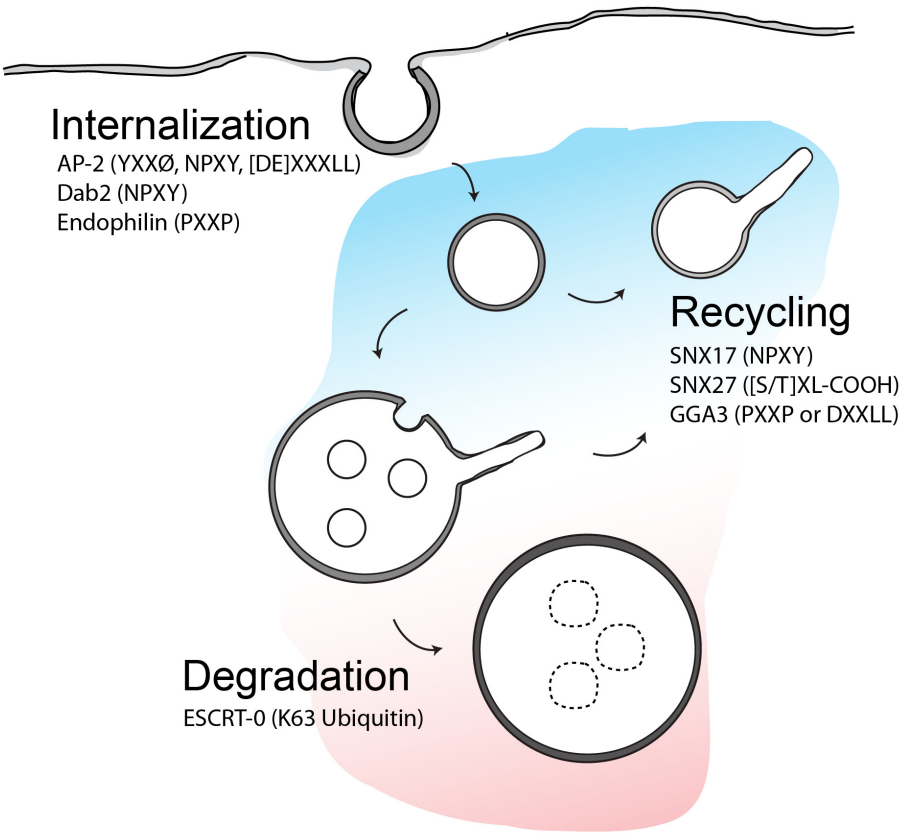


**Figure 1.3 Sorting motifs in the endolysosomal network.** Short sequences of peptides are recognized in cargo by endosomal adaptor proteins. Endosomal adaptors function at specific steps along the endolysosomal network and recognize specific sorting motifs.

#### 1.3.2.2 Receptor degradation

Lysosomal targeting of membrane bound receptors is a highly regulated process that can proceed via multiple routes. For cargo to be targeted for degradation through intra-lumenal vesicles (ILVs) it must first be poly-ubiquitinated with K63 ubiquitin chains. Ubiquitination is mediated by E3 ubiquitin ligases that specifically recognize cargo and catalyze the addition of a ubiquitin moiety to lysine residues. This process can be reversed through the action of de-ubiquitinase (DUBs) and the balance between ubiquitination and deubiquitination plays a key role of regulating receptor dynamics in the endolysosomal pathway. K63 ubiquitin chains are recognized by a processive series of complexes called the ESCRT machinery. The ESCRT complexes consist of

ESCRT I, II and III and act serially to recognize and sequester cargo into ILVs (Wollert et al., 2009). Receptors targeted for degradation are captured into flat clathrin-bilayer subdomains on the endosome and recognized by ESCRT-0 or other ESCRT associated proteins such as HD-PTP and ALIX (Ali et al., 2013; Pashkova et al., 2013; Raiborg, 2006). Cargo is then processively passed from ESCRT-0 to I and II. Whereas ESCRT-I and II recognize cargo, ESCRT-III, along with Vps4, drives membrane remodeling and budding of vesicles away from the cytosol (Adell et al., 2017; Chiaruttini et al., 2015; Schöneberg et al., 2017). At this stage, cargo is deubiquitinated and Vps4 catalyzes the ATP dependent scission of an ILV (Williams and Urbé, 2007). Incorporation into ILVs results in sequestration of cargo away from the cytosol and termination of signalling. Multivesicular bodies can then fuse with the lysosome and expose their contents to the proteases that mediate lysosome function.

In addition to ILV formation and degradation, cargo may be selected for degradation through autophagy. The best characterized form is macroautophagy whereby double-membrane organelles, called autophagosomes, capture proteins and organelles in the cytosol (Tooze et al., 2014). This pathway is upregulated under starvation conditions to provide building blocks for macromolecule biogenesis. It is generally initiated by the ULK1 complex that phosphorylates beclin 1 to promote local production of PI(3)P, recruitment of FYVE domain containing autophagy regulators and formation of the phagophore (Rubinsztein et al., 2012). The phagophore is a double layered, crescent-shaped structure that, once expanded, closes and forms the autophagosome. The autophagosome may then fuse with the lysosomes resulting in degradation of cargo. Cargo selected for macroautophagy include, but are not limited to, mitochondria, ribosomes, pathogens and signalling molecules (Stolz et al., 2014). Cargo selection is mediated by autophagy receptors that bridge cargo to the autophagosomal membrane through their LC3-interacting regions. Over two dozen autophagy receptors have been identified by yeast-two hybrid and proteomic approaches (Stolz et al., 2014). The most common motif recognized is ubiquitination, highlighting ubiquitin's important role in degradation.

Finally, recent work in yeast has identified a pathway dependent on fusion of vacuolar lysosomes (McNally et al., 2017a). This pathway is characterized by the formation of an IntraLumenal Fragment (ILF) that is formed when two vacuolar lysosomes fuse. Notably, it is selective since the lysosomal multicopper oxidase Fet5 was excluded from this fragment; however, the lysosomal iron transport Fth1 and other transporters were enriched. It will be important to

delineate the molecular determinants for selection into the ILF pathway, the biological processes that it regulates and the relevance of this pathway in mammalian and human cells.

#### 1.3.2.3 Receptor recycling

From an endosomal compartment, receptors undergoing recycling are sorted into tubes emanating from the vesicle. Enrichment of receptors over ligands occurs when membrane and fluid are separated due to the difference in surface area to volume ratio of tubes versus vesicles. Therefore, endosome geometry is one means of sorting cargo. *In vitro* experiments demonstrated that a diffusion barrier at the neck between the endosome and tubule could passively sort cargo (Aimon et al., 2014). This barrier does not apply to every cargo and therefore may provide a means to passively sort cargo. The identity of specific cargo and any molecular tools to explore this pathway *in vivo* remain unknown.

Regulating receptor activation is another mechanism of regulating receptor recycling. Unoccupied receptors recycle with a higher propensity than activated, ligand bound, receptors, presumably due to the propensity of ligand bound receptors to be ubiquitinated and targeted for degradation. Therefore, the strength of receptor:ligand complexes and RTK dimerization provides a mechanism to regulate receptor stability and recycling. As a receptor moves through the endolysosomal network and the varying pHs within the network, the ligand:receptor complex may dissociate. For example, EGF forms a stable complex with EGFR at a range of pHs, whereas transforming growth factor α (TGFα) dissociates at endosomal pH (French et al., 1995). Both EGF and TGFα induce EGFR internalization and localization to an endosomal compartment (Roepstorff et al., 2009). However, receptors activated by TGFα recycle at a higher rate, have prolonged stability and decreased ubiquitination compared to EGF stimulated EGFR (Roepstorff et al., 2009). This is consistent with the increased mitogenic effect of TGFα compared to EGF (Tomas et al., 2014). Epiregulin and epigen are weak EGFR agonists that also induce prolonged signalling and cell differentiation in MCF-7 and T47D breast cancer cell lines (Freed et al., 2017). Stronger agonists such as EGF and TGFα only show transient signalling and do not induce differentiation. Comparison of the EGFR dimerization interface induced by epiregulin or TGFα reveals significant conformational differences of the dimerization arm. In the TGFα interface H280 form a hydrogen bond with D279 of the opposing dimer. This bond is disrupted in the epiregulin interface and H280 is accessible. Since the $pK_a$ of histidine is ~6.0 (endosomal pH),

this may provide a molecular basis for prolonged epiregulin signalling and epiregulin dependent cell differentiation. These data support a model whereby targeting for receptor degradation is the key step in determining the fate of internalized receptors.

Evidence for active recycling based on specific recognition of linear sorting motifs came from studying GPCR trafficking (Hsu et al., 2012). Whereas the β2-adrenergic receptor (B2AR) is recycled via tubules, the δ-opioid receptor (DOR) is targeted for degradation (Cao et al., 1999; Whistler et al., 2002). On the same endosome, B2AR is enriched in recycling tubules but not DOR (Puthenveedu et al., 2010). These tubules are mostly distinct from bulk recycling as only 24% of Transferrin receptor tubules contained B2AR. B2AR recycling is mediated by a PDZ-interacting motif (Puthenveedu et al., 2010). This motif is recognized by SNX27 which is required for B2AR sorting and recycling (Lauffer et al., 2010; Temkin et al., 2011). SNX27 couples B2AR to the retromer complex and actin machinery to mediate sequence dependent sorting. Sequence dependent sorting is not limited to B2AR, since Wntless also undergoes retromer dependent sorting, however this is mediated by two ØX[L/M] motifs (Varandas et al., 2016). These data argue that sequence dependent sorting represents a distinct mode of recycling that is dependent on the retromer complex, however the molecular determinants that couple cargo to retromer are cargo specific.

### 1.3.3 Cargo sorting adaptors

Clearly subcellular localization of membrane receptors and signalling complexes must be tightly controlled during cancer cell migration. While any given signal may be present at one time, it is the coordination of many inputs that ultimately determines the ability of a cell to migrate. Cargo sorting proteins are well positioned to coordinate membrane traffic and send the right signal to the proper compartment. While many do not contain enzymatic activity, these proteins may contain protein interaction motifs, lipid binding domains and, importantly, recognize cargo.

#### 1.3.3.1 AP Complexes

The tetrameric AP complexes interact with clathrin and bridge clathrin coats and the membrane. There are five AP complexes (1,2,3,4 and 5) (Hirst et al., 2013). AP-2 is the best described AP family member and the main non-clathrin constituent of clathrin coated vesicles.

Studies of AP-2 have formed the basis of many hypotheses for cargo sorting in the endolysosomal network (Traub, 2009). AP-2 is composed of two large subunits (β2, and α), one medium (μ2) and one small (σ2) subunit. The μ2 subunit recognizes YXXØ motifs on cargo and $PI(4,5)P_2$ on the plasma membrane and can sort cargo into CCPs (Höning et al., 2005; Ohno et al., 1995; Owen, 1998). The σ2 subunit can also recognize [DE]XXXL[LI] motifs (Kelly et al., 2008). AP-2 acts as a key regulator of cargo transport by interacting with cargo, clathrin and many accessory proteins that contribute to efficient CME. More recently, in cells migrating in 3D, AP-2 and clathrin lattices have been observed along the cytosolic surface of collagen fiber contact sites (Elkhatib et al., 2017). These stabilize membrane protrusions and regulate cell migration. Therefore, AP complexes are important hubs for cargo transport and clathrin at the plasma membrane.

#### 1.3.3.2 ESCRT targeting proteins

Cargo targeted for ESCRT-dependent degradation can be recognized by the ESCRT-0 complex (Saksena et al., 2007). ESCRT-0 is composed of Hrs (hepatocyte growth factor-regulated tyrosine kinase substrate) and STAM (signal transducing adaptor molecule). Hrs is recruited to the membrane through PI3P binding of its FYVE domain (Raiborg et al., 2001). Hrs also recruits STAM to endosomes where, together, they recognize ubiquitin. Both proteins contain multiple ubiquitin interaction motifs and a single ESCRT-0 complex can interact with multiple ubiquitin moieties at once (Wollert et al., 2009). This is thought to be essential for cargo recognition since the affinity for any single ubiquitin interacting motif is low. Multiple interactions would allow for high avidity substrate recognition while allowing for cargo to be passed onto ESCRT-I. Hrs interacts directly with the ESCRT-I subunit, TSG101, highlighting a central role for Hrs in membrane recruitment, cargo recognition and transition to ESCRT-I.

#### 1.3.3.3 GGA proteins

The GGA proteins recognize and sort cargo at endosomes and the trans-Golgi network. GGA1, GGA2 and GGA3 are made of up a VPS-27, Hrs and STAM (VHS), GGA and Tom1 (GAT) and gamma adaptin ear (GAE) domains, with a hinge region separating the GAT and GAE domains (Bonifacino, 2004). GGA1 and 3 share more homology to one another than GGA2. The VHS domain recognizes dileucine based sorting motifs in M6PR, sortillin, furin receptor, PI4KIIIβ and others. The GAT domain of GGA3 also binds ubiquitin and promotes EGFR degradation

(Puertollano and Bonifacino, 2004). The GAE domain binds to accessory proteins containing the DFXØ motif such as Rabaptin-5 (Miller et al., 2003). The hinge region interacts directly with clathrin heavy chain (Puertollano et al., 2001b). Overexpression of GGA proteins promote clathrin recruitment to the trans-Golgi and GGAs colocalize with clathrin at the trans-Golgi and endosomes. Together these data argue that GGAs may function as coat proteins similar to AP-2 in clathrin mediated endocytosis. However, GGAs exist as monomers in solution and structural insight into the architecture of the GGA coat is currently lacking. Therefore, as well as cargo recruiters, GGA proteins are multi-valent scaffolds for several proteins involved in membrane trafficking.

GGA proteins are recruited to the membrane through a direct interaction with GTP-loaded Arf proteins (Boman et al., 2000; Dell'Angelica et al., 2000; Hirst et al., 2000; Poussu et al., 2000). A co-crystal structure of the GGA1 GAT domain and Arf1 revealed the importance of a GGA1 asparagine residue at the interface between these two proteins (Shiba et al., 2003). This asparagine residue is conserved between all GGA family members and mutation (N194A) abrogates Arf binding of all GGA family members. GFP-tagged GGA1, 2 and 3 appear as punctate structures at the Golgi and endosomes. However, asparagine mutant GGA proteins are predominantly cytosolic, highlighting a key role Arf proteins in GGA membrane recruitment. Through this wide variety of interactions, GGA proteins, recognize and select cargo to promote proper localization of a variety of membrane proteins at the trans-Golgi network and endosomes.

#### 1.3.3.4 Retromer

Retromer is an endosomal complex that mediates membrane tubulation, cargo selection and trafficking from endosomes to the plasma recycling or trans Golgi network. It is composed of two subcomplexes. A heterotrimer of Vps26, Vps29 and Vps35 and SNX protein heterodimer (Cullen and Korswagen, 2011). Vps35 interacts with the WASH complex. WASH promotes actin nucleation on endosomes and regulates protein sorting (Derivery et al., 2009). SNX proteins all contain a Phox-homology (PX) domain (Worby and Dixon, 2002). Many also contain a Bin/amphiphysin/Rvs (BAR) domain, Src-homology 3 (SH3) domains, a PDZ domain or a Band4.1/ezrin/radixin/moesin (FERM) domain. Via their PX domain they recognize phosphoinositides (mainly PI3P). The BAR domain can sense membrane curvature and oligomerization of SNX dimers promotes membrane bending and tubulation (Peter, 2004). Therefore, together with the WASH complex, retromer drives membrane tubulation. Cargo

recognition domains, such as the PDZ and FERM domain, regulate retromer dependent trafficking of many receptors. Notably, SNX27 is a PDZ domain containing protein that recognizes and interacts with B2AR, GLUT1 and glutamate receptors, among other proteins, to mediate their trafficking (Steinberg et al., 2013; Temkin et al., 2011; Wang et al., 2013). SNX27 is downregulated in patients with Down's syndrome and SNX27 overexpression rescues cognitive defects in a mouse model of Down's syndrome (Wang et al., 2013). Therefore, retromer and sorting nexins represent cargo recognition modules that link cargo to membrane tubules and the actin cytoskeleton.

## 1.4 Lipid recognition and Phosphoinositides

Cargo and signalling complexes are polarized during cancer cell migration and the endolysosomal network plays a key role in these processes. Endolysosomal trafficking of signalling receptors can regulate receptor stability and localization. In addition, different compartments of the endolysosomal network have distinct chemical characteristics that can be recognized by cytoskeletal regulators. Therefore, chemical modification of lipid headgroups allows for coordinated crosstalk between signalling molecules and the machinery that regulates cancer cell migration. At the interface between organelles and the cytoskeleton are proteins that recognize specific chemical properties of lipid headgroups on the cytosolic face of the membrane.

### 1.4.1 Phospholipid modifications

The inner leaflet of the plasma membrane is negatively charged due to the enrichment of phosphatidylserine and phosphatidylinositol (Leventis and Grinstein, 2010). Phosphatidylinositol is a minor component of the plasma membrane; however, it plays an important role in signal transduction (Fruman et al., 2017; Schink et al., 2016). Phosphatidylinositol is made up of a glycerol backbone, linked to two fatty acid chains and an inositol headgroup. Inositol is a six-carbon ring with a hydroxyl group at each carbon position. In phosphatidylinositol, inositol is linked to the glycerol backbone at the 1' carbon position. Combinations of mono-, di- or tri-phosphorylation of the 3, 4 and 5 hydroxyl groups of the inositol head group generates 7 distinct polyphosphoinositides. The most abundant phosphoinositides and first to be identified is $PI(4,5)P_2$. $PI(4,5)P_2$ is enriched on the inner leaflet of the plasma membrane. $PI(4,5)P_2$ can be recognized by a pleckstrin homology (PH) domain containing proteins and as such has been implicated in diverse

cellular processes including endocytosis, $Ca^{2+}$ signalling and cytoskeletal rearrangements (Czech, 2000).

Whereas $PI(4,5)P_2$ localizes primarily to the plasma membrane, specific phosphatidylinositol species localize to specific subcellular localizations. Studies of the subcellular localization and dynamics of lipid modifications were aided by the development of fluorescent proteins fused to lipid binding domains that specifically recognize different phospholipids (Balla and Varnai, 2009). For example, the PI4P-binding FAPP1 PH domain localizes predominantly to the Golgi, PI3P-binding Hrs FYVE domain localizes to early endosomes and $PI(4,5)P_2$-binding phospholipase C $\delta_1$ PH domain localizes to the plasma membrane. Despite levels of $PI(3,4,5)P_2$ representing less than 1% of the plasma membrane, it plays a central role in cell signalling and migration.

### 1.4.2 Lipid recognition domains

Clearly, specific recognition of different phosphoinositide species will be a key determinant in the subcellular localization and function of a particular effector. Phosphoinositide recognition is mediated by protein domains with polybasic patches or grooves, that together with geometric constraints, can selectively recognize different phosphoinositide head groups. The following is an introduction of a subset of lipid recognition domains that are relevant to this thesis.

#### 1.4.2.1 FERM Domain

The Band4.1, Ezrin, Radixin and Moesin (FERM) domain is a multifunctional domain that consists of 3 subdomains (A, B and C). The FERM domain of Ezrin binds $PI(4,5)P_2$, whereas the Kindlin2 FERM domain binds $PI(3,4,5)P_3$. The FERM-like domain of SNX17 interacts with the distal NPXY motif of β1 integrin receptor (Böttcher et al., 2012; Steinberg et al., 2012) and the FERM domain of FAK is implicated in FAK autoinhibition (Frame et al., 2010). A number of FERM domains also contain nuclear export sequences and nuclear import sequences and localize to the nucleus under specific conditions. Therefore, while FERM domains may recognize phosphoinositides, they can also function to recognize signalling complexes and integrate multiple signals.

#### 1.4.2.2 PX and FYVE Domains

Both Fab1, YOTB, Vac1, EEA1 (FYVE) and PX domains preferentially interact with PI3P (Balla and Varnai, 2009). The FYVE domain is approximately 60-70 amino acids and consists of two β-hairpin zinc fingers followed by an α-helix. The PX domain is approximately 130 amino acids. FYVE domains and most PX domains alone are not sufficient to bind PI(3)P. They require dimer or oligomer formation in order to specifically promote membrane recruitment (Kutateladze, 2007). FYVE domains are predominantly found in proteins that regulate membrane trafficking. PX domains are also found in proteins that regulate membrane trafficking but more specifically proteins belonging to the sorting nexin (SNX) family.

#### 1.4.2.3 PH domain

There are approximately 250 human genes that encode PH domains (Lemmon, 2007). PH domains are about 120 amino acids and consist of a 7-stranded beta-sandwich followed by an alpha helix. Early work showed that the PH domain of phospholipase C-$\delta_1$ binds specifically to PI(4,5)$P_2$ and crystallography studies provided the structural basis for clear stereospecificity of this PH domain (Ferguson et al., 1995; Lemmon et al., 1995). This provided the initial framework for studying PH domains and their ability to bind phosphoinositides. However, it should be noted that only about 10-20% of PH domains bind phosphoinositides with high affinity or specificity. In recent years, analysis of PH domains has expanded beyond phosphoinositides to interactions between PH domains and GPCRs and other protein:protein interactions. However, based on *in silico* predictions, these represent the minority of interactions whereas most PH domains associate with the membrane, albeit weakly and with low specificity. The β1/ β2 loop represents an important element of these interactions. Specific and strong interactions (low μM to nM affinities) between PH domains and phosphoinositides have been identified for PI(4,5)$P_2$, PI(3,4)$P_2$, and PI(3,4,5)$P_3$. Strong and specific interactions depend on hydrogen bonds formed between the headgroup and basic side chains in the binding pocket. Intriguingly, mutation of a glutamic acid in the Akt1 PH domain to lysine (E17K) results in constitutive recruitment of Akt1 to the membrane (Carpten et al., 2007). Mutant Akt1 (E17K) recognizes PI(4,5)$P_2$, is constitutively phosphorylated and induces leukaemia in mice (Carpten et al., 2007; Landgraf et al., 2008). This highlights the critical role for specific recognition of phosphoinositides in cell homeostasis.

### 1.4.3 Phospholipid based signal transduction

Enzymatic control of signalling activation through lipid kinases and phosphatases provide a rapid way to localize and regulate signalling. Trafficking of activated receptors through the endolysosomal network provides an additional level of control. Given that different phospholipid species localize to distinct subcellular localizations and protein domains exist that recognize these phospholipids, it should be straightforward to conclude that modification of lipid head groups represent an important mechanism for recruiting signalling effectors. By controlling the duration and localization of phospholipid species, lipid modifying enzymes represent important regulators of cell signalling. Several cell surface receptors, through lipid modifications transmit signals across the plasma membrane to the inner leaflet of the cell. This provides a mechanism for signal transduction from the outside of the cell to the inside.

#### 1.4.3.1 PI3K/Akt Signalling

There are three classes of PI3K enzymes, each with a different substrate specificity but all modify the 3' hydroxyl group of the inositol ring of phosphatidylinositol. Class I PI3Ks are multisubunit enzymes that can phosphorylate PI(4,5)$P_2$ to generate PI(3,4,5)$P_3$ (Vanhaesebroeck et al., 2012; Whitman et al., 1988). Class II enzymes generate PI(3)P and PI(3,4)$P_2$ and class III PI3K generates PI(3)P. The sole member of Class III is Vps34. Vps34 regulates membrane trafficking and is the only PI3K member to be evolutionarily conserved from yeast to humans. As discussed earlier, PI(3)P accumulates of early endosomes. This promotes early endosome homotypic fusion through recruitment of the FYVE domain containing tether, EEA1. Accumulation of PI(3)P also promotes endosome maturation. Vps34 is also an important initiation factor of autophagy and Vps34 inhibition blocks autophagy initiation.

The PI3K/Akt pathway is arguably the most intensely studied lipid signalling pathways (Manning and Toker, 2017). Class I PI3K enzymes are composed of a regulatory subunit, p85, and a catalytic subunit, p110. Multiple isoforms of each subunit exist with specific tissue expression patterns. The PI3K pathway is activated downstream of many receptor tyrosine kinases including Met, insulin receptor and platelet-derived growth factor receptor (Auger et al., 1989; Maroun et al., 1999b; Ruderman et al., 1990). Generation of PI(3,4,5)$P_3$ or PI(3,4)$P_2$ at the plasma membrane promotes recruitment of Akt, that, in turn, promotes cell survival and growth through phosphorylation of over 200 substrates (Manning and Toker, 2017). Akt negatively regulates cell

death through phosphorylation of Bcl2 antagonist of cell death (BAD), promotes cell cycle progression by inactivating GSK3β and promoting translation through mTOR activation. Therefore, tight control of $PIP_3$ generation and localization is critical for cellular homeostasis.

#### 1.4.3.2 PTEN

Phosphatase and tensin homolog deleted on chromosome 10 (PTEN) is a lipid and protein phosphatase that catalyzes the conversion of $PI(3,4,5)P_3$ to $PI(4,5)P_2$ and thereby negatively regulates PI3K signalling (Maehama and Dixon, 1998). PTEN may also negatively regulate Akt activation and cancer cell invasion by acting as a phosphatase for $PI(3,4)P_2$ (Malek et al., 2017). Loss of PTEN leads to accumulation of $PI(3,4,5)P_3$, enhanced Akt signalling, cell growth, proliferation and survival. PTEN was identified as a tumour suppressor in multiple cancers, is one of the most frequently mutated genes in human cancer and hereditary mutations in PTEN predisposes individuals to cancer (Li, 1997; Liaw et al., 1997; Marsh et al., 1997; Steck et al., 1997). PTEN consists of a N-terminal $PI(4,5)P_2$ binding domain, phosphatase domain, a phospholipid binding C2 domain and C-terminal tail that regulates PTEN stability (Song et al., 2012). PTEN is predominantly cytosolic, however PTEN SUMOylation regulates trafficking to the nucleus where it protects cells from DNA damage (Bassi et al., 2013). Therefore, PTEN localization has an important role in determining PTEN function.

#### 1.4.3.3 PI3K Signalling and cell migration

A paradigm for $PI(3,4,5)P_3$ signalling and cell migration was established in *D. Discoideum* and mammalian neutrophils (Artemenko et al., 2014). The soil amoeba *D. Discoideum* undergoes chemotaxis, or migration towards a chemical gradient, during its life cycle. In response to linear gradients of cyclic AMP (cAMP), $PI(3,4,5)P_3$ is generated at the leading edge (Parent et al., 1998). Generation of $PIP_3$ regulates cell speed and deletion of PI3K enzymes in *D. Discoideum* reduces their ability to migrate, however it is not absolutely required for chemotaxis (Hoeller and Kay, 2007). To maintain a stable $PI(3,4,5)P_3$ gradient, PTEN acts globally and this balance between local excitation and global inhibition allows for efficient *D. Discoideum* migration (Levine et al., 2006). A $P(3,4,5)IP_3$ gradient is also observed in migrating neutrophils (Servant, 2000; Yoo et al., 2010). Generation of $PI(3,4,5)P_3$ at the leading edge of neutrophils promotes rearrangement of the actin cytoskeleton, extension of the plasma membrane in the direction of chemoattractant and cell

migration. Actin rearrangement is dependent on activation of Rac1 and recruitment of the Arp2/3 complex to the leading edge (Weiner et al., 1999). Using an optogenetic system to acutely generate local $PI(3,4,5)P_3$, polarized $PI(3,4,5)P_3$ production in neutrophils is sufficient to reorient the leading edge and promote Rac1 activation (Graziano et al., 2017). Rac1 activation, in this context, was dependent on the Rac1 GEF, P-Rex1. Clearly therefore, $PI(3,4,5)P_3$ signalling extends beyond the well-studied PI3K/Akt paradigm and dysregulated PI3K activation.

Cancer cell migration and metastasis is also dependent on PI3K signalling (Sawyer et al., 2003; Simond et al., 2017). Upon stimulation of MTLn3 carcinoma cells with EGF, PI3K is activated and new sites of actin polymerization (actin barbed-ends) are generated at the leading edge (Mouneimne et al., 2004). Inhibition of PI3K reduces the number of barbed-ends and lamellipodia protrusion. In MV3 melanoma cells migrating in 3D, $PI(3,4,5)P_3$ is polarized at the leading edge of the cell, however its function in this context or in cells migrating in 3D is unknown (Welf et al., 2016). Clearly, the focus of PI3K signalling in cancer has focused on a role for Akt in promoting cell survival and proliferation and there is a lack of understanding of how dysregulated PI3K signalling in cancer contributes to cancer cell migration (Fruman et al., 2017; Lien et al., 2017). Many GEFs and GAPs of small GTPases are PI3K effectors and have lipid binding domains that specifically recognize $PIP_3$ suggesting a potential mechanism for coupling $PIP_3$ signalling to the actin cytoskeleton (Gambardella and Vermeren, 2013). Studies from model systems such as *D. Discoideum* and neutrophils provide a strong basis for further studies. These data highlight the important role for extracellular chemical cues in promoting cell migration through activation of intracellular signalling pathways in multiple organisms and in healthy and diseased tissue.

## 1.5 Small GTPases

There are over 150 human proteins in the Ras small GTPase superfamily and many of these have evolutionarily conserved orthologs (Wennerberg, 2005). These are subdivided into five subfamilies based on sequence similarity and that broadly regulate different aspects of the cell. Broadly, the Ras family regulates cell signalling; the Rab family regulates membrane trafficking; the Rho family regulates the cytoskeleton; the Ran family regulates nucleocytoplasmic shuttling; and the Arf/Arl family that regulates both membrane trafficking and the cytoskeleton. Together, the Ras superfamily has been implicated in normal development and mutations of specific

members have been associated with cancer. However, despite their clear functional role, specific inhibition of these enzymes has met with limited success.

The Ras superfamily of small GTPases hydrolyze GTP to GDP and release one inorganic phosphate molecule. However, their function is not thought to reside in their ability to regulate GTP or GDP levels in the cell but rather as molecular switches, whereby their "active" or "inactive" state is defined by being bound to GTP and GDP respectively. In their active state, small GTPases recruit effectors. GTPase activity and conversion of GTP to GDP releases effectors and results in the "inactive" state of the enzyme. The ratio of GTP to GDP in the cell is approximately 10:1. Therefore, release of GDP allows for GTP binding and reactivation of the enzyme. Activation and subsequent inactivation of small GTPases can be thought of as a cycle and GTPases that are undergoing multiple rounds of activation and inactivation are said to be "cycling" (Figure 1.3).

The crystal structure of the G-domain of HRAS in complex with a non-hydrolyzable GTP analog, provided the first glimpse into the significance of GTP binding for Ras activation (Milburn et al., 1990; Pai et al., 1989). In the GTP bound state, hydrogen bonds form between the γ-phosphate of GTP and both Thr35 in the switch-I region and Gly60 in the switch II region. The threonine residue also interacts with a $Mg^{2+}$ ion. These interactions stabilize the switch-I and switch-II regions, allowing for specific recognition of Ras by effector proteins. Upon GTP hydrolysis, the γ-phosphate is released, and the switch regions return to a flexible state. Regulation of small GTPase cycling is achieved by GEFs that promote release of GDP and GAPs that promote GTPase enzymatic activity (Cherfils and Zeghouf, 2013).

### 1.5.1 ADP-ribosylation factor small GTPases.

There are 6 known members of the Arf family of small GTPases, termed Arf1 through Arf6, however Arf2 has been lost in humans (Jackson and Bouvet, 2014). Arf proteins are 20kDa proteins, divided into 3 classes based on sequence homology. Arf1-3 make up class I; Arf 4 and 5 make up class II; and Arf6 is the lone member of class III. Arf1 was originally identified as a protein factor required for the ADP-ribosylation of Gsα by cholera toxin (Kahn and Gilman, 1986). However, it has been widely recognized that their main cellular function is in membrane trafficking and regulation of the actin cytoskeleton (Donaldson and Jackson, 2011). Class I and II Arf proteins localize predominantly to the Golgi body and regulate Golgi trafficking. The Class III Arf, Arf6, is found at the plasma membrane and early endosomes. Arf6 is arguably the best

characterized Arf family member, followed by Arf1. In contrast to other small GTPases, Arf proteins possess a N-terminal amphipathic helix that is indispensable for membrane insertion (Antonny et al., 1997). In addition to the amphipathic helix, Arf proteins have a N-myristoyl group that anchors these proteins to the membrane.

Arf6 regulates membrane trafficking and the actin cytoskeleton by recruiting effectors to specific subcellular domains in an activation dependent manner. For example, recruitment of the lipid kinase, PIP4K5 and phospholipase D1 (PLD) to the plasma membrane generates $PI(4,5)P_2$ and phosphatidic acid respectively (Cockcroft, 2009; Honda et al., 1999; Jenkins and Frohman, 2005). This in turn may regulate CCPs or activation of small GTPases that are involved in actin rearrangement such as Rac1. Arf6 may interact with AP2 and localizes to CCPs to regulate transferrin receptor trafficking (Montagnac et al., 2011; Paleotti et al., 2005). Arf6 is also required for Rac activation through the Rac1 GEF, DOCK180 (Santy et al., 2005). It is clear that an important function for Arf6 is regulation of the actin cytoskeleton. However, it is less clear whether this is as a result of an effector that regulates Rac1 directly or whether lipid modification by effectors is sufficient to promote actin rearrangement through Rac1. The GGA proteins are also important effectors of Arf proteins and regulate selective cargo trafficking at the trans-Golgi or endosomes, as discussed earlier.

Similar to Ras, Arf proteins have distinct structural conformations in GDP and GTP bound forms. In the switch I region, Thr44 interacts with the γ-phosphate of GTP and $Mg^{2+}$ (Pasqualato et al., 2001). Gly66 also interacts with GTP. These interactions stabilize the switch I and II regions allowing specific recruitment of downstream effectors. A crystal structure between Arf1 and the N-terminal portion of the GAT domain of GGA1 revealed the structural basis for membrane recruitment of GGA proteins (Shiba et al., 2003). This structure revealed contacts between GGA1 and both switch regions of Arf1 as well as the interswitch region. In this structure, GGA1 Asp194 forms two hydrogen bonds with Arf1 Phe51 at the junction between switch I and the interswitch region. GGA1 and GGA3 mutants (N194A) are no longer able to bind GTP-loaded Arf, bind to membrane and are cytosolic (Parachoniak and Park, 2012; Puertollano et al., 2001b). Clearly therefore an understanding of the regulators of Arf6 activation will contribute to our understanding cell migration.

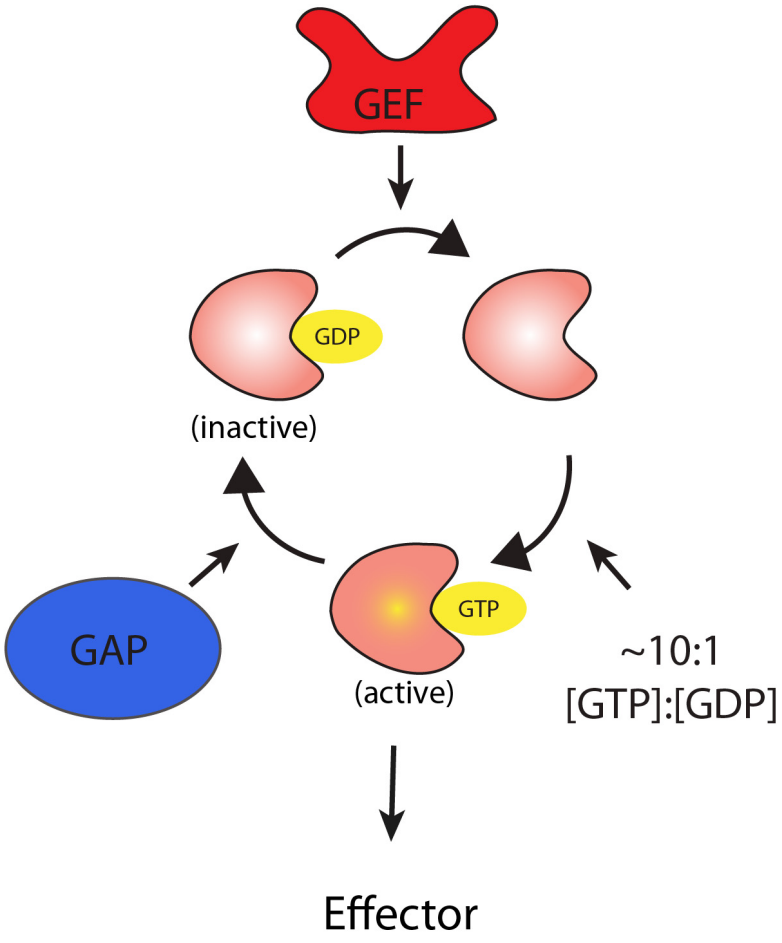


**Figure 1.4 Outline of small GTPase cycling.** A small GTPase may exist in one of three states (GDP-bound, nucleotide-free or GTP-bound). Release of GDP is regulated by GEF. The ratio of GTP to GDP in the cytosol is roughly 10:1 and therefore GTP will have a much higher likely hood of binding the nucleotide free small GTPase relative to GDP. GTP hydrolysis is catalyzed by GAPs. It is in the GTP bound state where a small GTPase can be recognized by effectors and is considered "active".

### 1.5.2 Arf Guanine Nucleotide Exchange Factors

Arf GEFs specifically regulate GDP release from Arf small GTPases through their Sec7 domain (Chardin et al., 1996). Arf GEFs are organized into 7 broad families subfamilies including the IQSEC proteins (1-4), the PSD proteins (1-4) and the cytohesin family (1-4) (Cox et al., 2004). The IQSEC, PSD and cytohesin proteins are putative Arf6 GEFs that have been implicated in angiogenesis, lymphoid cell adhesion, bacterial invasion and cancer cell migration (Casalou et al., 2016; D'Souza and Casanova, 2016; Gamara et al., 2015; Hongu et al., 2015; Humphreys et al., 2016). The Sec7 domain is a unique fold that contains 10 α-helices that form a hydrophobic

groove, as well as hydrophilic residues that form the edges of the groove (Cherfils et al., 1998). This groove and its adjacent edges form the interface that mediates GDP release from the target Arf protein. The hydrophilic interface contains a "glutamic finger" that displaces the $Mg^{2+}$ ion and β-phosphate of GDP to destabilize the GDP:Arf interaction. Mutation of this glutamate residue to lysine abolishes GEF activity. Identification of the Cytohesin-2 Sec7 domain facilitated the identification of other Sec7 domain containing Arf GEFs (Chardin et al., 1996).

#### 1.5.2.1 Cytohesins

There are four members in the Cytohesin family (Cytohesin-1, 2, 3 and 4). Cytohesin-1 was the first to be identified as a transcript highly enriched in NK cells over T cells (Liu and Pohajdak, 1992). Shortly thereafter, it was identified in a screen for interactors for the leukocyte specific β2 integrin (Kolanus et al., 1996). However, Cytohesin-1 protein is expressed in other cell types that do not express β2 integrin. Cytohesin-1 also had a high degree of homology to ARNO and Grp1. Therefore, ARNO and Grp1 were termed cytohesin-2 and 3 respectively. Cytohesin-4 was later identified as a brain specific transcript (Ogasawara et al., 2000). The cytohesin proteins consist of a coiled-coil domain, Sec7 domain and a PH domain. Consistent with a role for Arf activation, cytohesins regulate membrane trafficking of integrin receptors, adhesion of leukocytes and myelination (azreq and Bourgoin, 2011; Oh and Santy, 2010; Yamauchi et al., 2012). *In vitro* cytohesin proteins can activate Arf1, 3 and 6 to varying degrees, however the specificity of cytohesin proteins *in vivo* has not been well defined (Cohen et al., 2007; Meacci et al., 1997).

A decade after their initially discovery, it was found that each cytohesin protein has two splice variants (Ogasawara et al., 2000). These isoforms differ by the inclusion of a 3-nucleotide exon whose inclusion results in an additional glycine residue in the β1/2 loop of the PH domains. Addition of a glycine residue in cytohesin-2 changes phosphoinositide selectivity *in vitro* (Cronin et al., 2004). Whereas the diglycine variant of cytohesin-2 binds $I(1,3,4,5)P_4$, the triglycine variant binds $I(1,4,5)P_3$. A study of whole brain cDNA found that the predominant transcripts of cytohesin-1 and 2 were the triglycine variants, whereas the cytohesin-3 diglycine transcript was predominant (Ogasawara et al., 2000). This led to the assumption that cytohesin-1 and 2 acted downstream from $PI(4,5)P_2$ whereas cytohesin-3 functioned downstream from $PI(3,4,5)P_3$. Studies on cytohesin splice variants have been limited to *in vitro* experiments and a functional consequence of cytohesin splice variants is currently lacking.

### 1.5.3 Arf GTPase Activating Proteins

Small GTPases have low intrinsic GTPase activity. GTP hydrolysis by Arf proteins is required for signal termination and dissociation of effectors. There are 31 human Arf GAP genes identified that fall into 11 subfamilies based on domain architecture and phylogeny (Jackson and Bouvet, 2014). They all share a common Arf GAP domain. Many also contain membrane binding domains such as a PH domain or BAR domain (Spang et al., 2010). Arf GAPs stimulate GTP hydrolysis by stabilizing a transition state through an arginine finger. In the crystal structure of ASAP3 and Arf6, the catalytic Arg residue is positioned to stabilize the γ-phosphate of GTP allowing for GTP hydrolysis by Arf6 (Ismail et al., 2010). Mutation of the ASAP3 catalytic Arg residues abrogates Arf6 GTP hydrolysis. However, Arf GAPs are not only thought to attenuate Arf activation but also to promote GTPase cycling. Arf GAPs have been implicated in many processes regulated by Arf small GTPase such as coat assembly at the Golgi, receptor trafficking, focal adhesion dynamics and membrane ruffling (Cukierman et al., 1995; Etoh and Fukuda, 2015; Kowanetz et al., 2004; Randazzo et al., 2000).

### 1.5.4 Arf6 in cancer

Cancer cell migration and invasion are processes that depend on Arf6 and are required for cancer progression (Hongu et al., 2016; Li et al., 2017). A definitive role for Arf6 in human cancer remains to be defined, however Arf6 promotes tumour invasion and metastasis in mouse and tissue culture models. Arf6 protein levels are higher in breast cancer cell lines with increased invasive capacity and silencing Arf6 reduces their capacity to invade (Hashimoto et al., 2004). Upon injection into athymic male mice, melanoma cells overexpressing dominant active Arf6 (Q67L) are more invasive compared to parental cell lines or dominant negative Arf6 (T27N) expressing cells (Muralidharan-Chari et al., 2009). However, cells overexpressing either dominant active or inactive Arf6 mutants have impaired tumour growth suggesting that Arf6 cycling may be important for tumour progression (Muralidharan-Chari et al., 2009). Indeed, overexpressing a hyperactive mutant (T157A) of Arf6 in MDA-MB-231 breast cancer cells induces rearrangement of the actin cytoskeleton, formation of actin-rich rosettes and degradation of extracellular matrix (Marchesin et al., 2015).

A role for Arf6 in the tumour microenvironment has also been proposed. In contrast to the whole-body knockout, which is embryonic lethal, mice with endothelial cell specific conditional

knockout of Arf6 (EC-*Arf6* cKO) are healthy and fertile despite a mild defect in vascular length of the dorsal torso (Hongu et al., 2015). In contrast, tumours that formed upon orthotopic injection of B16 melanoma or Lewis lung carcinoma cell lines had reduced blood vessel number and area (Hongu et al., 2015). A reduction in tumour growth in EC-*Arf6* cKO mice was also observed. Using immortalized endothelial cell lines from these mouse models, Arf6 was shown to be required for HGF-dependent angiogenesis and cell spreading. These data demonstrate that Arf6 has a pleotropic role in many models of tumour progression and cancer cell invasion. A greater understanding of the mechanisms that regulate Arf6 activation and the downstream effectors of Arf6 would clearly improve our current understanding of tumour biology.

## 1.6 The HGF/Met Receptor Signalling Axis

RTKs are type I transmembrane receptors that regulate cell proliferation, survival, migration among other functions (Lemmon and Schlessinger, 2010). There are 58 receptors in humans that are divided into 20 subfamilies. Met receptor tyrosine kinase is part of a RTK family that includes RON (recepteur d'origine nantais) and c-Sea (Huff et al., 1993; Ronsin et al., 1993). Like many other RTKs, Met is activated upon ligand mediated dimerization (Koschut et al., 2016; Schlessinger and Ullrich, 1992). Along with its ligand, hepatocyte growth factor (HGF), Met elicits a morphogenic response that promotes cell migration, invasion and cellular characteristics associated with EMT (Birchmeier et al., 2003). Met receptor is predominantly expressed in epithelial cells, including hepatocytes, but may also be activated in endothelial cells, as well as neutrophils. HGF is secreted by mesenchymal cells. Together, HGF and Met maintain organismal homeostasis in response to injury and drive cancer progression when dysregulated. Currently, therapeutic interventions targeting Met are being tested in cancer settings (Bradley et al., 2017; Gherardi et al., 2012).

### 1.6.1 Hepatocyte Growth Factor

In the 1980s, two groups independently identified a fibroblast derived "scatter factor" and a serum-derived mitogenic factor for hepatocytes termed "hepatocyte growth factor" (Gherardi et al., 1989; Miyazawa et al., 1989; Nakamura et al., 1984; Stoker et al., 1987). Shortly thereafter, "scatter factor" and "hepatocyte growth factor" were found to be the same molecule and this was

the ligand for the Met RTK (Bottaro et al., 1991; Gherardi and Stoker, 1990; Naldini et al., 1991b; Weidner et al., 1991). HGF binds directly to the extracellular region of Met with high affinity ($K_d$ ~0.2 nM) and with a 2:2 stoichiometry (Gherardi et al., 2006). Despite structural studies of ligand bound to receptor, it is still unclear how HGF induces Met dimerization. HGF is synthesized as a single polypeptide precursor and cleaved into α and β subunits with molecular weights of 69 and 34 kDa respectively. Cleavage of the precursor may be mediated by urokinase-type plasminogen activator (uPa), tissue-type plasminogen activator (tPa), HGF activator (HGFA), kallikrein, coagulation factor Xia, hepsin or matriptase (Herter et al., 2005; Kirchhofer et al., 2005; Lee et al., 2000; Mars et al., 1993; Peek et al., 2002; Shimomura et al., 1995). The α chain contains a N-terminal domain and four Kringle domains (Figure 1.5). The β chain contains a serine proteinase domain that lacks enzymatic activity (Lokker et al., 1992). The subunits are linked through a disulfide bridge.

Mice with homozygotic ablation of *HGF* die *in utero* between E13.5 and E15.5 with placental defects and reduced liver size (Schmidt et al., 1995; Uehara et al., 1995). Consistent with a function in liver regeneration, HGF is elevated in the serum of rats with 70% hepatectomy and blocking HGF with anti-HGF antibody diminishes hepatocyte proliferation after liver injury (Burr et al., 1998; Nakamura et al., 1984). HGF is also required for the migration of myogenic precursor cells into the developing limb bud (Bladt et al., 1995; Dietrich et al., 1999). In adults, HGF also promotes regeneration of epithelium upon injury in kidneys after acute renal failure, lung tissue after pneumonectomy and liver tissue after partial hepatectomy (Ishiki et al., 1992; Kawaida et al., 1994; Sakamaki et al., 2002).

Levels of HGF range from 0.26 to 0.39 ng/mL in human serum (Funakoshi and Nakamura, 2003). However, levels can be elevated in patients with different diseases including cancer. Higher concentrations can also be found in the cerebral cortex (9.6 ng/mL) and urine (19.3 ng/mL). Elevation of HGF levels and processing may represent an important mechanism to promote Met signalling. Plasminogen activators are elevated during kidney repair and in highly metastatic tumours, along with overexpression of Met. This suggests that concomitant Met elevation and HGF processing could promote Met signalling and represent a pathway to tumour progression.

### 1.6.2 Met receptor tyrosine kinase

Full-length Met receptor was identified in 1987 (Park et al., 1987) and, in 1991, HGF was identified as the physiological ligand for Met (Bottaro et al., 1991; Naldini et al., 1991b). Deletion of *Met* results in embryonic lethality of homozygotic mice (Bladt et al., 1995). These mice die between E14.5 to E16.5. *Met* -/- embryos had reduced liver size, damage to the liver parenchyma and defects in placental development. Additionally, myogenic precursor cells did not migrate to the developing limb bud in *Met* -/- embryos. These phenotypes bear a striking resemblance to those observed in *HGF* -/- mice and argue that HGF is the sole ligand for Met *in vivo*.

Met is synthesized as a single chain precursor (Figure 1.5). The precursor is cleaved between residues 307-308 and the cleavage products are an extracellular α subunit and membrane spanning β chain. These are linked through a disulfide bond (Tempest et al., 1988). The extracellular portion of Met is composed of a Sema domain, a short cysteine-rich domain and four immunoglobulin-like (Ig-like) domains (Figure 1.5) (Gherardi et al., 2003). The Sema domain is a β-propeller that is made up of the alpha chain and the first 212 amino acids of the β chain (Stamos et al., 2004). Amino acids 933 to 955 form a transmembrane helix. The intracellular portion of Met is made up a juxtamembrane region (a.a. 961-1077), the kinase domain (1078-1345) and a multisubstrate docking site at the C-tail (a.a. 1346-1390). The Sema domain, IgG3 and IgG4 interact with HGF NK domains. The Sema domain may also have a role in receptor dimerization independent of its ability to bind HGF and therefore may provide a mechanism for coupling ligand binding to dimerization and activation of Met (Kong-Beltran et al., 2004). However, this is currently untested. That being said, it is clear that Met dimerizes and is phosphorylated upon ligand binding.

Upon HGF binding and Met dimerization, phosphorylation of Met occurs rapidly and in trans. The kinase domain of Met folds into separate N-terminal and C-terminal lobes that are connected through an activation loop (Rickert et al., 2011; Schiering et al., 2003). This domain contains catalytic activity and promotes phosphorylation of Met and its effectors. Phosphorylation of three tyrosine residues in the activation loop (Y1230, Y1234 and Y1235) promotes Met activation (Longati et al., 1994; Naldini et al., 1991a). The juxtamembrane domain of Met contains two known regulatory sites, S985 and Y1003. Phosphorylation of S985 is associated with reduced tyrosine phosphorylation and is a putative negative regulatory site (Gandino et al., 1994). S985 phosphorylation is catalyzed by PKC and increased during oxidative stress, suggesting that PKC

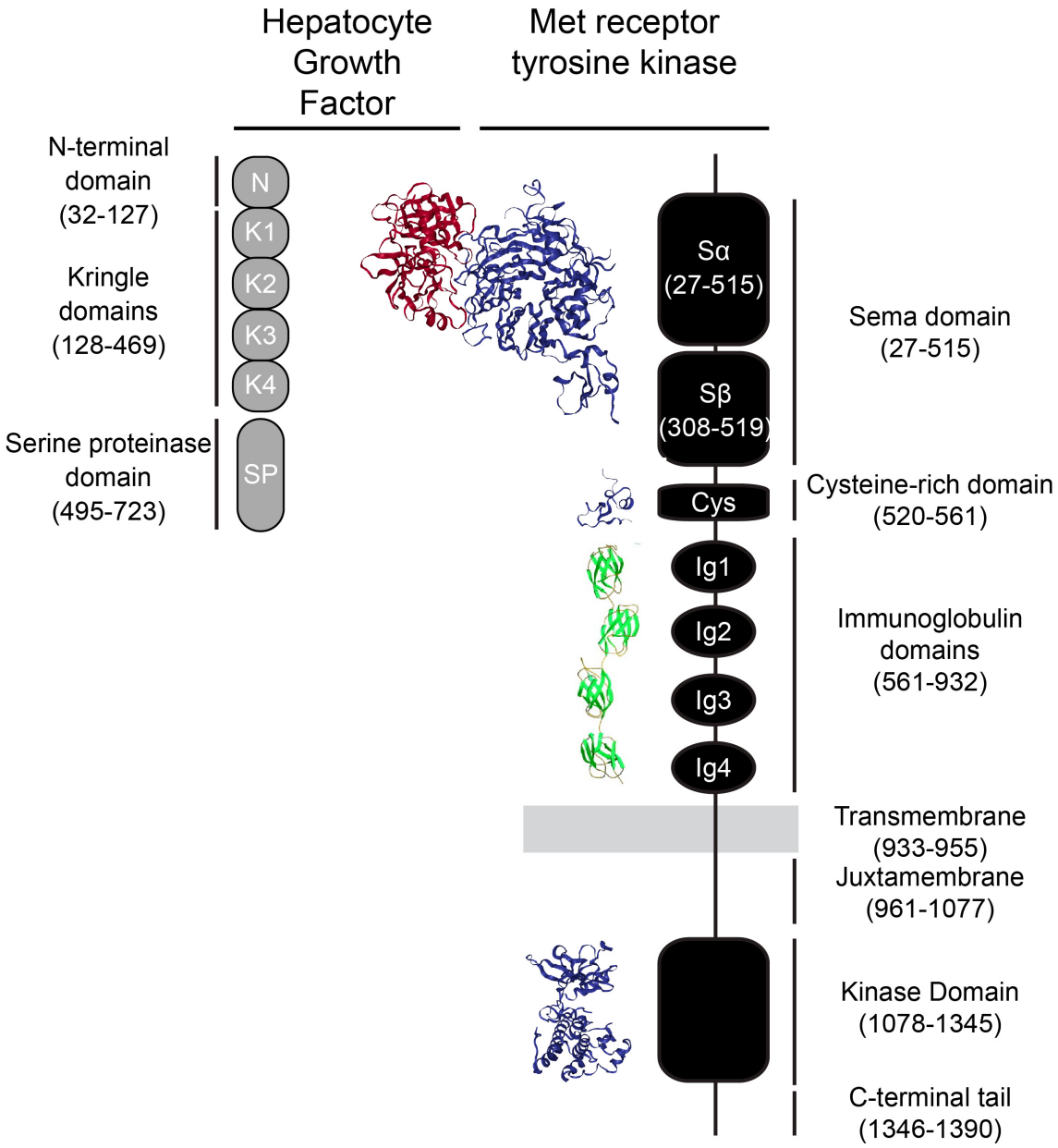


**Figure 1.5 Structural overview of HGF and Met.** Shown are the structural domains of HGF and the Met receptor tyrosine kinase. Amino acids are indicated in brackets. Crystal structures of HGF bound to the Sema domain (1SHY) and the kinase domain (3Q6U) were obtained from the Protein Data Bank. The NMR structure of the cysteine-rich domain (1SSL) was obtained from the Protein Data Bank. Structural models of Imunnoglobulin domains were obtained from Gherardi et al., 2003.

can downregulate HGF signalling during conditions of oxidative stress (Hashigasako et al., 2004). However, the mechanism of negative regulation on Met activity by S985 phosphorylation is unknown. Phosphorylation of Met Y1003 enhances receptor degradation. The c-Cbl E3 ubiquitin ligase specifically recognizes phosphorylated Y1003 through its tyrosine kinase binding domain (Peschard et al., 2004). Phosphorylation of Y1003, in turn, promotes receptor ubiquitination and phosphorylation of the ESCRT targeting protein, Hrs (Abella et al., 2005). Mutation of this site (Y1003F) uncouples Met from c-Cbl, enhances Met dependent signalling and reduces its ubiquitination (Peschard et al., 2001). Consequently, Met Y1003F has prolonged signalling and enhanced oncogenic activity. Therefore, our current understanding of the juxtamembrane domain of Met supports its role as a negative regulator of Met dependent biology.

The C-tail of Met forms a multi-functional docking site that is necessary for all known biological functions dependent upon Met activation (Figure 1.6) (Maina et al., 1996; Ponzetto et al., 1994). The docking site centers around Y1349 and 1356. Upon phosphorylation, these sites recognized by proteins containing src homology 2 (SH2) and protein tyrosine binding (PTB) domains that specifically recognize phosphorylated tyrosine-based motifs. Due to steric constraints, two proteins cannot occupy both phosphorylated Y1349 and Y1356 of the same Met molecule simultaneously. Adaptors and enzymes that have been shown to associate with the multifunctional binding site include Grb2 (Fixman et al., 1996; Ponzetto et al., 1996), p85 subunit of PI3K (Fixman et al., 1995; Ponzetto et al., 1993), PLCγ (Ponzetto et al., 1994), c-Src (Ponzetto et al., 1994), Shc (Fixman et al., 1996; Pelicci et al., 1995), Shp2 (Fixman et al., 1996), SHIP-1 (Koch et al., 2005; Stefan et al., 2001) and Grb2 associated binder 1 (Gab1) (Weidner et al., 1996). Y1356 is recognized by Grb2 and Shc adaptor proteins (Ponzetto et al., 1996). Grb2 can indirectly recruit Gab1 and Cbl to the activated receptor (Lock et al., 2000). Therefore, by recruiting effectors, Met promotes localized activation of signalling pathways that promote cell proliferation, breakdown of cell-cell junctions and cell migration.

### 1.6.3 HGF/Met signalling

Upon Met activation, epithelial cells undergo several changes that promote cell motility. In 2 dimensions, cells will undergo rearrangement of the actin cytoskeleton, breakdown of cell-cell junctions and scattering. In 3 dimensions, epithelial cysts will form branching tubules in response to HGF and cancer cells will invade the local microenvironment. These processes depend

on the activation of multiple signalling cascades including the Ras/MAPK, PI3K, multiple small GTPases and others (Figure 1.6). Activation of these pathways and their biological readouts have been used to identify determinants of Met signalling.

#### 1.6.3.1 The Gab1 scaffold

The major phosphorylated protein downstream of the Met receptor tyrosine kinase in epithelial cells is the scaffold Gab1 (Nguyen et al., 1997). Deletion of *Gab1* is embryonic lethal and Gab -/- mice have defects in migration of myogenic precursor from the somites into the developing limb bud, reduced liver size and placental defects (Sachs et al., 2000). These phenotypes bear a striking resemblance to those of *HGF* and *Met* knockout mice and highlight an essential role for Gab1 in Met dependent processes. Upon HGF stimulation of cells, Gab1 forms a signalling complex with Met (Weidner et al., 1996). Gab1 is indirectly recruited to Met, as well as EGFR, via Grb2 (Lock et al., 2000). However, in contrast to other RTKs, Gab1 can also recognize Met directly through a unique 13 amino acid sequence called the Met binding domain (MBD) (Lock et al., 2002; Schaeper et al., 2000; Weidner et al., 1996). This polyproline sequence in Gab1 recognizes Met sequences that encompass the C-lobe of the kinase domain and phosphorylated Met Y1349 (Lock et al., 2003). The MBD is not found in other Gab family members and, when combined with enhanced membrane targeting, is sufficient to confer direct Met binding to the related Gab2 protein and promote lamellipodia formation (Frigault et al., 2008). Direct recruitment of Gab1 to Met prolongs Met signalling relative to EGFR. Notably, it is only HGF and not EGF that promotes epithelial tubulogenesis of MDCK cells (Maroun et al., 1999a). Therefore, studying the signalling pathways downstream from Met may provide a unique insight into the molecular requirements for cell migration.

Gab1 contains an amino terminal PH domain followed by a large flexible region. Recruitment of Gab1 to the plasma membrane is mediated by specific recognition of $PI(3,4,5)P_3$ by the PH domain (Maroun et al., 1999a; Rodrigues et al., 2000). Mutation of residues W26 and R29 in the PH domains or deletion of the PH domain abrogates membrane recruitment and HGF dependent cell scattering and tubulogenesis (Maroun et al., 1999b). Supporting the hypothesis that Gab1 membrane recruitment regulates Met dependent biology, addition of the Src kinase myristoylation sequence to a Gab1 mutant lacking the PH domain, rescues membrane recruitment and HGF-dependent tubulogenesis (Maroun et al., 2003).

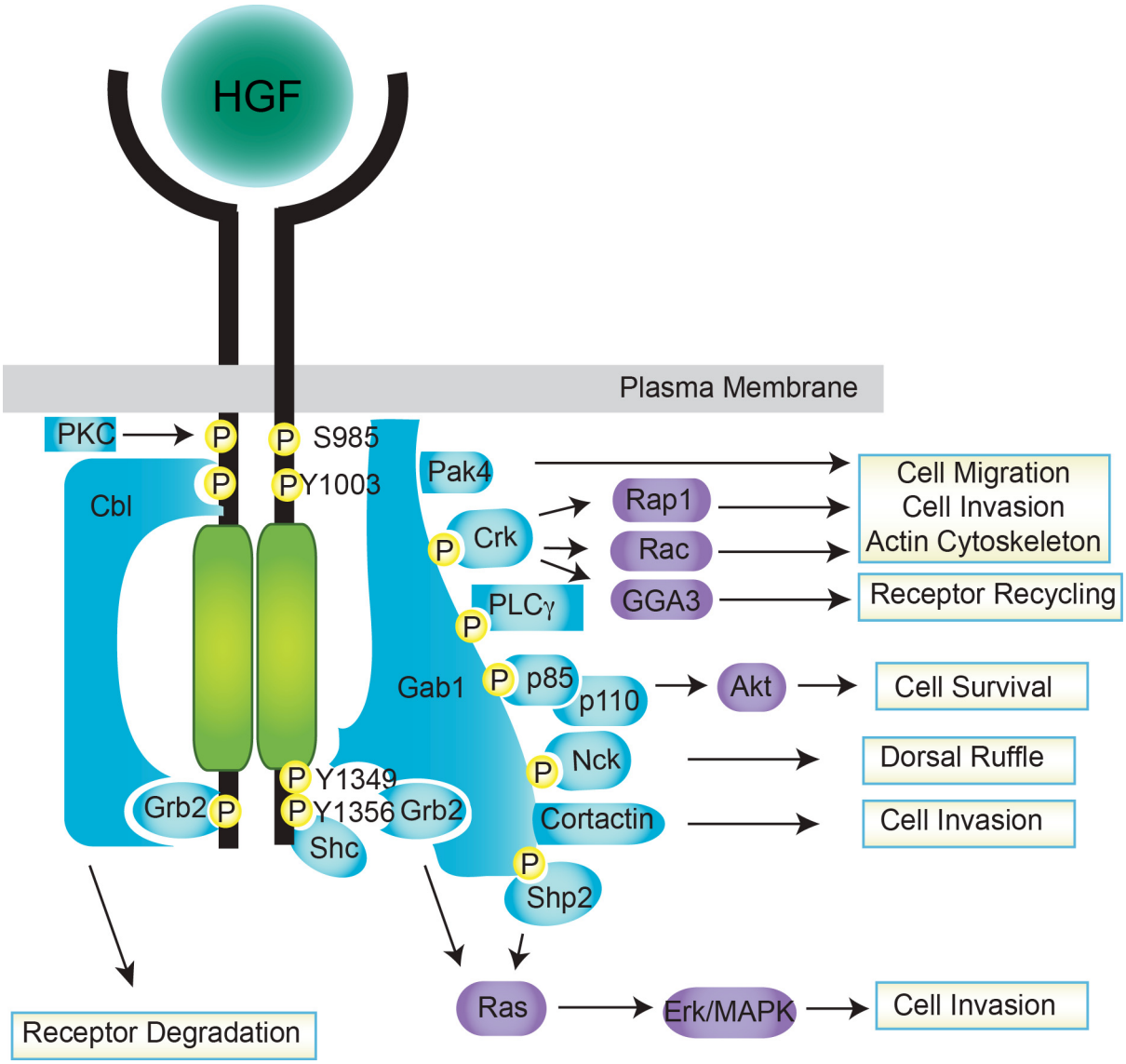


**Figure 1.6 Overview of Met signalling.** Upon binding of HGF, Met dimerizes and is phosphorylated. Downstream effectors recognize specific phosphorylated residues and activate signalling pathways to promote several cellular processes.

The large flexible region of Gab1 contains multiple phosphotyrosine and polyproline binding sites for SH2/PTB- and SH3-containing proteins respectively. Signalling proteins that interact with and act downstream from Gab1 include PAK4 (Paliouras et al., 2009), Crk (Schaeper et al., 2000), Crk L (Schaeper et al., 2000), PLC γ (Gual et al., 2000), Nck (Abella et al., 2010b), p85 (Schaeper et al., 2000), Shc (Schaeper et al., 2000), Shp2 (Schaeper et al., 2000), N-WASP (Abella et al., 2010b) and cortactin (Rajadurai et al., 2012). Together, these signalling adaptors

and regulators promote rearrangement of the actin cytoskeleton and cell signalling downstream from HGF. Collectively, these works highlight the importance of Gab1 and its direct recruitment to Met for HGF-dependent biology.

#### 1.6.3.2 Met activation of the PI3K pathway

In response to HGF, p85 binds Met directly through SH2 dependent recognition of phosphorylated Met Y1349 (Ponzetto et al., 1993). Active Ras may also activate PI3K through direct binding of the p110 subunit (Rodriguez-Viciana et al., 1994). While both of these pathways are potentially responsible for initial PI3K activation downstream from Met, most HGF-dependent PI3K activity is associated with Gab1 (Maroun et al., 1999a). Mutation of the Gab1 binding site (Y1356) uncouples Met from p85 and PI3K activity is required for HGF dependent cell scattering (Fixman et al., 1995; Ponzetto et al., 1994; Royal and Park, 1995). Inhibtion of PI3K reduces HGF dependent actin rearrangement, Rac1 activation and dorsal ruffle formation (Abella et al., 2010b; Royal et al., 2000). These data highlight a central role for Gab1 in PI3K activity downstream from Met. Gab1 has 3 YXXM p85 binding sites suggesting that it can amplify PI3K signalling. The Gab1 PH domain also specifically recognizes $PI(3,4,5)P_3$ and mediates a positive feedback loop that prolongs RTK signalling (Rodrigues et al., 2000). Notably, in contrast to EGF where PI3K activation returns to baseline within 15mins, HGF-dependent PI3K activity is prolonged for up to 60 mins (Maroun et al., 1999a). This correlates with direct recruitment of Gab1 to Met and the ability of HGF to promote rearrangement of the actin cytoskeleton, cell scatter and tubulogenesis in MDCK cells.

#### 1.6.3.3 Met activation of Arf6 and Rac1

Arf6 knockout in mice is embryonic lethal and reduced liver size is observed at embryonic day 10.5 (Suzuki et al., 2006). The phenotype of Arf6 knockout mice closely resembles that of HGF, Met and Gab1 knockout mice. Apoptosis of liver cells and hypocellularity due to impaired branching of the hepatic diverticulum and formation of the hepatic cord results in a small liver in Arf6 -/- mice. Arf6 -/- fetal hepatocytes fail to form cord-like structures in response to HGF, supporting a requirement for Arf6 in Met dependent biology.

Cell migration in response to HGF depends on activation of Rac1, Cdc42 and Arf6 (Palacios and D'Souza-Schorey, 2003; Ridley et al., 1995; Royal et al., 2000; Tushir and D'Souza-

Schorey, 2007). In turn, these small GTPases regulate rearrangement of the actin cytoskeleton to promote membrane ruffling and generation of a leading edge. Rac1/Cdc42 are active at the leading edge and overexpression of either dominant active Rac1 or Cdc42 blocks actin rearrangement (Royal et al., 2000). In response to HGF, Gab1 interacts directly with the Rac1 GEF Pak4, which is also required for HGF-dependent actin rearrangement and cell migration (Paliouras et al., 2009). The Rac1 GEFs, TIAM1 and Vav2, have also been shown to regulate HGF dependent cell migration from an endosomal compartment (Ménard et al., 2014; Palamidessi et al., 2008). Activation of Rac1 is also dependent of Arf6 since a dominant negative Arf6 mutant blocks Rac1 activation and translocation to the plasma membrane (Palamidessi et al., 2008). This suggests that there exists a Met-dependent small GTPase cascade that promotes cell migration. To support this, the Rac1 GEF, DOCK180, is recruited to the plasma membrane in response to HGF (Koubek and Santy, 2017). DOCK180 also acts downstream from the Arf GEF Cytohesin-2 to promote actin rearrangement and cell migration (Santy et al., 2005). HGF-dependent activation of Rac1 and lamellipodia formation is inhibited by PI3K inhibition (Royal et al., 2000). However, prior to this thesis the molecular link between Met and Arf6 in cancer cells was unknown.

### 1.6.4 Endolysosomal trafficking of Met

Internalization, trafficking and recycling are key components in regulating receptor stability, signal amplitude and duration. The Bergeron laboratory showed that signalling molecules were present on endosomal compartments by injecting rats with EGF and co-purifying Grb2 and son of sevenless (SOS) with an endosomal fraction (Di Guglielmo et al., 1994). Signalling from endosomes is tightly regulated. EGFR is phosphorylated to different extents in response to increasing concentrations of EGF. However, the amount of phosphorylated EGFR in a given endosome remains constant despite changes in total EGFR (Villaseñor et al., 2015). Increasing concentrations of EGF increase to number of endosomes containing phosphorylated EGFR arguing that the endolysosomal system can function as an analogue to digital switch to regulate EGFR signalling. However, signalling from the plasma membrane has also been demonstrated by recruitment of tagged endogenous H-Ras to the plasma membrane in response to EGF (Pinilla-Macua et al., 2016). Together these data support a model where different signalling pathways are activated at distinct steps during endolysosomal trafficking. However, the biological significance of individual signalling microdomains is unknown.

Incorporation of Met into the endolysosomal network is a key regulatory determinant of Met signalling and biology (Hammond et al., 2003; Mak et al., 2007). While activation of Met promotes recruitment of the signalling adaptors described above, Met also engages with components of the endolysosomal machinery, including Eps15, Cbl and GGA3 (Parachoniak and Park, 2009; Parachoniak et al., 2011; Peschard et al., 2004). Each of these regulates a distinct aspect of Met trafficking arguing that Met dynamically engages with multiple complexes throughout the pathway. Upon HGF binding, Met is rapidly internalized and enters the early endosomal compartment within 5 minutes (Li et al., 2005b; Petrelli et al., 2002). This may occur through dynamin-dependent endocytosis or dorsal ruffles (Abella et al., 2010a; Li et al., 2007b). The adaptor protein tensin-4 bridges an indirect complex between β1 integrin and Met to stabilize Met at the plasma membrane (Muharram et al., 2014). Tensin-4 silencing increases Met endocytosis. Tensin-4 through its SH2 domain, recognizes phosphorylated Y1313 and the multifunctional docking site encompassing Y1349 and Y1356 of Met. Tensin-4 promotes Met stability and signalling and overexpression of a mutant that no longer binds Met (R474A) enhances Met localization with a degradative lysosomal compartment. Tensin-4 and Met expression also correlate in colorectal and ovarian tumours. Endocytosis is required for Met signalling and inhibition of endocytosis inhibits tumour growth of Met-dependent cell lines in mouse xenografts (Joffre et al., 2011).

Upon internalization, Met enters early endosomes and engages with endosomal sorting proteins (Abella et al., 2005; Parachoniak and Park, 2009; Parachoniak et al., 2011; Sangwan et al., 2011). Disruption of early endosome fusion is sufficient to promote prolonged receptor stability and signalling (Sangwan et al., 2011). From early endosomes Met recycles back to the plasma membrane in a Rab4 recycling pathway or is targeted for degradation by ubiquitination and degraded via the lysosome (Abella et al., 2005; Hammond et al., 2001; Parachoniak et al., 2011). Phosphorylation of Met on Y1003 promotes recruitment of c-Cbl and Cbl-b E3 ubiquitin ligases. Cbl family E3 ligases catalyze Met ubiquitination and the Cbl binding site is required for Met ubiquitination and downregulation. A Met Y1003F mutant is no longer ubiquitinated, does not phosphorylate the ESCRT-0 component, Hrs, and has a prolonged stability compared to the wild-type receptor (Abella et al., 2005). Tumours arising from cells expressing the mutant receptor are also more aggressive and grow at an increased rate, highlighting the role of membrane trafficking in tumourigenesis.

While escape from degradation is one mechanism for oncogenic activation, enhanced Met receptor recycling can also promote invasive properties of cancer cells (Parachoniak and Park, 2012). Recycling of Met prolongs Met signalling and is required for HGF-dependent cell migration. The endosomal adaptor GGA3 specifically regulates Met receptor recycling upon HGF stimulation (Parachoniak et al., 2011). By forming a direct complex with the signalling adaptor, Crk, GGA3 functions as a molecular switch to direct Met into a recycling pathway back to the plasma membrane. Depletion of GGA3 by siRNA reduces Met half-life, Met phosphorylation and Erk1/2 phosphorylation. Moreover, the GGA3:Crk complex is required for GGA3 recruitment to Met carrying endosomes and HGF-dependent cell migration. Similar to integrins, Met also engages with the endosomal adaptor RCP in p53 mutant breast cancer cell lines (Muller et al., 2013). This complex promotes Met recycling through a Rab11 compartment and enhances HGF-dependent cell migration. However, the molecular basis for this interaction has not been defined.

### 1.6.5 Deregulation of Met trafficking in cancer

The Met receptor tyrosine kinase has received attention in the past two decades as a potential therapeutic target in lung, breast and gastric cancer, among others (Bradley et al., 2017; Gherardi et al., 2012). Met inhibitors have entered pre-clinical and clinical trials, however none have entered clinical practice, highlighting a requirement for a greater understanding of Met dependent cancer biology and the opportunities for therapeutic intervention.

Prior to identification of the full-length receptor, Met was identified as the oncogenic product of human osteosarcoma cells (HOS) treated with the chemical carcinogen N-Methyl-N'-nitro-N-nitrosoguanidine (Cooper et al., 1984). Carcinogen treated HOS cells had a genetic fusion between the coiled-coiled domain of *Tpr* on chromosome 1 and the kinase domain of *MET* on chromosome 7 (Park et al., 1987; 1986). The Tpr-Met gene product could transform NIH3T3 fibroblasts and was sufficient to promote tumourigenesis *in vivo* (Cooper et al., 1984; Liang et al., 1996). The Tpr region encodes a leucine zipper motif that drives Met dimerization and constitutive activation (Pal et al., 2017; Rodrigues and Park, 1993). Tpr-Met is cytosolic, lacks the Cbl ubiquitination site and is uncoupled from c-Cbl (Mak et al., 2007; Peschard et al., 2001). However, addition of the juxtamembrane domain that lacks the Cbl ubiquitination site, and consequently increased Tpr-Met ubiquitination, is not sufficient to suppress the transforming ability of Tpr-Met (Mak et al., 2007). Addition of both the interleukin 2-α leader sequence and transmembrane

domain (Tac), and the Met juxtamembrane region is required to suppress oncogenic transformation. The Tac-Tpr-Met fusion protein is efficiently ubiquitinated and active, supporting a role for membrane trafficking and ubiquitination in Met signal attenuation. While Tpr-Met has not been found in human tumours or cell lines other than the original chemically transformed HOS cells, Met genomic rearrangements similar to Tpr-Met have been identified in human melanoma, spitz tumours and pediatric glioblastoma (International Cancer Genome Consortium PedBrain Tumor Project, 2016; Wiesner et al., 2014). 6 melanoma and spitz tumour cases were identified where a fusion product between a multimerization domain and exons 15-21 of Met occurred. 5 of 6 of the multimerization domains were coiled-coil domains, similar to the Tpr coiled-coil found in Tpr-Met. It was reported that *TRIM4-MET* and *ZKSCAN1-MET* are constitutively active; *TRIM4-MET* and *ZKSCAN1-MET* increase p-Erk, p-Akt and p-PLCγ1; and *ZKSCAN1-Met* is tumourigenic. These data provide clinical evidence that escape from the endolysosomal network and degradation enhance Met signalling and its tumourigenic properties.

Activating mutations in the Met kinase domain have also been identified in cases of sporadic and hereditary human renal papillary carcinoma (Olivero et al., 1999; Schmidt et al., 1999; 1998; 1997). These include V1110I, M1268T and D1246N. All these mutations are within the kinase domain of Met and cluster around the ATP binding pocket or the activation loop. When overexpressed in HGF producing NIH3T3 fibroblasts, mutant receptors are hyper-phosphorylated (Olivero et al., 1999). In one study, it was shown that M1268T and D1246N mutant receptors are internalized and recycled at a higher rate than wild type receptor (Joffre et al., 2011). Blocking endocytosis reduces remodeling of the actin cytoskeleton in these Met-mutant expressing cell lines and reduces their transforming capacity. However, it is unclear whether this is strictly due to endocytosis of the Met receptor *per se*, a global inhibition of endocytosis or reduced Met signal transduction.

The most common Met mutation found in cancer are splice site mutations that result in skipping of exon 14. These occur in approximately 3-4% of lung adenocarcinoma patients (Frampton et al., 2015; Paik et al., 2015). Exon 14 encodes part of the juxtamembrane domain that contains S985 and Y1003. Therefore, escape from endolysosomal downregulation represents an important oncogenic event for Met receptor. In contrast to activating mutations in small GTPases or other RTKs, these data argue that escape from ubiquitination and membrane targeting of Met is

an oncogenic event in a subset of cancer patients, highlighting a role for Met trafficking in cancer cell migration (Figure 1.7).

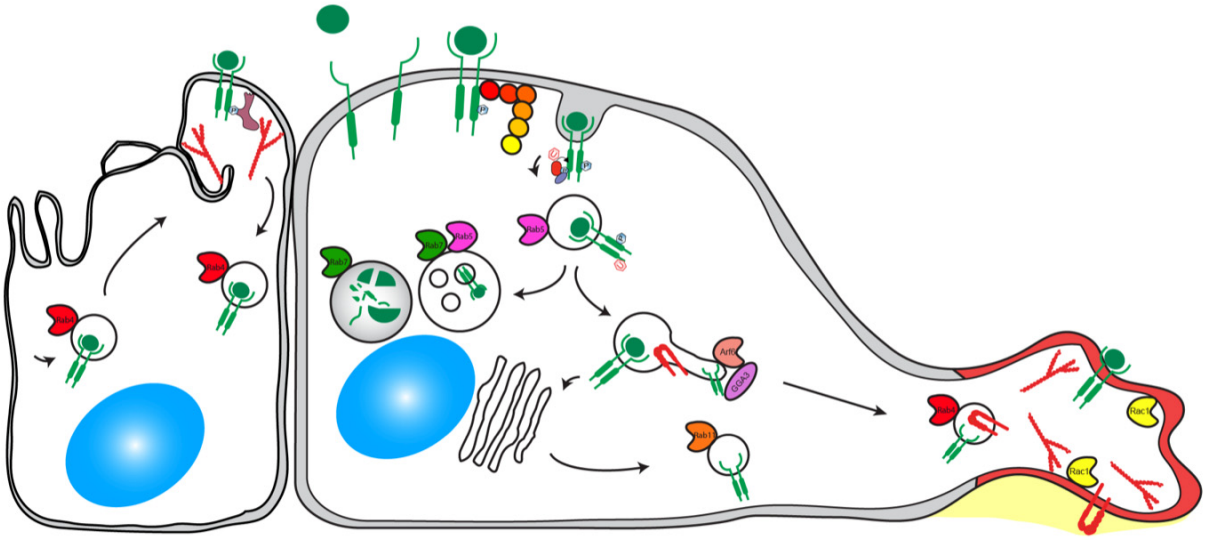

**Figure 1.7 Subcellular localization of Met regulates cancer cell migration**

# Chapter 2

## 2 Regulation of cell migration and β1 integrin trafficking by the endosomal adaptor GGA3

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*authors contributed equally to this work

## 2.1 Preface

At the time of this work, integrin receptor stability was known to be regulated by a balance between recycling to the plasma membrane and degradation. Dysregulated recycling of integrin receptors was an important mechanism to promote cell migration. Genes implicated in integrin recycling were associated with disease progression, however a cargo sorting adaptor for integrins into the recycling pathway was unknown. Building on this work, we show that GGA3 also regulates the stability of a subset of integrin subunits and cell migration. We show that membrane recruitment of GGA3 by Arf6 is required for these functions. Finally, we show that GGA3 interacts with the cargo adaptor, SNX17 to regulate integrin trafficking.

## 2.2 Abstract

The integrin family of cell adhesion receptors link extracellular matrices to intracellular signalling pathways and the actin cytoskeleton and regulate cell migration, proliferation and survival in normal and diseased tissues. The subcellular location of integrin receptors is critical for their function and dysregulated trafficking is implicated in various human diseases. Here we identify a role for GGA3, in regulating trafficking of β1 integrin. GGA3 knockdown reduces cell surface and total levels of α2, α5 and β1 integrin subunits, inhibits cell spreading, reduces focal adhesion number, as well as cell migration. In the absence of GGA3, integrins are increasingly retained inside the cell, traffic towards the perinuclear lysosomal compartment and their degradation is enhanced. Integrin traffic and maintenance of integrin levels are dependent on the integrity of the Arf binding site of GGA3. Furthermore, sorting nexin 17 (SNX17), a critical regulator of integrin recycling, becomes mislocalized to enlarged late endosomes upon GGA3 depletion. These data support a model whereby GGA3, through its ability to regulate SNX17 endosomal localization and through interaction with Arf6 diverts integrins from the degradative pathway supporting cell migration.

## 2.3 Introduction

Cell migration involves a coordinated interaction with the underlying matrix via cell surface receptors called integrins. The integrin family of receptors link extracellular matrix to intracellular signalling pathways that together regulate actin remodeling required for cell migration. This family of receptors comprises 26 subunits (18 α and 8 β) that form 24 known heterodimeric pairs, each with a different specificity for extracellular matrix (Caswell et al., 2009). β1 integrin can heterodimerize with α1, α2 and α5 integrin subunits, amongst others, to form functional receptors and recruit signalling molecules such as paxillin, focal adhesion kinase or vinculin (Harburger and Calderwood, 2008).

While β1 integrin functions and signals at the plasma membrane, it can be internalized via clathrin- or caveolin-dependent endocytosis and enter an early endosomal compartment (Arjonen et al., 2012). Once inside the cell, β1 integrin may be targeted for degradation via late endosomes/lysosomes or returned to the plasma membrane via an endosomal recycling compartment. Lysosomal degradation of β1 integrin is modulated by ubiquitination of the receptor,

recognition by the ESCRT machinery and is inhibited by direct binding of SNX17 to the receptor on early endosomes supportive of receptor recycling (Böttcher et al., 2012; Kharitidi et al., 2015; Lobert et al., 2010; Steinberg et al., 2012). Integrins recycle via spatially and temporally distinct pathways regulated by Rab and Arf proteins. β1 integrin can recycle through an Arf6-positive endosomal recycling compartment and disruption of the Arf6 GTP-GDP cycle results in a buildup of integrin receptors in vesicles below the plasma membrane and a reduction in recycling (Brown et al., 2001; Powelka et al., 2004). Each of these steps supporting integrin traffic and recycling, contribute to integrin mediated cell migration and focal adhesion turnover.

The 6 mammalian Arf small GTPases fall into three subclasses based on sequence homology. Arf1, Arf2, Arf3 fall under class I, Arf4 and Arf5 are class II and Arf6 is the only known class III member. While Arf1-5 regulate the secretory pathway and Golgi function, Arf6 localizes to the plasma membrane and peripheral puncta. In endosomes, Arf6 regulates dynamic clathrin structures called G-clathrin and recycling of multiple cargoes, including the major histocompatibility complex I, transferrin and the Met RTK (Luo et al., 2013; Parachoniak et al., 2011; Radhakrishna and Donaldson, 1997; Zhao and Keen, 2008). Arf6 regulates actin dynamics, cell-cell junctional integrity and membrane protrusions, as well as vesicle dynamics via the GGA proteins (Luo et al., 2013; Palacios and D'Souza-Schorey, 2003; Song et al., 1998).

The GGA family of proteins (GGA1, GGA2 and GGA3 in humans) are evolutionarily conserved endosomal adaptor proteins, originally identified as Arf effectors, that bind specifically to the GTP loaded form(Boman et al., 2000; Dell'Angelica et al., 2000; Hirst et al., 2000; Poussu et al., 2000). Upon recruitment to membrane, GGA proteins promote clathrin assembly and mediate intracellular transport of receptors, such as the mannose-6-phosphate receptor, sortillin and the Met receptor tyrosine kinase (Nielsen et al., 2001; Parachoniak et al., 2011; Puertollano et al., 2001a; 2001b). GGA proteins contain several functional domains (Bonifacino, 2004). The VHS domain, is involved in di-leucine motif recognition. The GAT domain is required for membrane recruitment of GGAs, binding ubiquitin and Arf proteins. The hinge region encompasses a proline rich Crk SH3 binding motif and a recognition motif for clathrin and the GAE domain binds accessory proteins that modulate membrane trafficking such as Rabaptin-5. While all three GGA proteins localize to the trans-Golgi network, GGAs have been observed on

early endosomes as well as recycling endosomes. Recent work from our group and others has established a role for GGA1 and GGA3 in an endosomal recycling pathway for the transferrin receptor (GGA1) and Met receptor tyrosine kinase (GGA3) (Parachoniak et al., 2011; Puertollano and Bonifacino, 2004; Zhao and Keen, 2008). Met recycling is dependent on an interaction between GGA3 with Arf6 as well as the Crk adaptor protein(Parachoniak et al., 2011). Both GGA1 and GGA3 also localize to a sub-population of G-clathrin structures and Arf1 and Arf6 depletion abolishes G-clathrin (Luo et al., 2013). Despite detailed structural data on the modular domains of the GGA proteins and mounting information on regulation of various GGA cargo proteins, less is known about the requirements for GGA proteins in integrin traffic.

Here, we report that GGA3 is a novel regulator of integrin dependent cell migration and integrin trafficking in cancer cell lines. We find a selective requirement for GGA3 in diverting β1 integrins from a degradative trafficking pathway, supporting enhanced integrin stability, focal adhesion number and cell migration. This is specific to a subset of integrin subunits and is dependent on Arf6 and the Arf binding site of GGA3.

## 2.4 Results

### 2.4.1 GGA proteins are required for efficient cell migration

GGA proteins are increasingly implicated in Arf regulated processes and trafficking. Our earlier work demonstrated a role for GGA3 in cell migration in response to hepatocyte growth factor (HGF), the ligand for the Met RTK (Parachoniak et al., 2011). To establish if GGA proteins modulate HGF-independent cell migration, we selectively depleted GGA1, 2, or 3 and examined migration of HeLa cells on a collagen matrix over a 12-hour period. Whereas depletion of GGA1 or GGA2 resulted in a modest decrease in cell speed to 66% and 69% of control levels, respectively (Figure 2.1A, B), a marked decrease in speed was observed in GGA3-depleted HeLa cells to 36% of control levels (Figure 2.1A, B). This was predominantly due to a reduction in speed of migration as directional persistence was not diminished upon GGA silencing (Figure 2.1A, C). To establish if GGA3 was required for migration of other highly invasive cancer cells, we depleted GGA3 in highly migratory and invasive basal breast cancer cells, MDA-MB-231. Consistent with HeLa cells, GGA3 depletion reduced migration of MDA-MB-231 cells to 54% of control cells and diminished both speed of migration as well as directional persistence (Figure 2.1D). Together,

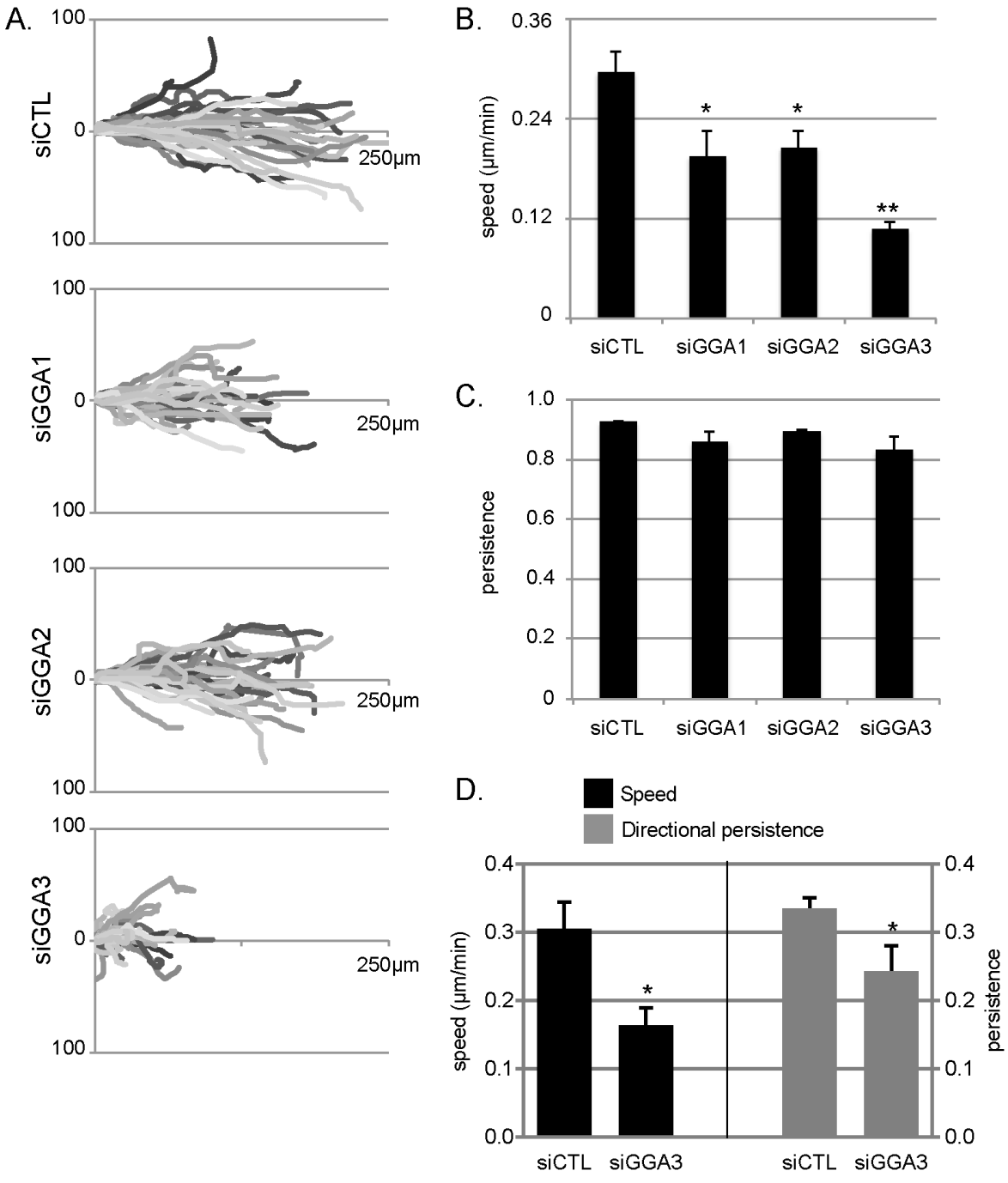



**Figure 2.1 GGA3 depletion severely impairs cell migration.** (A) Migration tracks of HeLa cells treated with siRNA for 24hours, replated in a collagen-coated well containing Ibidi cell-culture insert. After 16 hours insert was removed and images were captured every 15mins for 12 hours. (B & C) Quantification of HeLa cell speed and persistence. (D) Quantification of MDA-MB-231 random cell migration speed and persistence. Mean of 4 experiments is shown. 30 cells analyzed per experiment. Error bars represent standard error of the mean. *p<0.05. **p<0.01

these data demonstrate that GGA3 may regulate processes required for cell migration.

### 2.4.2 GGA3, but not GGA1 or 2, modulates β1 integrin protein levels

Coordinated trafficking of integrins through the endolysosomal network is required for efficient cell migration and defective integrin traffic may enhance integrin degradation (Böttcher et al., 2012; Steinberg et al., 2012; Tiwari et al., 2011). Consistent with reduced cell migration, depletion of GGA3 but not of GGA1 or GGA2, decreased steady state levels of the mature form of β1 integrin (Figure 2.2A), the collagen binding integrin α2, and the fibronectin-binding integrin α5 subunits, both of which heterodimerize with β1 integrin (Figure 2.2A, Supplemental Figure 2.1). A similar decrease in α2, and the mature form of β1 integrin was observed upon depletion of GGA3 in MDA-MB-231 cells, however α5 levels were unaffected in this cell line (Figure 2.2B). In addition to the reduction of total levels of β1 integrin, we also found that knockdown of GGA3 reduced surface β1 integrin to 66% of siCTL cells (Supplemental Figure 2.1B). This is consistent with a specific role for GGA3, amongst GGA family members, in regulating the protein levels of a subset of β1 integrin heterodimers.

### 2.4.3 GGA3 regulates cell surface levels of a subset of integrins

Integrin trafficking can be differentially regulated depending on the heterodimer (De Franceschi et al., 2015) but also on the basis of receptor activity and ligand engagement (Arjonen et al., 2012). To test whether the ability of GGA3 to regulate integrin stability is dependent on ligand engagement, GGA3-depleted cells were plated on different matrices and individual integrin subunits were analyzed by flow cytometry. GGA3 silencing significantly reduced cell surface levels of α2β1 and α5β1 integrins in HeLa cells plated on collagen (adhesion mediated by α2β1 and α1β1 integrins), fibronectin (adhesion mediated by α5β1 and αv-integrins) or vitronectin (adhesion mediated by αv-integrins) but not αV or α1 integrin levels (Figure 2.2C, D, E). These data suggest that GGA3 regulates the stability of a subset of integrin subunits. However, the mechanistic basis for the selectivity remains to be identified.

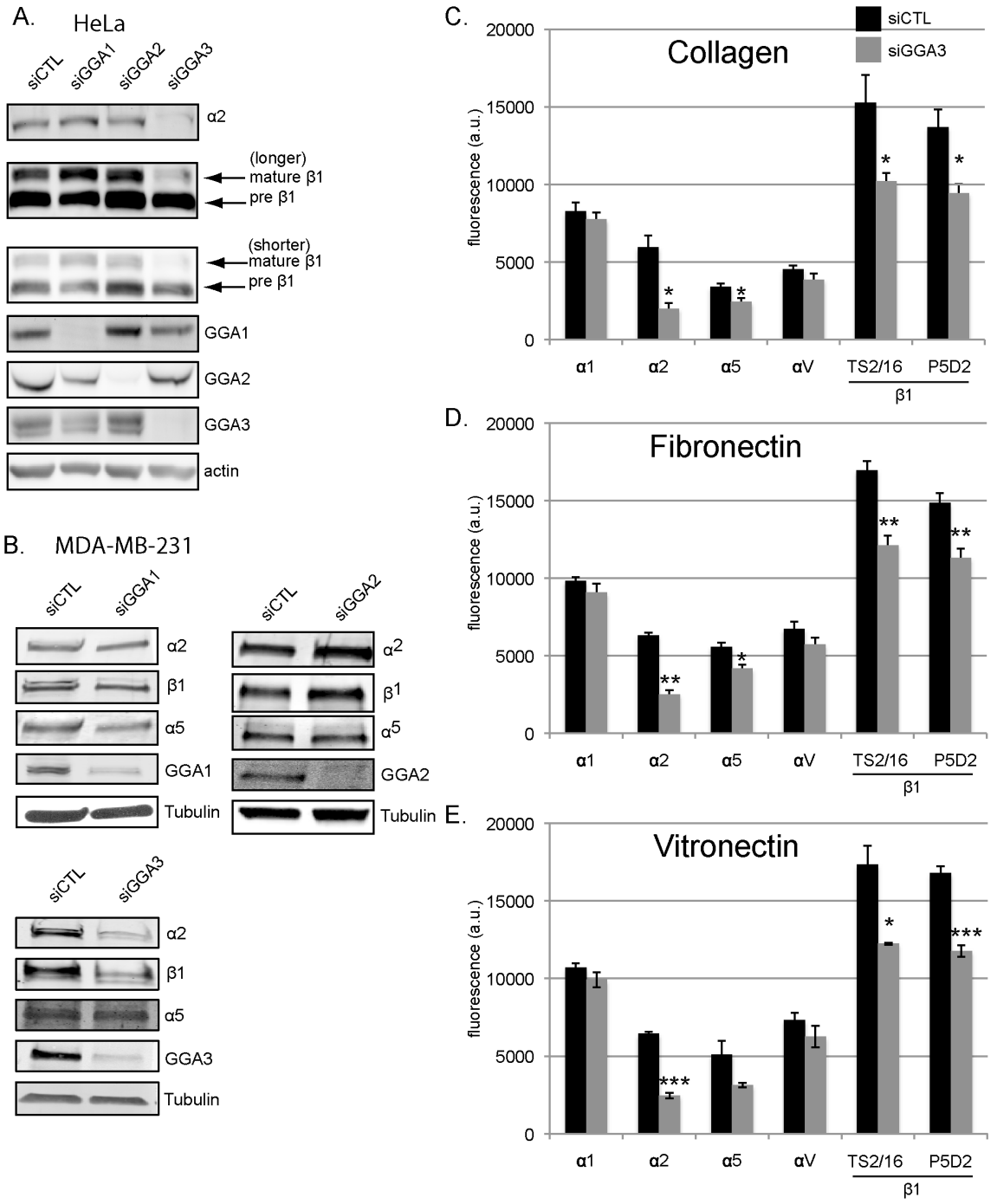



**Figure 2.2 GGA3 regulates a subset of integrins levels irrespective of the underlying matrix.** Whole cell lysates prepared from (A) HeLa cells seeded on collagen or (B) MDA-MB-231 cells and GGA family members depleted were immunoblotted with the indicated antibodies. Longer and shorter denotes the relative sensitivity of exposure. Surface levels of integrin subunits of siRNA treated HeLa cells seeded on plates coated with either (C) 25µg/mL collagen, (D) 10µg/mL fibronectin or (E) 2 µg/mL vitronectin as measured by flow cytometry. Mean of 3 or 4 experiments is shown. Error bars represent stand error of the mean. *p<0.05. **p<0.01. ***p<0.001

### 2.4.4 GGA3 and β1-integrin colocalize in cytoplasmic punctae

Previous reports have described the ability of GGA3 to localize to peripheral recycling endosomes where it promotes clathrin assembly (Puertollano et al., 2001b) and recycling of the Met receptor to the plasma membrane (Parachoniak et al., 2011). To investigate whether GGA3 localizes with integrin β1 in endosomes, constructs expressing β1 integrin-GFP and mCherry-GGA3 were transiently cotransfected and cells plated on a collagen matrix. Whereas, β1 integrin-GFP localized to the plasma membrane and in punctae throughout the cell, mCherry-GGA3 was observed only in punctae (Figure 2.3A). Notably, 40% of β1 integrin-GFP puncta localized with mCherry-GGA3 and these move together (Figure 2.3A, Panel B) providing evidence that GGA3 localizes at β1 integrin positive endosomes consistent with a role for GGA3 in regulating β1 integrin trafficking.

### 2.4.5 GGA3 is required for trafficking of β1 integrin via a Rab4 compartment

Upon internalization, β1 integrins undergo distinct fates. The endocytosed receptors continue to signal from EEA1-positive endosomes (Alanko et al., 2015), the receptors are recycled back to the plasma membrane or they may be targeted to the lysosome for degradation. To visualize the effect of GGA3 depletion on β1 integrin trafficking, we labeled surface integrin with anti-β1 integrin antibody at 4$^{o}$C and observed its localization at different time points after transferring the cells to 37°C. β1 integrin is internalized and localizes to puncta by 30 mins in both control and GGA3 knockdown cells. However, in control cells, β1 integrin localized to the cell periphery and in puncta after 60 mins of antibody chase. Whereas, in GGA3 KD cells, 60 mins post-antibody chase, β1 integrin localized to a perinuclear compartment (Figure 2.3B). β1 integrin has been demonstrated to recycle via both Rab4- and Rab11-positive recycling pathways. To test whether GGA3 was required for β1 integrin trafficking via one of these recycling compartments we expressed GFP-Rab4 or GFP-Rab11 in control or GGA3 depleted cells. We observed some overlap of β1 integrin with GFP-Rab11 in control cells and GGA3 silencing had no significant effect (19.1% control versus 20.8% GGA3 KD) (Supplemental Figure 2.2A, B). Interestingly, depletion of GGA3 reduced localization of β1 integrin with GFP-Rab4 (39.8% control vs 26.0% GGA3 KD) (Figure 2.3C, D). These data support a role for GGA3 in the trafficking of β1 integrin via a Rab4 positive compartment.

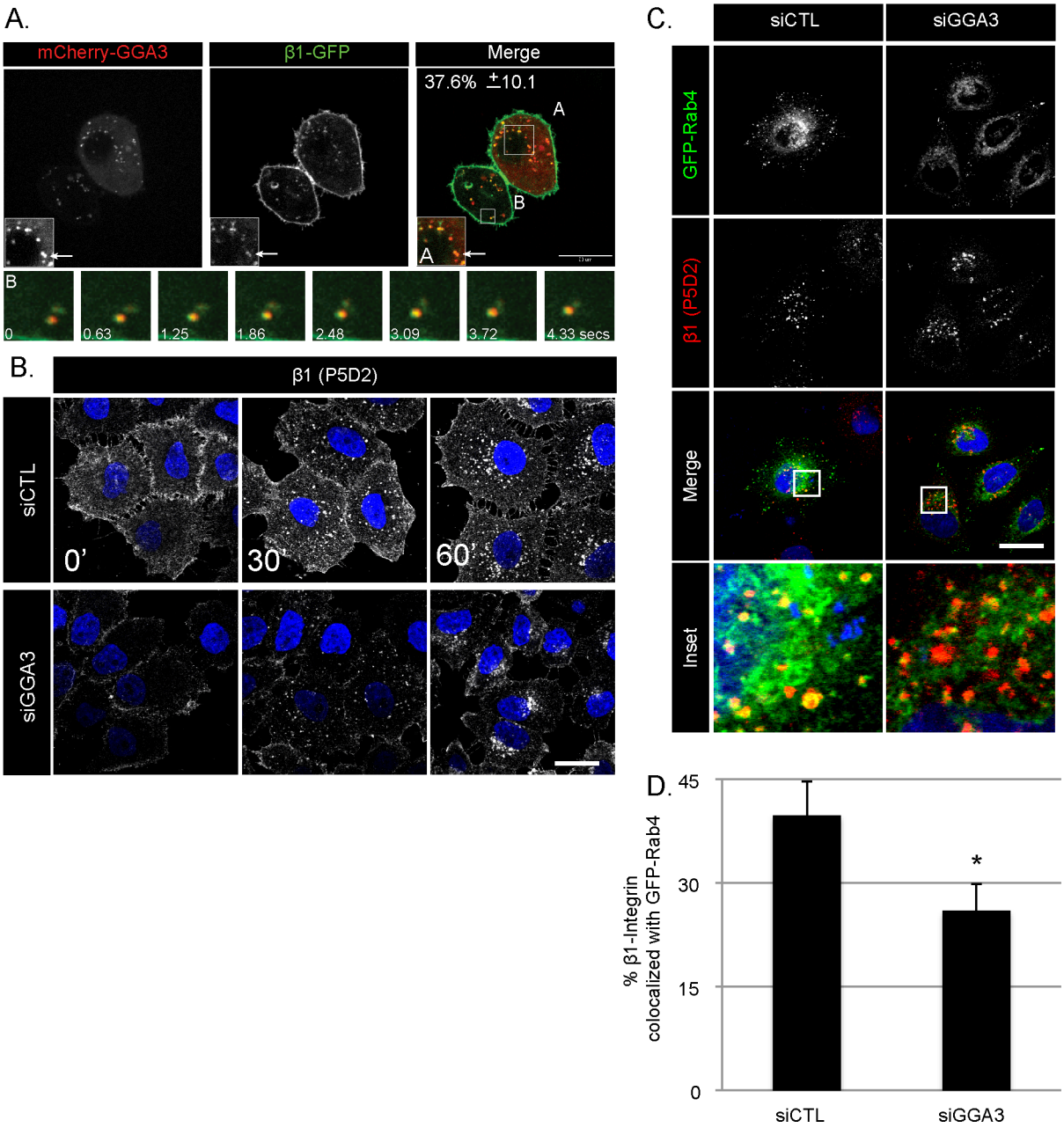



**Figure 2.3 GGA3 regulates β1 integrin trafficking via a Rab4 compartment.** (A) SKBR3 cells were transfected with mCherry-GGA3 and β1-eGFP, plated on 35mm collagen coated dish and imaged after 48 hours by spinning disc confocal microscopy. (B) HeLa cells were plated on collagen-coated glass cover slips and treated with control or GGA3 siRNA for 72 hours. Surface integrins were labeled with anti-β1 integrin antibody P5D2 at 4°C for 30 min, washed and fixed with 4% PFA or allowed to internalize for 30 or 60 minutes at 37°C before being fixed. Cells were permeabilized, stained by indirect immunofluorescence and images captured by confocal microscopy. (C) HeLa cells were plated on collagen-coated glass cover slips and treated with control or GGA3 siRNA for 72 hours. GFP-Rab4 was transfected 48 hours prior to the experiment. Surface integrins were labeled with anti-β1 integrin antibody P5D2 at 4°C for 30 min internalize for 60 minutes at 37°C before removal of surface-bound antibody by acid wash and fixation. Cells were permeabilized, stained by indirect immunofluorescence and images were captured by **Figure**

**2.3 (continued)** confocal microscopy. (D) Quantification of β1 integrin colocalization with GFP-Rab4. Mean from 3 independent experiments. 10 cells were analyzed per experiment. *p<0.05

To quantify the dynamics of integrin trafficking, we used a biotin-based surface labeling and internalization assay (Roberts et al., 2001). GGA3 depletion increased the percentage of internalized β1 integrin from 13.2% observed in control cells at 20 min to 25.9% in GGA3 depleted HeLa cells (Figure 2.4A, B). A similar trend was observed in MDA-MB-231 cells, where the amount of internalized β1 integrin increased significantly in GGA3 knockdown cells when compared with control cells at 20 min (20% in GGA3 KD cells vs 11% in CTL) and 30 min timepoints (20% in GGA3 KD cells vs 10% in CTL) (Figure 2.4D, E). The lysosomotropic amine, primaquine, blocks endosomal recycling, including recycling of β1 integrin (Arjonen et al., 2012; Reid and Watts, 1990; Schwartz et al., 1984). Although primaquine altered the overall endocytosis kinetics, primaquine treatment reproducibly diminished the effect of GGA3 KD on the levels of internalized β1 integrin (Figure 2.4A, C, D, F). Together, this supports a role for GGA3 in recycling of β1 integrin.

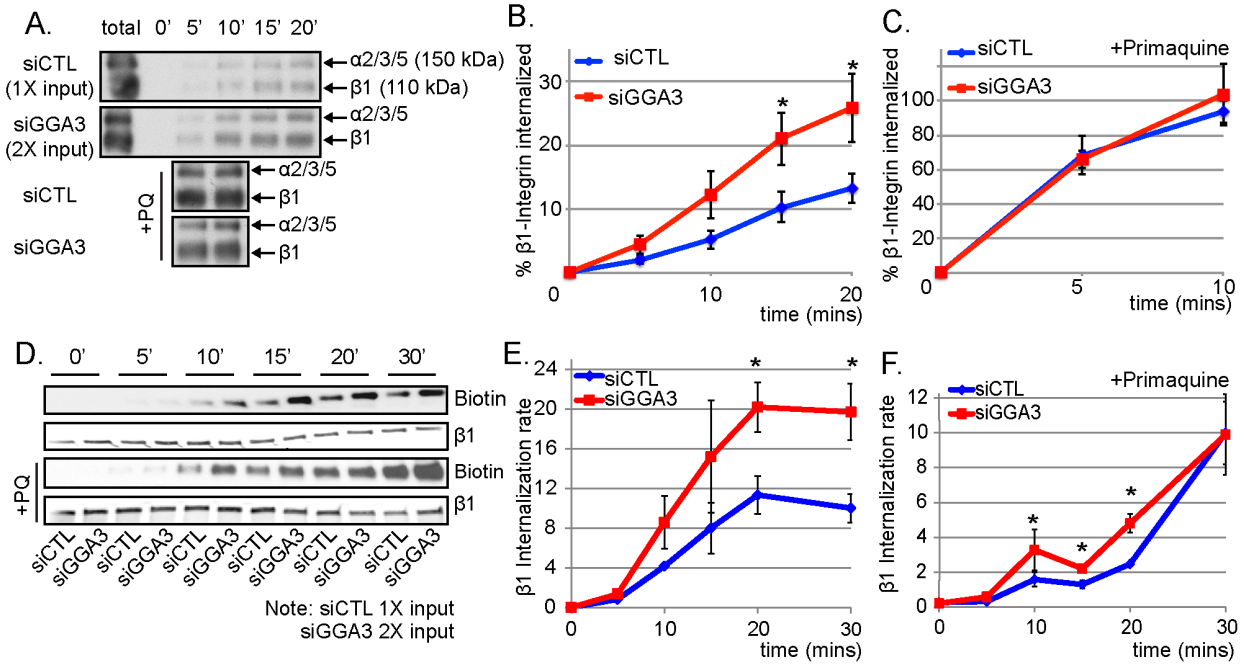



**Figure 2.4 GGA3 regulates accumulation of internalized β1 integrin.** Internalization of biotinylated β1 integrin was assessed in (A) HeLa and (D) MDA-MB-231 cells. Representative blots are shown. Quantification of internalized β1 integrin in HeLas in the (B) absence or (C) presence of primaquine. Quantification of internalized β1 integrin in MDA-MB-231 cells in the

(E) absence or (F) presence of primaquine. Band intensity normalized to end point [30 min]), relative to control-silenced cells. Lysate input in GGA3 silenced cells equivalent to 200% of control input. Mean from 3 independent experiments. Error bars indicate standard error of the mean. *$p < 0.05$

### 2.4.6 A GGA3-Arf interaction mediates β1 integrin sorting

GGA family proteins bind specifically to active GTP-loaded Arf proteins (Boman et al., 2000; Dell'Angelica et al., 2000; Hirst et al., 2000). Introduction of a single amino acid substitution (N194A) uncouples GGA3 from Arf proteins. Integrin receptor recycling is regulated by Arf6 and the Arf-binding site in GGA3 is required for GGA3 recruitment to membranes (Powelka et al., 2004). We tested whether the Arf binding site of GGA3 is required for its ability to regulate β1 integrin protein levels using HeLa cells stably expressing siRNA resistant wild type (WT) GFP-GGA3 or mutant N194A GFP-GGA3. Depletion of endogenous GGA3 reduces mature β1 integrin protein levels by 79% when compared to control cells. Expression of WT GFP-GGA3 efficiently rescues mature β1 integrin levels to 109% of control cells (Figure 2.5A, B) and the N194A GFP-GGA3 mutant had no effect (47% relative to control) (Figure 2.5A, B). Consistent with previous studies that β1 integrin degradation is dependent on the lysosome (Lobert et al., 2010), the v-ATPase inhibitor bafilomycin A1, but not the proteasome inhibitor lactacystin, rescued total levels of mature β1 integrin (Figure 2.5C, D & Supplemental Figure 2.3). To study this further, we analyzed β1 integrin localization upon GGA3 depletion and observed a marked increase in β1 integrin overlap with the lysosomal marker, LAMP1, from 20.2% in control cells versus 44.4% in GGA3 depleted cells (Figure 2.5E,F). Hence, in the absence of GGA3, trafficking of internalized β1 integrin is enhanced towards a degradative lysosomal compartment. The increased overlap of β1 integrin with LAMP1 observed in GGA3-depleted cells is rescued by expression of WT GFP-GGA3, but not the GFP-GGA3 N194A mutant uncoupled from Arf (Figure 2.5E, F). We confirmed this observation using another anti-β1 integrin antibody (Supplemental Figure 2.4). Based on these data we propose that the GGA3 Arf-GTP binding site is required for maintenance of integrin levels and regulates integrin trafficking away from a degradative lysosomal compartment.

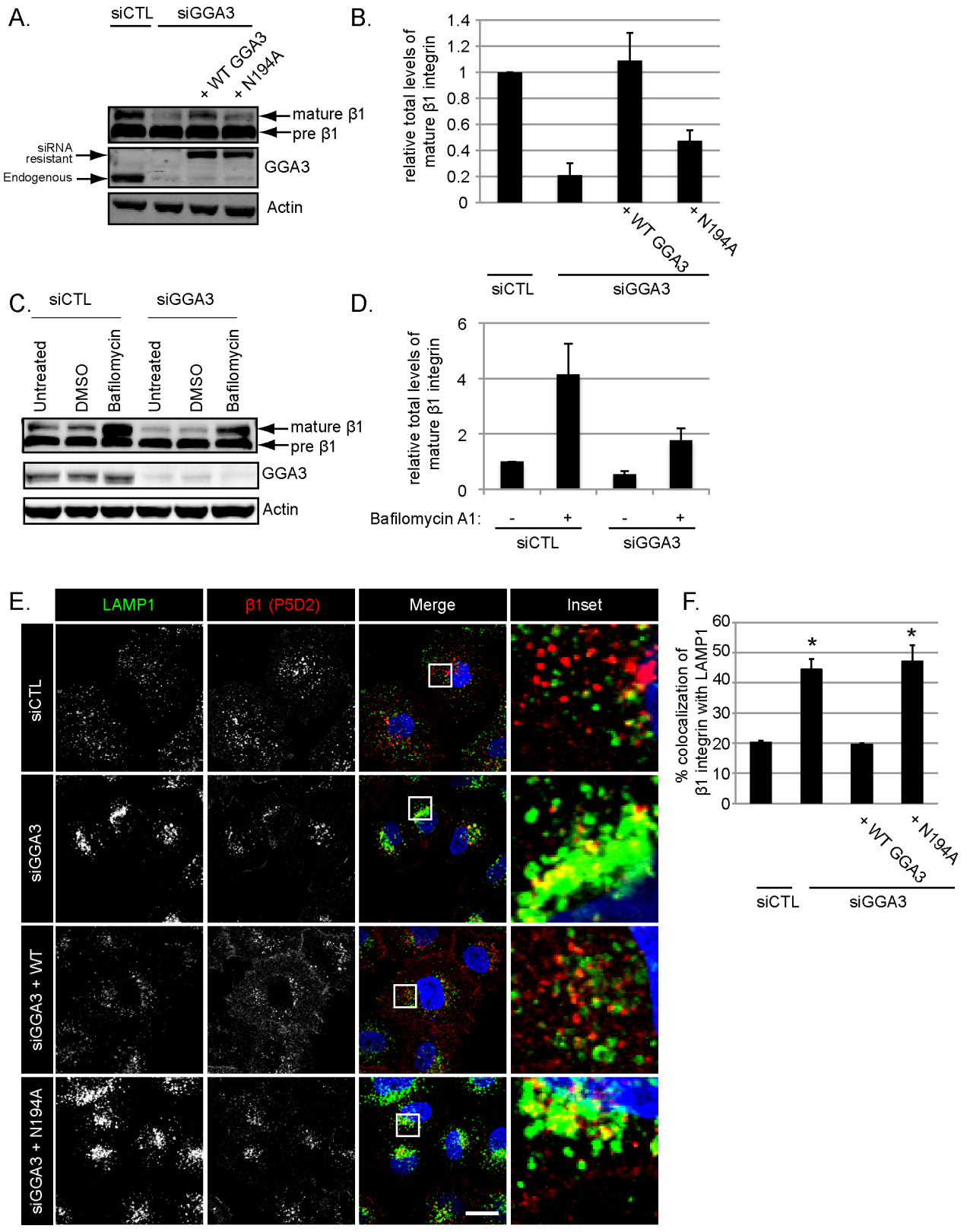



**Figure 2.5 GGA3 interaction with Arf is required for maintenance of β1 integrin levels and trafficking away from the lysosome.** (A) Stable cell lines expressing siRNA resistant WT GFP-

**Figure 2.5 (continued)** GGA3 or a mutant uncoupled from Arf proteins (N194A) were generated. Whole cell lysates of HeLa cells or stable cell lines seeded on collagen and treated with control or GGA3 siRNA and analyzed by immunoblotting. (B) Quantification of relative mature β1 levels normalized to actin loading control. The mean from 3 independent experiments is shown. (C) HeLa cells were untreated, treated with vehicle control or 0.1μM Bafilomycin A1 for 16 hours, lysed and 50ug of protein was analyzed by immunoblotting. (D) Quantification of vehicle or Bafilomycin A1 siCTL or siGGA3 cells. (E) HeLa cells or stable cell lines were plated on collagen-coated glass cover slips and treated with control or GGA3 siRNA for 72 hours. Surface integrins were labeled with anti-β1 integrin antibody P5D2 at 4ºC for 30 min, washed and fixed with 4% PFA or allowed to internalize for 60 minutes at 37ºC before being fixed. Cells were permeabilized and stained for β1 integrin or LAMP1 via indirect immunofluorescence and images were captured by confocal microscopy. (F) Quantification of β1 integrin colocalization with LAMP1. Mean from 3 independent experiments. 5 independent fields of view were quantified per experiment. Error bars indicate standard error of the mean. *$p<0.05$

### 2.4.7 A GGA3-Arf interaction regulates cell spreading and focal adhesions

In order to spread efficiently, a cell must form new contacts with the underlying matrix. Integrin recycling supports this process and disruption of recycling may impair the ability of a cell to form new focal adhesions. To assess whether GGA3 depletion and decreased β1 impaired cell spreading, the ability of cells to spread on a collagen matrix was examined. Cells were allowed to adhere for 15 minutes onto collagen coated wells and the surface area of adherent cells was measured over the course of 2 hours. GGA3 knockdown reduced cell spreading to 57% of control cells after 2 hours (Figure 2.6A, B). Moreover, whilst the expression of WT GFP-GGA3 efficiently rescued cell spreading in GGA3 depleted cells (99% of control), expression of the N194A GFP-GGA3 mutant did not (69% of control). Furthermore, depletion of GGA3 reduced the number of vinculin-positive focal adhesions to 30% of control cells (Figure 2.6D). This was rescued by re-expression of WT GFP-GGA3 (101% of control) but not N194A GFP-GGA3 as cells had only 26% of focal adhesions relative to control (Figure 2.6D). Notably, in contrast to β1 integrin, total levels of vinculin were not altered by depletion of GGA3 (Figure 2.6C). Together these data demonstrate that GGA3 and a GGA3-Arf interaction is required for efficient integrin dependent cell spreading and focal adhesion formation on collagen matrix.

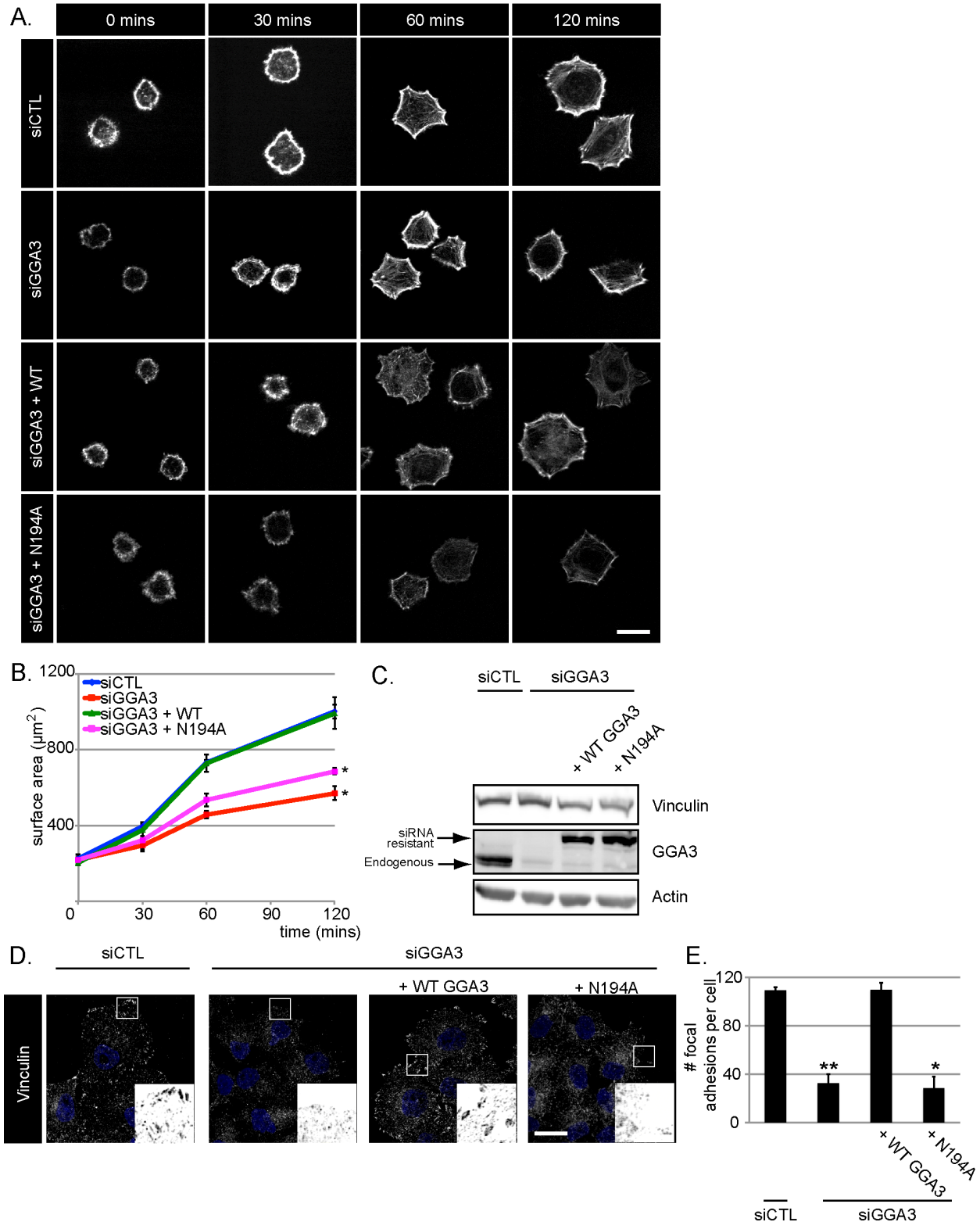



**Figure 2.6 GGA3 Arf-binding site is required for cell spreading and focal adhesion number.** (A) HeLa cells or stable cell lines treated with control or GGA3 siRNA for 72 hours were trypsinized and replated on a collagen coated ibidi μ-slide for 15mins at 37ºC. Non-adherent cells were washed off and pre-warmed media was replaced. After the indicated time periods cells were

**Figure 2.6 (continued)** fixed with 4% PFA, stained with Phalloidin and imaged by confocal microscopy. (B) Mean of the cell surface area from 3 independent experiments is shown. (C) Whole cell lysates of HeLa cells or stable cell lines seeded on collagen and treated with control or GGA3 siRNA for 72 hours and analyzed by immunoblotting. (D) HeLa cells or stable cell lines were plated on collagen coated glass cover slips and treated with control or GGA3 siRNA for 72hours. Cells were then fixed with 4% PFA, immunostained by indirect immunofluorescence and images were captured by confocal microscopy. Insets show higher magnification and inverted contrast. Error bars indicate standard error of the mean. *$p<0.05$.

### 2.4.8 Arf6 is required for cell spreading and β1 integrin trafficking

The requirement for Arf6 in β1 integrin trafficking has been rigorously characterized. However, recently it was shown that both Arf1 and Arf3 may recruit GGA3 to endosomes (D'Souza et al., 2014). We identified siRNA duplexes that specifically targeted Arf1, Arf3 or Arf6 and tested their effect on integrin mediated cell spreading (Figure 2.7A). Arf1 or Arf3 depletion had no significant effect on cell spreading on a collagen matrix (Arf1 102% and Arf3 105% of control) (Figure 2.7B, C). However, Arf6 depletion significantly reduced cell spreading to 59% of control cells after 2 hours Figure 2.7B, C). In addition to cell spreading we also found that Arf6 silencing led to a perinuclear localization of internalized β1 integrin antibody (Figure 2.7D). We observe that Arf6 silencing phenocopies GGA3 depletion in a manner similar to the expression of the N194A GGA3 mutant. Therefore, we conclude that Arf6 is the predominant Arf-family binding partner of GGA3 that regulates cell spreading and β1 integrin trafficking.

### 2.4.9 The GGA3 Arf-binding site is required for efficient cell migration

To test whether impaired integrin trafficking and defects in focal adhesions might contribute to the decreased cell migration observed on a collagen matrix, we performed scratch assays as in Figure 2.1. Consistent with our previous data, control HeLa cells filled the scratch and migrated with an average speed of 0.27μm/min, whereas GGA3 depletion resulted in reduced cell speed to 0.12μm/min and decreased ability to close the wound (Figure 2.8). Expression of siRNA-resistant WT GGA3 rescued migration to 0.21μm/min (77% of control), whereas, expression of GGA3 (N194A) failed to rescue cell migration (0.13μm/min) (48% of control). This data demonstrates that recruitment of GGA3 through its Arf-binding site is required for cell migration.

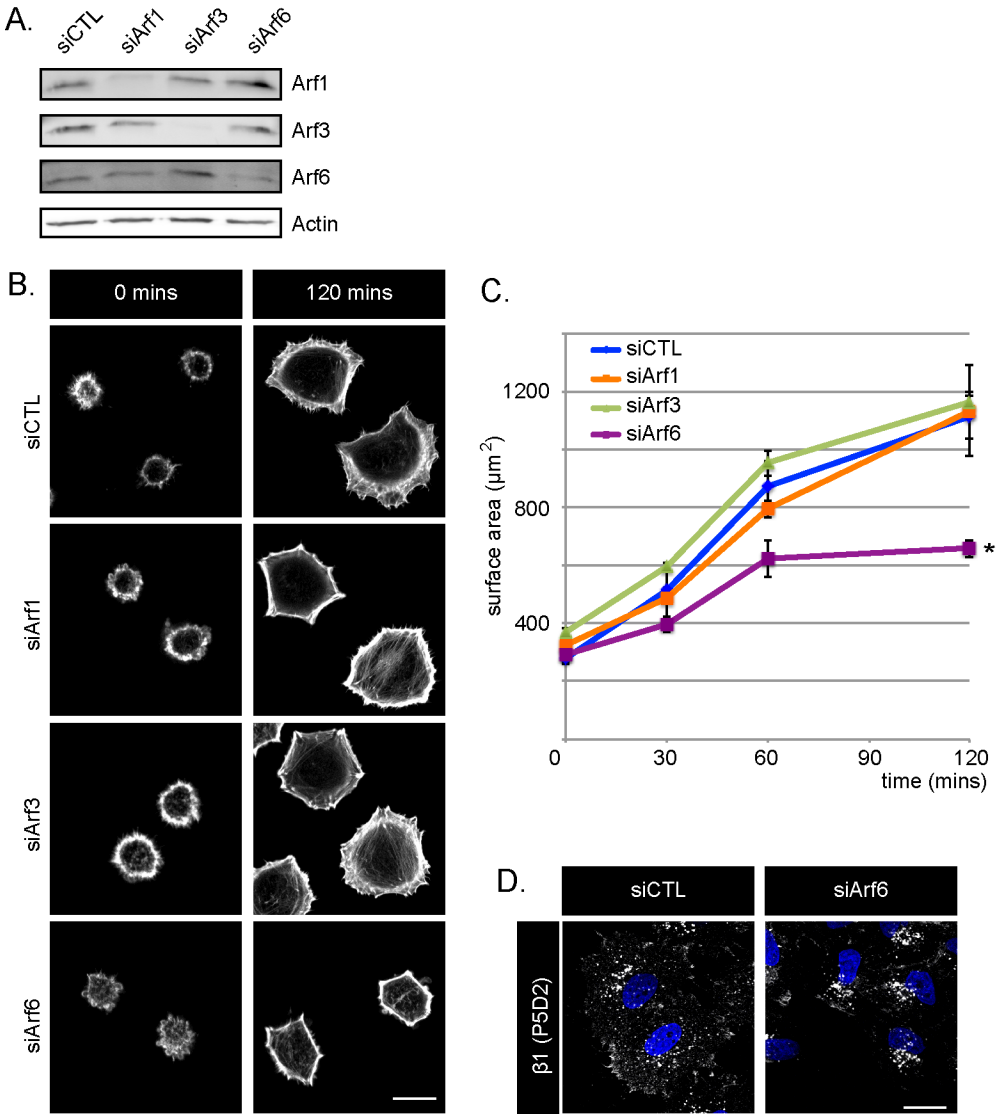



**Figure 2.7 Arf6 regulates cell spreading and integrin trafficking.** (A) Whole cell lysates of HeLa cells seeded on collagen and treated with control, Arf1, Arf3 or Arf6 siRNA for 72 hours and analyzed by immunoblotting. (B) HeLa cells treated with control, Arf1, Arf3 or Arf6 siRNA for 72 hours were trypsinized and replated on a collagen coated ibidi µ-slide for 15mins at 37°C. Non-adherent cells were washed off and pre-warmed media was replaced. After the indicated time periods cells were fixed with 4% PFA, stained with Phalloidin and imaged by confocal microscopy. (C) Mean of the cell surface area from 3 independent experiments is shown. (D) HeLa cells were plated on collagen-coated glass cover slips and treated with control or Arf6 siRNA for 72 hours. Surface integrins were labeled with anti-β1 integrin antibody P5D2 at 4°C for 30 min, washed and fixed with 4% PFA or allowed to internalize for 60 minutes at 37°C before being fixed. Cells were permeabilized and stained by indirect immunofluorescence and images were captured by confocal microscopy. Error bars represent stand error of the mean. *$p<0.05$.

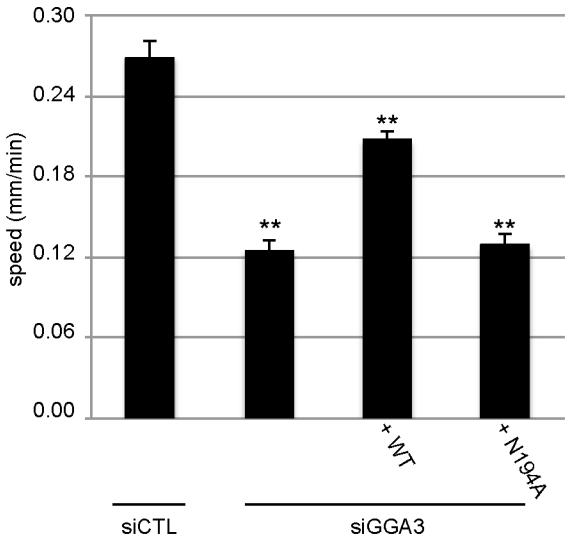



**Figure 2.8 The GGA3 Arf binding site is required for efficient cell migration.** Quantification of speed of HeLa cells or stable cell lines treated with control or GGA3 siRNA for 24hours and replated in a collagen coated well containing ibidi cell-culture insert. After 16 hours insert was removed, media replaced, and images were captured every 15mins for 12 hours. Mean of 3 experiments. 30 cell tracks per experiment were analyzed from 3 independent fields of view. Error bars represent stand error of the mean. **$p<0.01$.

### 2.4.10 GGA3 regulates the subcellular localization of sorting nexin 17

GGA3 regulates an Arf6 dependent β1 integrin trafficking pathway through Rab4 recycling endosomes. This is similar to the SNX17 adaptor protein that binds directly to the β1 integrin C-terminal tail and is also required for trafficking through Rab4 endosomes. To examine the relationship between SNX17 and GGA3 we investigated the effect of GGA3 knockdown on SNX17. Steady state SNX17 protein levels and SNX17 association with cellular membrane fractions are unaffected following GGA3 knockdown (Figure 2.9A). However, the subcellular localization of SNX17 is altered following GGA3 knockdown whereby SNX17 levels are decreased in an EEA1-positive endosomal compartment and increased in a LAMP1-positive compartment (Figure 2.9B, C). The enlarged SNX17-positive endosomes were also strongly positive for β1-integrin (Figure 2.9D, E). Therefore, in the absence of GGA3, both β1 and SNX17 relocalize to an enlarged LAMP1-positive compartment. In line with the co-operative role for GGA3 and SNX17 in integrin recycling, we found that both proteins associate with β1-integrins based on GFP pull-downs and that endogenous SNX17 and GGA3 co-precipitate in MDA-MB-231 cells (Figure 2.9F, G). Together, these data support a model whereby GGA3, SNX17 and β1-

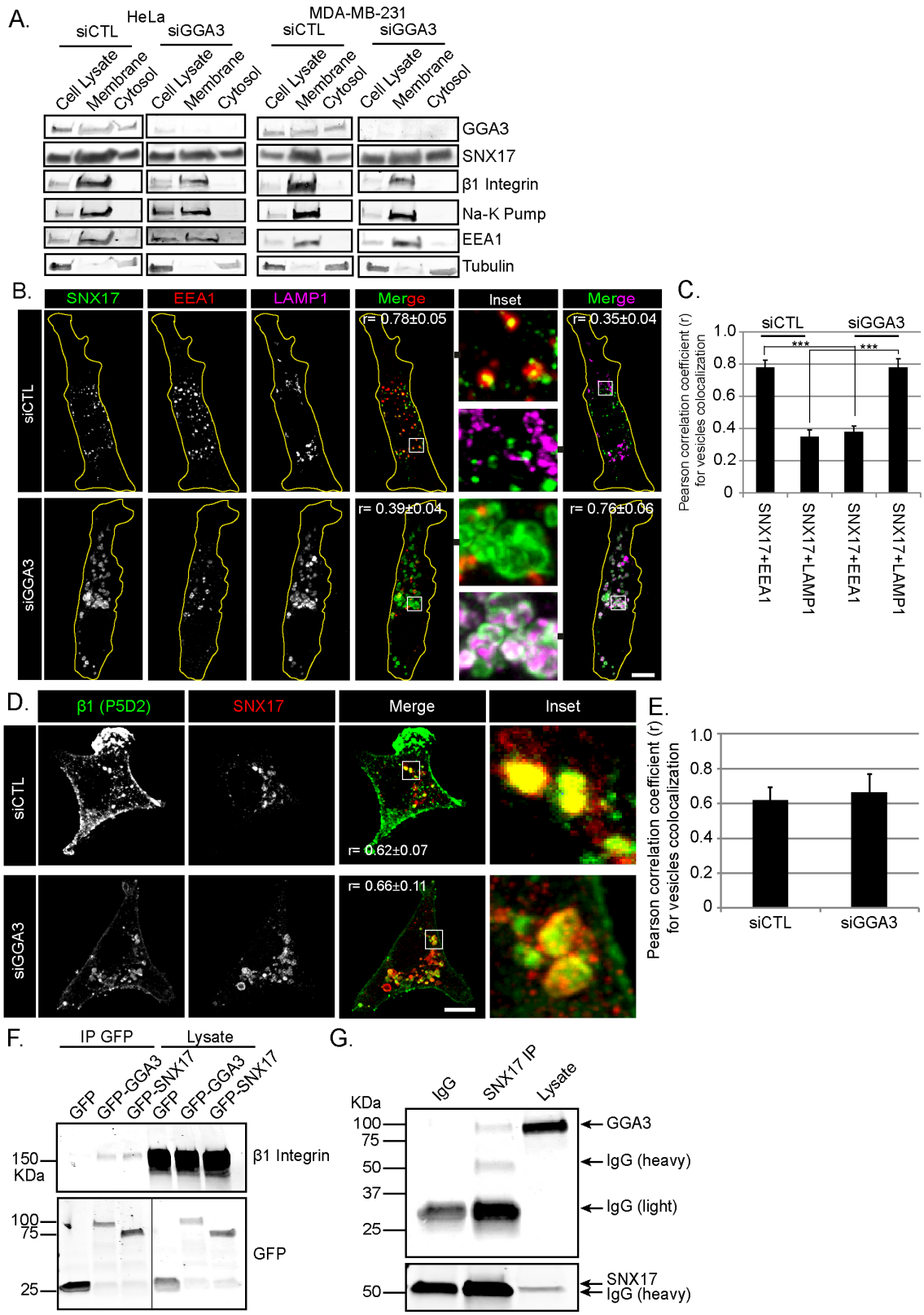



**Figure 2.9 GGA3 regulates SNX17 localization and forms a complex with SNX17 and β1 integrin.** (A) Subcellular fractionation derived from control or GGA3 silenced HeLa and MDA-
Figure 2.9 (continued) MB-231 cells. β1 integrin, Na+-K+ pump and EEA1-positive control for

**Figure 2.9 (continued).** membrane fraction. Tubulin-positive control for cytosolic fraction. (B) Representative single channel and merged images of subcellular localization of SNX17 staining with EEA1 or LAMP1 stainings in control and GGA3 silenced MDA-MB-231 cells. Insets highlight regions of overlap (yellow for SNX17 and EEA1, white for SNX17 and LAMP1). scale bar, 10μm. (C) Quantification of respective colocalization, r=Pearson's correlation coefficient between the indicated vesicular stainings (mean±SD; n=30 cells; *p<0.001). (D) Representative single channel and merged images of β1 integrin and SNX17 subcellular localization in control and GGA3 silenced MDA-MB-231 cells. Insets highlight regions of overlap (yellow). scale bar, 10μm. (E) Quantification of β1 integrin and SNX17 colocalization, r=Pearson's correlation coefficient for colocalization (mean±SD; n=30 cells). (F) GFP-TRAP pulldown in MDA-MB-231 cells transfected with GFP, GFP-GGA3 or GFP-SNX17. (G) Endogenous SNX17 immunoprecipitation from MDA-MB-231 cell extracts.

integrins, associate in cells and GGA3 is required for correct SNX17 targeting and SNX17-dependent trafficking of β1 integrin to enable cell migration.

## 2.5 Discussion

Endocytic adaptors and their effectors can regulate β1 integrin receptor trafficking, however the functional consequences of disrupting these processes and their specificity are poorly understood (De Franceschi et al., 2015; Parachoniak and Park, 2012). Here we describe a novel role for GGA3, specifically amongst GGA family members, in regulating SNX17 subcellular localization and subsequently β1 integrin trafficking to the cell periphery and β1 dependent cellular processes, including cell spreading, formation of focal adhesions and cell migration. This was established by multiple approaches, including examining the rate of cell movement on collagen, total and surface levels of β1 integrin under conditions in which GGA3 was depleted and rescue experiments, as well as β1 integrin and SNX17 localization upon rescue with WT or mutant GGA3.

These multiple approaches yielded complementary and quantitative results supporting a model whereby, in a migrating cell, internalized β1 integrin enters a GGA3- and SNX17-positive compartment that promotes entry into an endosomal recycling compartment rather than entry into a degradative compartment. In the absence of GGA3, SNX17 becomes associated with enlarged LAMP-1 positive structures and β1 integrin traffics in part to this lysosomal compartment where it is degraded. Entry of β1 integrin into a Rab4-positive recycling compartment is enhanced upon a GGA3-Arf interaction. GGA3-dependent trafficking of β1 integrin is required for formation of focal adhesions and efficient cell migration.

β1 integrins recycle via an Arf6-positive compartment and recycling is tightly coupled to the activity of Arf6 (Powelka et al., 2004). Here we confirm the requirement for Arf6 in cell spreading and integrin trafficking, identify that the Arf6 effector GGA3 is required for recycling of β1 integrin and that mutation of the Arf binding site in GGA3 abrogates this leading to enhanced entry of β1 into a degradative lysosomal compartment. While both the dominant active and dominant negative forms of Arf6 colocalize with integrin positive vesicles in cells, overexpression of these proteins block integrin recycling (Brown et al., 2001; Eva et al., 2012; Powelka et al., 2004). Depletion of Rab35, a negative regulator of Arf6 activation, increases the proportion of GTP-loaded Arf6 and integrin recycling (Allaire et al., 2013). GGA3 binds specifically to GTP-loaded Arf6 (Boman et al., 2000; Dell'Angelica et al., 2000; Hirst et al., 2000) and we show that regulation of integrin levels and trafficking of β1 integrin is dependent on the integrity of the Arf binding site of GGA3. Together these studies support that Arf6 activation is required for integrin recycling. Consistent with this, overexpression of ACAP1, an Arf6 GAP that reduces the amount of active Arf6 while allowing for turnover, also increases integrin recycling (Li et al., 2005a). Hence it is likely that the rate of Arf GTP/GDP flux is a limiting step in β1 recycling, maintenance of integrin levels and efficient cell migration.

GGA family members were identified as Arf-GTP binding proteins that localize to the trans-Golgi network. They function in cargo recognition to select cargo into newly formed vesicles and as regulators of clathrin assembly at these sites. While both GGA1 and GGA3 are predominantly localized to the TGN, a subpopulation of both of these proteins are localized to recycling endosomes (Boman et al., 2000; Dell'Angelica et al., 2000; Parachoniak et al., 2011; Zhao and Keen, 2008). Whether these two GGA proteins participate in a single recycling pathway or parallel pathways has not been carefully investigated. We report here a specific role for GGA3 but not GGA1 for cell migration. Importantly, GGA3 depletion results in reduced cell migration and reduced α2, α5 and β1 integrin levels. Different heterodimeric integrin pairs are proposed to recycle via different recycling pathways (De Franceschi et al., 2016; Morgan et al., 2013). Given that GGA3 depletion promotes a decrease in β1 protein levels but not αV integrin, this reflects specificity in GGA3 dependent trafficking.

Integrin heterodimers can exist in multiple states including a closed or inactive conformation and an open or active confirmation. The proportion of each conformation is regulated by the presence of ligand or by "inside-out" signalling through binding of talin or kindlin promoting an active conformation, whereas SHARPIN, ICAP-1 and filamin act as negative regulators of integrin activation (Calderwood et al., 2013; Pouwels et al., 2012). The trafficking dynamics and localization of each of these conformation states of β1 within the cell has been probed using conformation-specific antibodies (Arjonen et al., 2012; Powelka et al., 2004). These studies have established that recycling of both the active and inactive forms of β1 are dependent on Arf6. This is consistent with our data supporting a role for GGA3 and a GGA3/Arf6 interaction in this process, where depletion of GGA3 decreases α2β1 levels irrespective of conformational change induced by matrix engagement. While GGA proteins are recruited to endosomes via Arf proteins, another function of GGA proteins is to promote clathrin assembly on membranes (Puertollano et al., 2001b). At steady state, the clathrin coat machinery that regulates integrin recycling has not been determined. While previous studies have identified a binding site for the Arf GAP ACAP1 on β1 integrin and a ACAP1/clathrin heavy chain interaction modulates recycling of β1 integrin (Li et al., 2005a; 2007a), this interaction is dependent on serum or growth factor induced phosphorylation of ACAP1 (Bai et al., 2012). At steady state a role for clathrin light chain (CLC) in regulation of β1 integrin recycling has been identified (Majeed et al., 2014). CLC KD reduced the abundance of rapidly recycling gyrating-clathrin structures labeled with GGA1, hence a role for GGA3 as an intermediate in clathrin vesicle genesis is consistent with a role in β1 trafficking.

Once a cell has adhered to the underlying substratum, new focal adhesions are formed and this is dependent upon trafficking of endosomal integrins to the cell periphery. An example of this has been demonstrated for PDGF-stimulated cells whereby overexpression of dominant negative Rab4 blocks cell spreading on vitronectin and recycling of αvβ3 heterodimers (Roberts et al., 2001). Here we show that depletion of GGA3 impairs the ability of cells to spread on a collagen matrix and this is dependent on the integrity of the GGA3 Arf binding site. Previously, we have also shown that GGA3 localizes to an early, Rab4, recycling compartment and this can be enhanced upon stimulation with HGF (Parachoniak et al., 2011). Here we show that β1 integrin recycling via a Rab4-positive compartment requires GGA3. While recycling of αvβ3 was found to

be independent of the Arf6 small GTPases, both the PDGF-stimulated αvβ3-pathway and β1 integrin pathway require Rab4 for efficient recycling, and consequently, cell spreading.

Our results demonstrate a role for GGA3 in cell migration, spreading and integrin trafficking to the cell periphery. Understanding the complexity of the molecular mechanisms regulating integrin recycling are critical for our understanding of how cells interact with their microenvironment. Given the importance of the microenvironment in cancer progression, it will be important to study the role of GGA3 and integrins in this context.

## 2.6 Acknowledgements

We would like to thank members of the Park lab and Dmitri Kharitidi for critically reading the manuscript; the McGill Advanced Bioimaging Facility and the Flow Cytometry Facility for technical assistance. We thank the CIC core-facility at Turku Centre for Biotechnology for their help with imaging and P. Laasola for technical assistance. C.D.H.R was supported by a Doctoral Award from Fonds de Recherche Québec – Santé, P.S. has been supported by Turku Doctoral Programme of Molecular Medicine (TuDMM) and C.A.P. was supported by a Canada Graduate Scholarship Doctoral Award from the Canadian Institutes of Health Research (CIHR). This research was supported by the Academy of Finland, and ERC Consolidator Grant (JI) and an operating grant to M.P. from CIHR (MOP-11545). M.P. holds the Diane and Sal Guerrera Chair in Cancer Genetics. The authors declare no conflict of interest.

## 2.7 Experimental Procedures

**Cell lines, antibodies, DNA constructs and chemicals**

HeLa cells were cultured in DMEM containing 10% FBS. Generation and maintenance of cell lines stably expressing siRNA-resistant GFP-GGA3 and GFP-GGA3 N194A was previously described (Parachoniak et al., 2011). Antibodies used for western blotting were α2 obtained from Millipore (Mab1936); α5 from Biolegend (328002); GGA3 (612310), Arf3 (610784) and β1 integrin (610468) from BD Biosciences; GGA1 (sc-30102), Arf6 (sc-7971) and EEA1 (sc-6415) and IgG rabbit (sc-2027) from Santa Cruz Biotechnology Inc.; GGA2 was a kind gift from Dr. Juan Bonifacino; Arf1 as described previously (Lamorte and Park, 2003); Na+K+ pump (a6F) from developmental studies hybridoma bank; and actin from Sigma-Aldrich. Antibodies used for

immunofluorescence and flow cytometry where indicated were TS2/16 (sc-53711), P5D2 (sc-13590) anti-β1 integrin obtained from Santa Cruz Biotechnology Inc.; LAMP1 from Abcam (AB24170 and AB25630); vinculin from Sigma-Aldrich (V-9131); α1, α2 and αV from Millipore (Mab1973, Cbl1477, 407286); α5 from Biolegend (328002); SNX17 (10275-1-AP) from Proteintech; GFP, Alexa-Fluor 488, 555, 647 and phalloidin-488 conjugated secondary antibodies from Molecular Probes. GFP-Rab4 and 11 were provided by Robert Lodge and β1 Integrin-GFP was described previously and was a kind gift from Martin Humphries (Manchester University) (Parsons et al., 2008). Bafilomycin A1 and Lactacystin was obtained from Calbiochem (196000).

**Matrices and Coating**

Where indicated, wells or 1.5 cover slips were coated with 25 μg/mL collagen, 10 μg/mL fibronectin or 2 μg/mL vitronectin for 1 h at 37°C and washed 2X with PBS. Cells were plated immediately after washing.

**siRNA transfection**

Unless otherwise noted, HeLa cells were seeded at $7.5x10^4$ cells per well in 6-well dishes and immediately transfected with 20nM siRNA using HiPerfect as per manufacturer's instructions (QIAGEN). All experiments, unless otherwise noted, were performed 72h post-transfection.

**Live-cell imaging and cell tracking**

After 24 hours, $1.5x10^5$ siRNA-treated cells were trypsinized, spun down, resuspended in 10% DMEM and counted. $2.5x10^4$ cells were replated in each well of a Ibidi cell culture insert placed in a coated 24 well dish. After 16 h, inserts were removed and 1 mL prewarmed 10% DMEM was added to each well. The dish was positioned on a motorized stage equipped with a Axiovert 200M inverted microscope (Carl Zeiss, Inc), LD A-Plan 20X/0.3 Ph1 objective lens, AxioCam HRM (Carl Zeiss, Inc) and digital camera; all contained within a transparent environment chamber Climabox (Carl Zeiss, Inc) maintained at 5% (v/v) $CO_2$ at 37°C. The microscope was driven by AxioVision LE software (Carl Zeiss, Inc). The motorized stage advanced to pre-programmed locations and images were captured every 15 mins for 12 h. Cell tracks were generated by manually tracking individual cells using MetaMorph software and data analyzed with Microsoft Excel. Dividing cells and cells making contacts were excluded from random cell migration analysis.

**Biochemical assays**

HeLa cells were harvested in TGH lysis buffer (50 mM HEPES, pH 7.5, 150mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 1% Triton X-100, 10% Glycerol, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium vanadate, 10 µg/ml aprotinin and 10 µg/ml leupeptin). Lysates were aliquoted and boiled for 5 mins with SDS sample buffer, resolved by SDS-PAGE and transferred to Immobilon-FL PVDF transfer membranes, blocked with LI-COR blocking buffer (LI-COR Biosciences), incubated with primary antibodies, followed by incubation with infrared conjugated secondary antibodies prior to detection and analysis on the Odyssey IR Imaging System (LI-COR Biosciences).

**Immunoprecipitation**

Cells were detached with trypsin, spun down and excess liquid was removed. 200 µl of lysis buffer (40 mM HepesNAOH, 75 mM NaCl, 2 mM EDTA, 1% NP40, protease – and phosphatase inhibitor pills) was added (for 10 cm plate). Tubes containing the lysates were rotated for 30 min at +4 °C. Lysates were then spun down at 13 000 rpm for 10 min, +4 °C and debris were discarded. 20 µl of supernatant was frozen as lysis control. The remainder of the sample was moved to new 1.5 ml tube and 1.5 µg of antibodies added per sample. The tubes were then incubated overnight at +4 °C. Lysates were immunoprecipitated with protein G Sepharose 4 Fast Flow beads (GE Healthcare) or incubated directly with GFP-Trap-A beads (Chromotek) for 1 h at 4ºC. Finally, immunoprecipitated complexes were washed 3 times with wash-buffer (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 % NP-40) followed by elution in reducing Laemmli buffer and denatured for 5 min at 95ºC for western blotting.

**Subcellular fractionation**

Briefly, the cells (10 cm dish) for each condition were washed with PBS and scraped with 500 µl of hypotonic lysis buffer (10 mM HEPES-KOH pH 7.2, 0.25 M sucrose, 1 mM EDTA, 1 mM MgOAc and protease and phosphatase inhibitor pills (PhosSTOP and Complete from Roche)). Cells were lysed with cell cracker about 30 times and 40 µl of total lysate was saved. The remaining lysate was centrifuged at 1000g for 10 min to remove nucleus and cell debris. The supernatant was then ultra-centrifuged at 100 000g to collect total membrane fraction (pellet) and cytosolic fraction

(supernatant). All fractionation steps were performed at 4 °C or on ice. Finally, the reducing Laemmli buffer was added to fractions and denatured for 5 min at 95ºC for western blotting.

**Internalization assays**

Biotin based internalization assays were performed as described previously (Muharram et al., 2014). Briefly, cells were rinsed once with cold PBS and surface bound proteins were labeled with 0.5 mg/mL EZ-link cleavable sulfo-NHS-SS-biotin diluted in Hank's balanced salt solution for 30 mins on ice. Unbound biotin was removed, and cells were allowed to internalize labeled cell surface proteins by addition of 10% FBS for indicated time points. Cells were then incubated with 60mM MESNA reducing agent for 30 mins on ice, followed by incubation with 100mM iodoacetamide quenching solution on ice for 15 mins. Cells were then lysed in lysis buffer (50mM Tris pH 7.5, 1.5% Triton X-100, 100mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium vanadate, 10 µg/ml aprotinin and 10 µg/ml leupeptin) and cleared by centrifugation (13 000rpm, 10mins, 4°C). Biotinylated integrins were immunoprecipitated from the lysate using P5D2 anti-β1 integrin and protein G sepharose beads.

**Cell spreading assay**

Cells were trypsinized, spun down, resuspended in DMEM + 10% FBS. $2.5x10^4$ cells were diluted in pre-warmed 500 µL media and plated in a collagen-coated 8-well Ibidi slide. Cells were allowed to adhere for 15 mins, wells were washed once with DMEM + 10% FBS and 500 µL media was replaced. After the indicated time points cells were fixed in 4% PFA and processed for immunofluorescence as previously described. Using MetaMorph software, individual cells were identified using an intensity threshold-based method and the surface area of individual cells was determined.

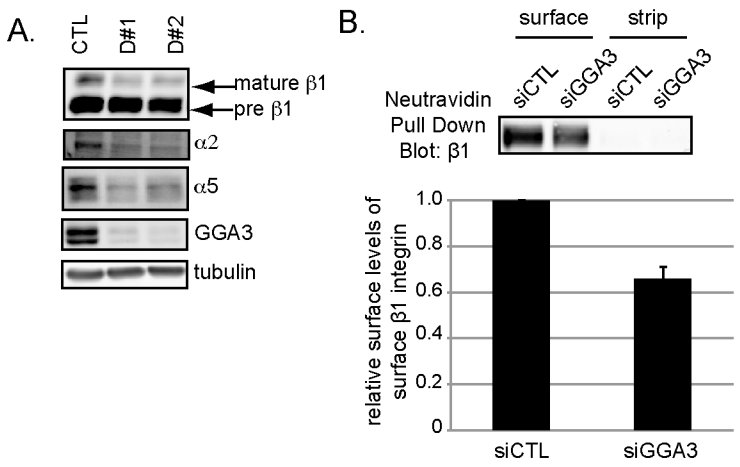
**Flow cytometry**

Cells were trypsinized, spun down, washed in FACS buffer (DMEM, 10% heat inactivated FBS, $CaCl_2$ and $MgCl_2$), incubated with dilutions of primary antibodies for 30 mins at 4°C, washed 3X, incubated with donkey anti-mouse Alexa647 for 45 mins at 4°C, washed 3X and 10 000 cells were analyzed using a LSR2 flow cytometer (BD Biosciences).

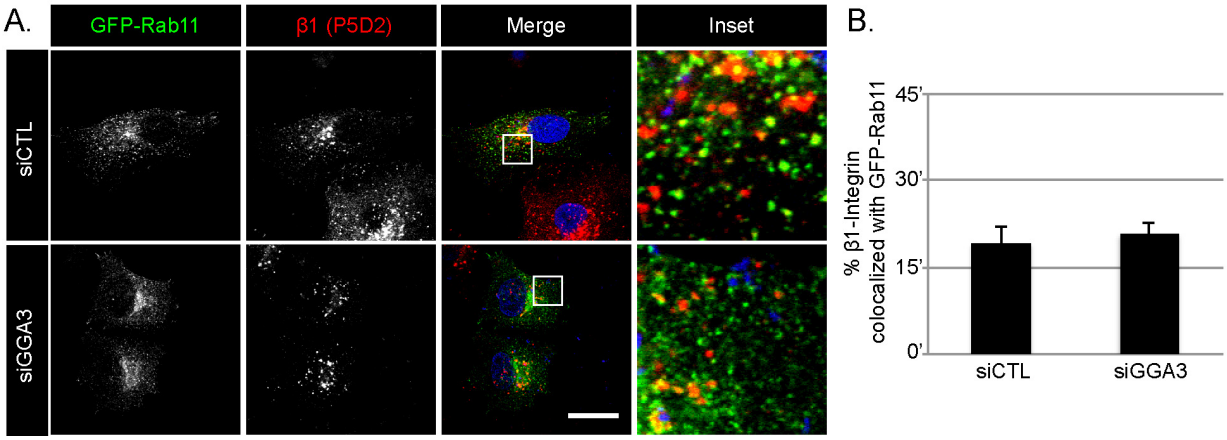
**Immunofluorescence studies**

Cells were seeded at $3.75x10^4$ in 6-well dishes containing glass coverslips (Bellco Glass Inc. Vineland, NJ) coated with collagen and treated with siRNA as previously described. Coverslips were then fixed with 4% paraformaldehyde (PFA; Fisher Scientific) in PBS for 20 mins. Cover slips were then washed four times in PBS and residual PFA was removed with 3 5-minute washes with 100 mM glycine in PBS. Cells were permeabilized with 0.1% Saponin/PBS and blocked for 30 mins with blocking buffer (5% bovine serum albumin, 0.05% Saponin, PBS). Coverslips were incubated with primary and secondary antibodies diluted in blocking buffer for 1 hour and 45 mins respectively, at room temperature. Coverslips were mounted with Immu-mount (Thermo-Shandon, Pittsburgh, PA). Confocal images were taken using a Zeiss 510 Meta laser scanning confocal microscope with 100X objective and 1X zoom and Zeiss 710 laser scanning confocal microscope with 63X objective and 1.5X zoom (Carl Ziess, Canada Ltd, Toronto, Ontario, Canada). Image analysis was carried out using the Zen 7.0 image browser, MetaMorph, ImageJ or Imaris 8.0.2 (Turku Centre for biotechnology license). GFP-Rab4 or GFP-Rab11 expressing cells were selected for imaging based on GFP-positivity alone and rolling ball background subtraction was used on images prior to analysis.

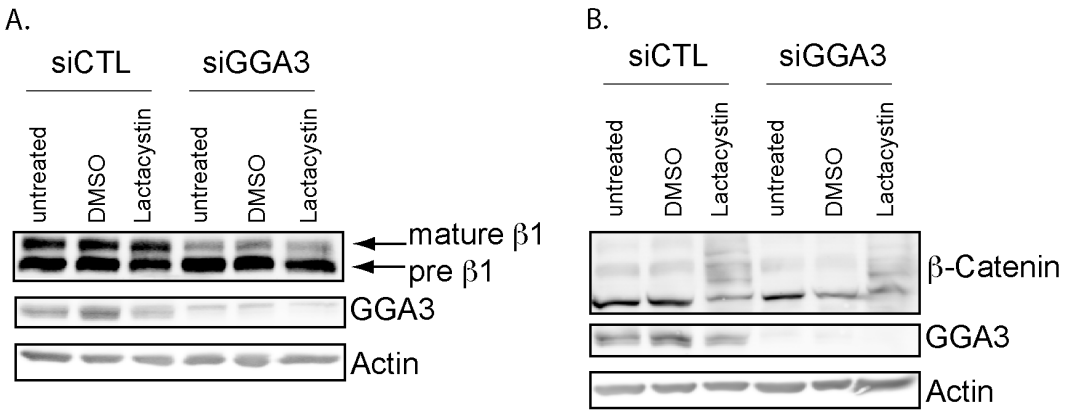
## 2.8 Supplemental Information




**Supplemental Figure 2.1 Multiple GGA3 siRNA duplexes affect α2, α5 and β1 integrin levels.** Whole cell lysates prepared from HeLa cells seeded on collagen and treated with indicated siRNA for 72 hours, were immunoblotted with the indicated antibodies.

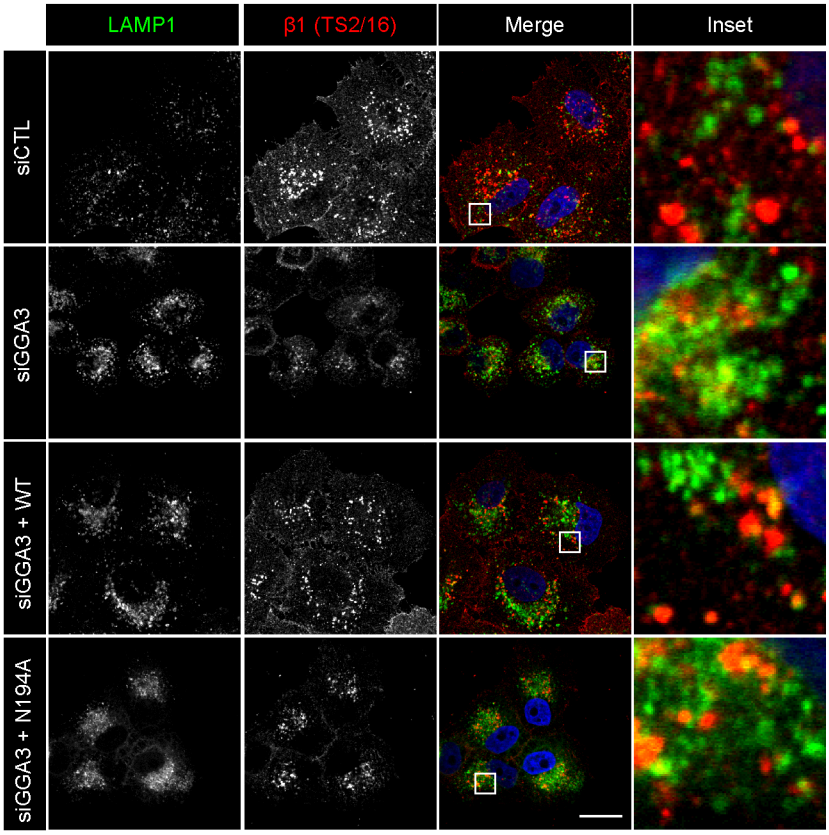



**Supplemental Figure 2.2 β1 integrin does not colocalize with a Rab11 compartment.** HeLa cells plated on collagen-coated glass cover slips and treated with control or GGA3 siRNA for 72 hours. After 24 hours GFP-Rab11 was transfected and media replaced. Surface integrins were labeled with anti-β1 integrin antibody P5D2 at 4°C for 30 min, washed and allowed to internalize for 60 minutes before being fixed with 4% PFA. Cells were permeabilized and stained for β1 integrin via indirect immunofluorescence and images were captured by confocal microscopy.

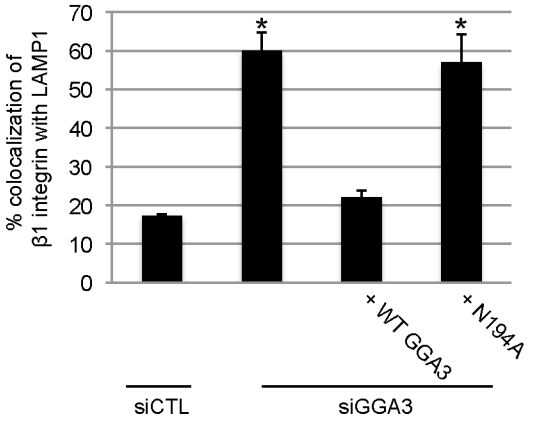



**Supplemental Figure 2.3 β1 integrin is not degraded via the proteasome in GGA3 depleted cells.** HeLa cells were untreated, treated with vehicle control or 10μM lactacystin for 16 hours, lysed and 50ug of protein was analyzed by immunoblotting.

A.




B.




**Supplemental Figure 2.4 GGA3 interaction with Arf is required β1 integrin trafficking away from the lysosome.** HeLa cells or stable cell lines were plated on collagen-coated glass cover slips and treated with control or GGA3 siRNA for 72 hours. Surface integrins were labeled with anti-β1 integrin antibody TS2/16 at 4ºC for 30 min, washed and fixed with 4% PFA or allowed to internalize for 60 minutes at 37ºC before being fixed. Cells were permeabilized and stained for β1 integrin or LAMP1 via indirect immunofluorescence and images were captured by confocal microscopy.

# Chapter 3

## 3 A functional role for cytohesin-1 microexon splice variants in Met receptor-dependent cell migration

Colin D.H. Ratcliffe, Nadeem Siddiqui, Nahum Sonenberg, Morag Park

## 3.1 Preface

In Chapter 2 we identified a role for the Arf6 effector, GGA3 in cancer cell migration. Arf6 had been associated with cell migration and invasion. Activation of the Met receptor tyrosine kinase promotes cell migration, in part, through Arf6. However, the mechanism for Met activation for Arf6 was unknown. We identified a specific splice variant of the Arf GEF cytohesin-1 that is important for HGF-dependent cell migration. Microexons are a recently described class of alternative splicing events, however specific functions for individual splice variants are largely unknown. In Chapter 3 we identify a functional role for cytohesin-1 splice variants that differentially regulate rearrangement of the actin cytoskeleton and cytohesin-1 localization.

## 3.2 Abstract

Differential inclusion or skipping of microexons is an increasingly recognized class of alternative splicing events. However, the functional significance of microexons and their contribution to signalling diversity is poorly understood. The Met receptor tyrosine kinase (RTK) modulates invasive growth and migration in development and cancer. Here we show that microexon switching in the Arf6 guanine nucleotide exchange factor, cytohesin-1, controls Met-dependent cell migration. We show that cytohesin-1 isoforms, differing by the inclusion of an evolutionarily conserved 3 nucleotide microexon in the pleckstrin homology domain, display differential affinity for $PI(4,5)P_2$ (triglycine) and $PI(3,4,5)P_3$ (diglycine). Selective phosphoinositide recognition by cytohesin-1 isoforms promotes distinct subcellular localizations, whereby the triglycine isoform localizes to the plasma membrane and the diglycine to the leading edge. These data highlight microexon skipping as a mechanism to spatially restrict signalling and provide a mechanistic link between RTK-initiated phosphoinositide microdomains and Arf6 during signal transduction and cancer cell migration.

## 3.3 Introduction

The Met RTK coordinates invasive growth in response to its ligand HGF. This is tightly regulated during development promotes a morphogenic program that is essential for liver development, migration of muscle precursors into the limb bud and wound healing in the adult (Gherardi et al., 2012). Underpathophysiological conditions, dysregulated signalling by the Met RTK leads to enhanced cell migration and the metastatic spread of cancer cells (Bradley et al., 2017; Gherardi et al., 2012; Knight et al., 2013; Parachoniak and Park, 2012).

Invasive properties of Met are tightly regulated by spatial localization of signalling complexes on subcellular compartments, including dorsal ruffles, invadopodia, lamellipoda and endosomes (Abella et al., 2010b; Ménard et al., 2014; Palamidessi et al., 2008; Rajadurai et al., 2012). Each of these compartments possess distinct morphological and molecular features. While Met recruits many different effectors, not all complexes are assembled at each subcellular location where Met is active and additional determinants must define the localization of different signalling complexes. For example, the plasma membrane is the predominant source of $PI(4,5)P_2$ in the cell, however this lipid may be modified by PI3K to locally generate $PI(3,4,5)P_3$ (Whitman et al., 1988).

The small GTPase, Arf6, is critical for Met dependent invasive growth, although, the molecular mechanisms that links Met to Arf6 activation in cancer cells is unknown (Tushir and D'Souza-Schorey, 2007). The Arf small GTPases are members of a superfamily of molecular switches that mediate changes in cell morphology, endomembrane traffic, and cell signalling (Gillingham and Munro, 2007; Simanshu et al., 2017). Arf6 is unique amongst Arf proteins in that it localizes primarily to the plasma membrane and endosomes, as opposed to Arf1 and Arf3 which localize predominantly to the Golgi apparatus (Donaldson and Jackson, 2011). Active GTP bound Arf6 modulates processes that are critical for cell migration and tumour metastasis (Muralidharan-Chari et al., 2009; Yoo et al., 2016). These include endosomal recycling of the Met RTK or integrin receptors, clathrin-independent endocytosis and rearrangement of the actin cytoskeleton (Eyster et al., 2009; Parachoniak et al., 2011; Powelka et al., 2004; Ratcliffe et al., 2016). Arf6 cycles between an “off” GDP-bound state and “on" GTP-bound state. Cycling between these states is enhanced by GEFs, which promote GDP release, and GAPs, that promote hydrolysis of GTP (Donaldson and Jackson, 2011; Simanshu et al., 2017). Subcellular localization of GEF and GAP proteins promotes recruitment and localized activity of small GTPases, however, the in vivo subcellular determinants for Arf6 activation remain to be fully defined.

Arf GEFs fall into 7 families and 3 of these encompass putative Arf6 GEFs. These families include Cytohesin (1-4), IQSEC (1-3) and PSD (1-4) (Casanova, 2007; Donaldson and Jackson, 2011). These families are defined by the presence of a Sec7 domain that enhances the release of GDP from Arf proteins. In addition, there are multiple splice variants of Arf GEFs. Some differ by an entire domain whereas others involve microexons (Fukaya et al., 2016; Ogasawara et al., 2000). The best characterized microexon splice variants are two isoforms of Cytohesin-2. These isoforms differ by a 3 nucleotide microexon, whose splicing leads to an additional glycine residue within the Cytohesin-2 pleckstrin homology (PH) domain (Cronin et al., 2004) yet a functional difference for these has not been tested.

Microexons are a recently described class of exons that are ≤27 nucleotides in size and are predominantly found in structured regions of proteins. Microexons are frequently identified in brain derived transcripts, including cytohesin-2, and are alternatively regulated in individuals with

autism spectrum disorder (Irimia et al., 2014). Despite decades of research on signalling from receptor tyrosine kinases, such as Met, the role for multiple isoforms of specific signalling molecules is largely unexplored. Here we demonstrate differential functions for cytohesin-1 isoforms whereby a splice variant of cytohesin-1 that lacks a 3 nucleotide microexon has distinct phospholipid binding and is uniquely required for HGF-dependent cell migration. We provide a mechanistic understanding into how microexon skipping controls HGF-dependent cell migration.

## 3.4 Results

### 3.4.1 Cytohesin-1 regulates HGF-dependent cell migration

Stimulation of epithelial and many cancer cells with HGF promotes activation of Met RTK and cellular morphological changes leading to enhanced cell migration. The invasive migratory program induced by Met RTK specifically requires the small GTPase Arf6, which in turn promotes rearrangement of the actin cytoskeleton (Supplemental Figure 3.1) yet how this is regulated by Met is unknown (Tushir and D'Souza-Schorey, 2007). To identify Arf GEFs required for HGF dependent cell migration, we first measured the expression of putative Arf GEFs, that are defined by the presence of a Sec7 domain (Donaldson and Jackson, 2011). We used HeLa cells which have been extensively studied for Met dependent migration and cell signalling (Frittoli et al., 2014; Palamidessi et al., 2008; Parachoniak et al., 2011). Out of 10 putative Arf GEFs, 6 were detectably expressed in HeLa cells, as assessed by qRT-PCR (Supplemental Figure 3.2A). These include Cytohesins 1,2 and 3; IQSEC 1 and 2; and PSD3.

To assess the effect of Arf GEF on cell migration, each Arf GEF was independently reduced by siRNA mediated silencing to $\leq 30\%$ (Supplemental Figure 3.2B) and cells were imaged in the presence and absence of HGF every 15 minutes for 24 hours. Cell speed was quantified between 16 and 24 hours post stimulation. Silencing cytohesin-1 and IQSEC2 reduced HGF-dependent cell migration, whereas silencing IQSEC1 (aka BRAG2) enhanced HGF-independent cell migration but no further increase of cell speed was observed following HGF treatment (Figure 3.1A). Enhanced cell speed in IQSEC1 silenced cells is likely due to enhanced integrin receptor surface levels (Dunphy et al., 2006; Moravec et al., 2012).

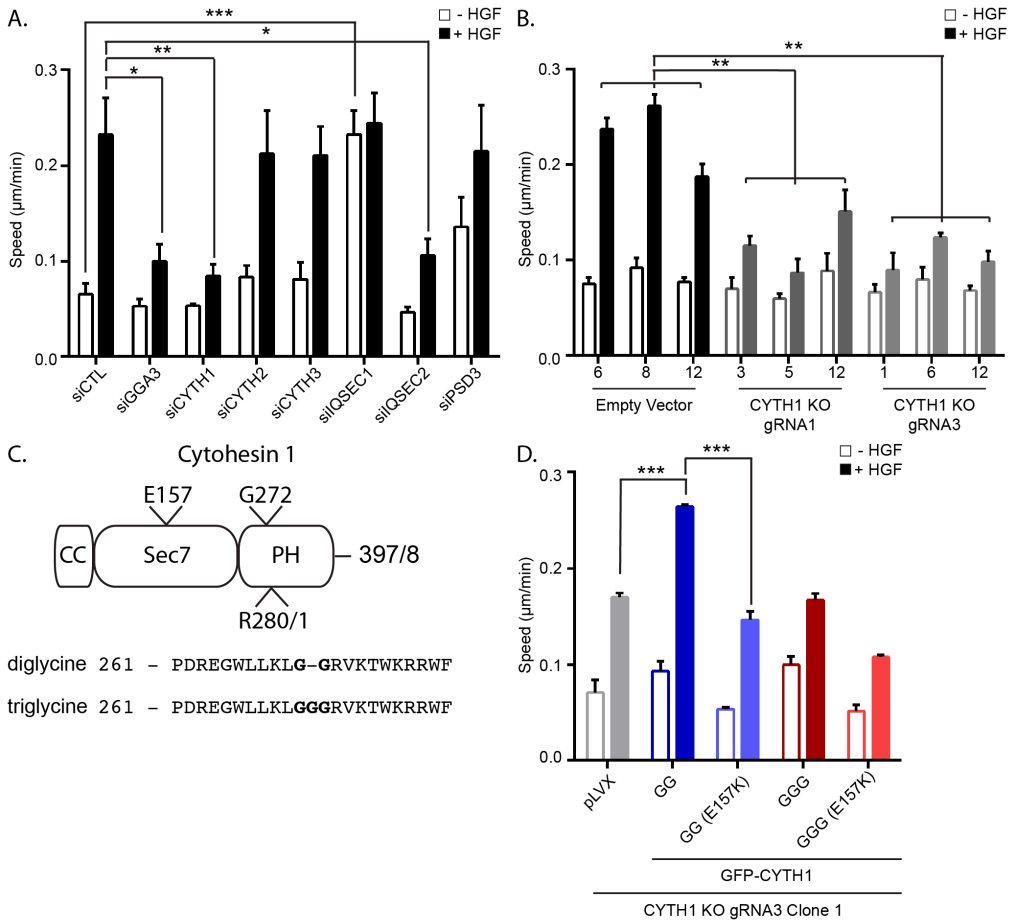



**Figure 3.1 Cytohesin-1 depletion reduces HGF-dependent cell migration.** (A) Random cell migration of HeLa cells treated with indicated siRNA smartpool and -/+ HGF. (B) Random cell migration of CYTH1 KO or lentiCRISPR v2 empty vector HeLa clones treated -/+ HGF. (C) Domain organization of CYTH1. (D) Random cell migration of pLVX empty vector or eGFP-CYTH1 variant expressing cells treated -/+ HGF. Error bars indicate standard error of the mean. * $p<0.05$ ** $p<0.01$ *** $p<0.001$.

IQSEC2 depleted cells appeared more spread and could readily be distinguished from control cells based on their morphology, cytohesin-1 depleted cells appeared morphologically indistinguishable from control cells but had diminished HGF induced cell migration. Hence, we focused our study on cytohesin-1. The decreased migratory phenotype observed by siRNA mediated depletion of cytohesin-1 was validated by generating HeLa cells with stable knock out (KO) of cytohesin-1 using the lentiCRISPR v2 system using 2 independent guide RNAs targeting exon 2 of CYTH1 (Supplemental Figure 3.2C). When HGF-dependent cell migration was compared in three control and CYTH1 KO clones, CYTH1 KO phenocopied siRNA mediated

silencing whereby all clones had reduced HGF-dependent cell speed compared to control clones (Figure 1B) confirming that cytohesin-1 regulates HGF-dependent cell migration.

Cytohesin-1 belongs to a family of 4 proteins (cytohesin-1,2 (aka ARNO), 3 (aka Grp1) and 4). These proteins consist of a coiled-coiled domain, a Sec7 domain, and a PH domain. The Sec7 domain has Arf GEF activity and the PH domain selectively recognizes phosphoinositides (Chardin et al., 1996). Two isoforms of Cytohesin-1 are expressed that differ by the inclusion of a evolutionarily conserved 3 nucleotide exon resulting in an additional glycine residue in the PH domain (Irimia et al., 2014; Ogasawara et al., 2000). We refer to these isoforms as the diglycine and triglycine variants (Figure 3.1C). To identify the splice variant that mediates HGF-dependent cell migration we generated a panel of stable cell lines expressing GFP-tagged isoforms or mutants of cytohesin-1 in the CYTH1 KO background (Supplemental Figure 3.2D). The diglycine GFP-CYTH1 but not triglycine GFP-CYTH1 was able to increase HGF-dependent cell migration compared to empty vector control (Figure 3.1D). This was dependent on the GDP exchange activity of the diglycine isoform as cells expressing a GFP-CYTH1 construct with a mutation of an essential glutamic acid (E157K) for exchange activity was unable to rescue HGF-dependent cell migration. Given these observations, we propose that the diglycine isoform of cytohesin-1 acts downstream from the Met receptor tyrosine kinase and is required for cancer cell migration.

### 3.4.2 Cytohesin-1 splice variants differentially mediate membrane ruffling

Cell migration requires the spatial coordination of multiple signals. Upon HGF stimulation, Met is rapidly internalized and a fraction of these receptors are recycled to the leading edge where Rac1 is active and induces rearrangement of the actin cytoskeleton (Ménard et al., 2014; Palamidessi et al., 2008; Parachoniak et al., 2011; Royal et al., 2000). Consistent with our data and previous reports, HGF induces a rearrangement in the actin cytoskeleton and peripheral actin ruffles (Figure 3.2A). In CYTH1 KO cells the percent of cells with HGF-induced peripheral actin ruffles was reduced (51% in CTL vs 23% in KO). This could be rescued by expression of diglycine GFP-CYTH1 (47%) but not the GEF exchange E157K mutant (22%) (Figure 3.2A, C).

Intriguingly, the majority of cells overexpressing the triglycine GFP-CYTH1 variant had peripheral actin ruffles (51%) in the absence of HGF-stimulation which were not increased

following HGF stimulation (Figure 3.2A, C). This suggests that while the triglycine variant was unable to rescue HGF-dependent cell migration, it is capable of promoting downstream signals that enhance membrane ruffling. Consistent with this, the GEF inactive E157K triglycine mutant did not promote peripheral actin ruffles (16%) (Figure 3.2A, C). These observations demonstrate a significant difference in cytohesin-1 isoform function and sensitivity to RTK stimulation.

To quantitatively assess the effect of HGF on plasma membrane dynamics we performed live-cell imaging in response to HGF stimulation and assessed the relative position of the plasma membrane every 15 secs between 15 mins and 60 mins post HGF stimulation. In control cells in response to HGF, lamellipodia are observed and a leading edge forms and moves forward with a velocity of 0.081 μm/min and maximum displacement of 5.74 μm when compared to 0.006 μm/min and 2.43 μm in the absence of HGF (Figure 3.2B, D, E). In contrast, in CYTH1 KO cells both the velocity and maximum displacement of membrane protrusions in response to HGF were reduced to 21.4% and 58% of control cells respectively (Figure 3.2B, D, E). Consistent with its ability to rescue cell migration, diglycine GFP-CYTH1 rescued membrane protrusion velocity (82% of control) and maximum displacement (104% of control). Rescue depended on the Arf GEF activity of diglycine Cytohesin-1 since cells expressing diglycine GFP-CYTH1 E157K failed to increase membrane velocity (35% of control) or maximum displacement (45% of control) relative to CYTH1 KO cells.

While there was no significant effect of the triglycine isoform on net membrane velocity, we observed an HGF-independent increase in the maximum displacement (193% of control), indicating that these cells were actively ruffling but failed to produce a stable leading edge (Figure 3.2B, D, E). This effect was also dependent on the Arf GEF activity of Cytohesin-1 as the E157K triglycine mutant did not increase the maximum displacement (Figure 3.2B, E). Together this demonstrates that the diglycine PH domain is required for HGF-dependent cell migration and establishment of a leading edge in a migrating cell and suggests that phosphoinositide recognition regulates these processes.

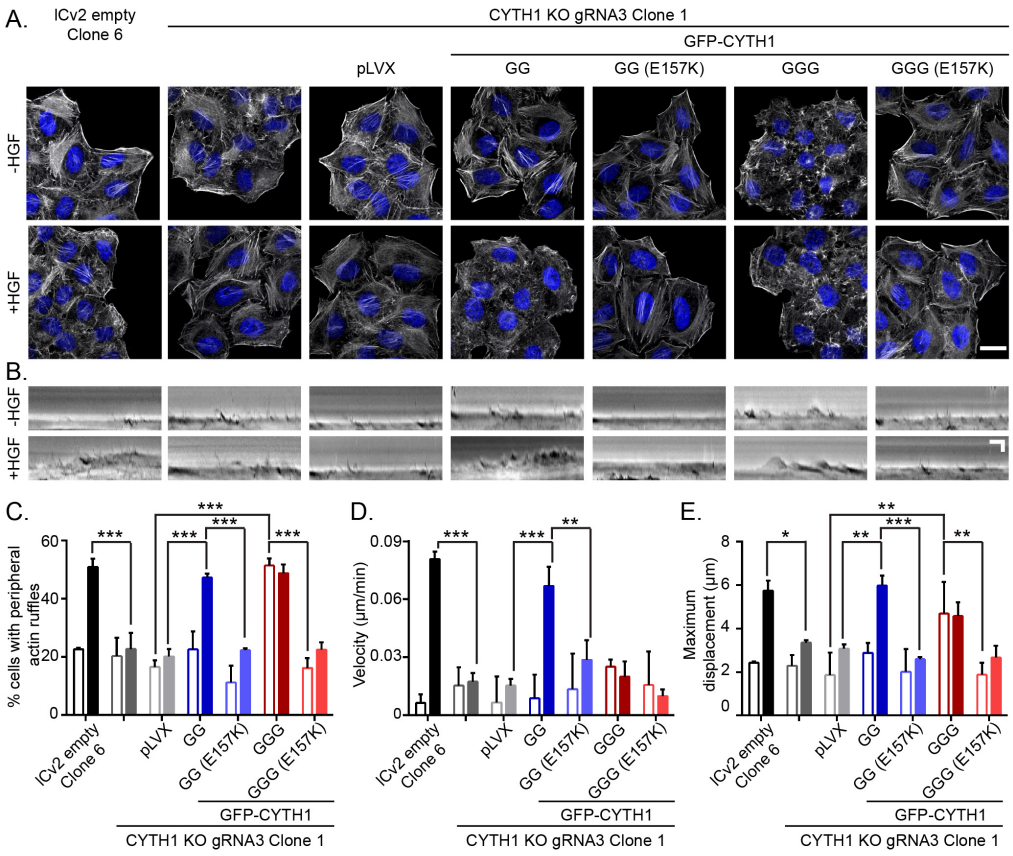



**Figure 3.2 Cytohesin-1 regulates membrane ruffling and actin cytoskeleton rearrangement.** (A) Confocal images of cells counter-stained with phalloidin (F-actin) and DAPI. -/+ HGF. (B) Kymographs were generated from linescans of the cells' leading edge imaged between 15 and 60 mins after HGF treatment. (C) Quantification of experiments (n=3) shown in (A). (D & E) Quantification of experiments (n=4) shown in (B). Scale bar, 20µm. Error bars indicate standard error of the mean. * $p<0.05$ ** $p<0.01$ *** $p<0.001$.

### 3.4.3 Cytohesin-1 variants differentially recognize phosphoinositide headgroups

The binding affinities of the PH domain of cytohesin-2 and cytohesin-3 to different inositol phosphate (IP) headgroups have been extensively characterized (Cronin et al., 2004; Klarlund et al., 2000). These studies have shown that the diglycine variant of cytohesin-2 has a significantly stronger affinity (14-fold) for I(1,3,4,5)$P_4$ relative to I(1,4,5)$P_3$ (Cronin et al., 2004), whereas the triglycine variant is less selective, binding to both I(1,3,4,5)$P_4$ and I(1,4,5)$P_3$ with similar affinities. To characterize the specificity of cytohesin-1 for I(1,3,4,5)$P_4$ and I(1,4,5)$P_3$ we performed isothermal titration calorimetry (ITC) using recombinant cytohesin-1 PH domain variants. We found that the diglycine cytohesin-1 PH domain bound to I(1,3,4,5)$P_4$ with a $K_d$ of 0.033 µM and I(1,4,5)$P_3$ with a $K_d$ of 21.05 µM (Figure 3.3A,B). This indicates that cythohesin-1 has a 640-fold

A.

| | I(1,4,5)P3 $K_d$ (μM) | I(1,3,4,5)P4 $K_d$ (μM) | Selectivity $K_{d\ IP4}$ / $K_{d\ IP3}$ |
|---|---|---|---|
| GG | 21.05 ± 0.33 | 0.033 ± 0.0003 | 640 |
| GGG | 7.23 ± 0.27 | 3.03 ± 0.15 | 2.4 |
| GG R280C | NB | NB | — |

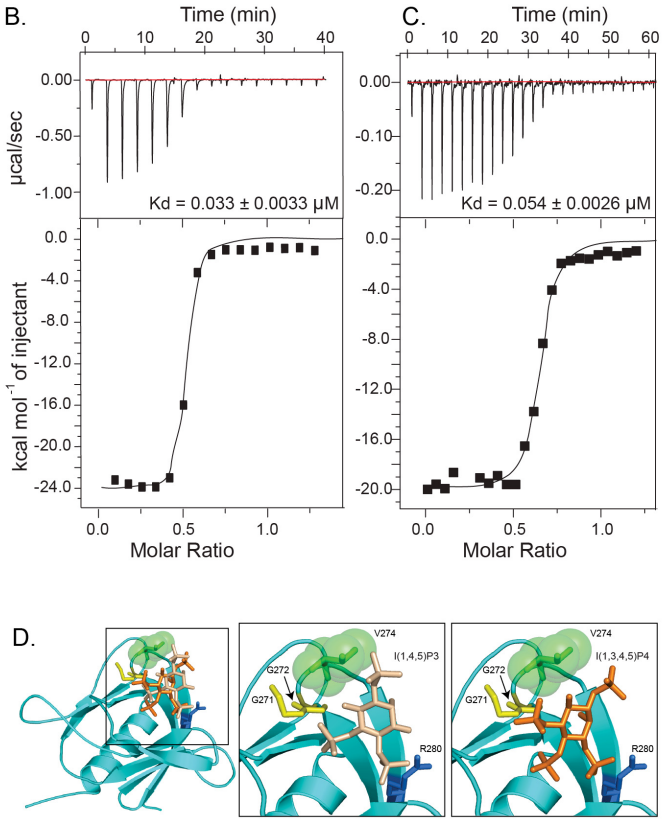



**Figure 3.3 G272 defines the phosphoinositide binding selectivity of Cytohesin-1.** (A) $K_d$ values measured by isothermal titration calorimetry. (B) Isothermal titration calorimetry trace of I(1,3,4,5)$P_4$ titrated into diglcyine CYTH1 PH domain. (C) Isothermal titration calorimetry trace of diglycine CYTH1 PH domain (a.a. 243-397) titrated into PI(3,4,5)P3 containing liposomes. (D) Molecular model of the diglycine CYTH1 PH domain bound to I(1,3,4,5)$P_4$ or I(1,4,5)$P_3$

greater affinity for I(1,3,4,5)$P_4$ over I(1,4,5)$P_3$. The triglycine variant of cytohesin-1 PH domain binds to I(1,3,4,5)$P_4$ with an affinity of 3.03 μM (~100 fold lower than diglycine) and I(1,4,5)$P_3$ with an affinity of 7.23 μM (~3-fold higher than diglcyine) (Figure 3.3). These results support that the diglycine variant of cytohesin-1 differentially interacts with PI(3,4,5)$P_3$ on membranes. To test this, we titrated PI(3,4,5)$P_3$-containing liposomes with the diglycine cytohesin-1 PH domain containing the C-terminal polybasic region and found that it bound with a comparable affinity (Kd

= 0.054 μM) to the head group alone (Figure 3.3C), confirming the ability of the diglycine PH domain to specifically recognize $PI(3,4,5)P_3$ in the context of a lipid membrane.

To gain further insight into the binding properties of the diglycine variant of cytohesin-1, we generated a homology model of its PH domain (Figure 3.3D). Since there is a ~90% sequence identity between the PH domains of cytohesin family members, we anticipate that the phosphoinositide binding pocket would be conserved. Based on previous studies, we predict that Arg280 forms contacts with the 3' phosphate of $I(1,3,4,5)P_4$ or 4' phosphate of $I(1,4,5)P_3$ and this site is required for a detectable interaction. Consistent with this model, an R280C mutation when introduced into diglycine Cytohesin-1 PH domain abrogated any interaction with $I(1,3,4,5)P_4$ or $I(1,4,5)P_3$ (Figure 3.3A). Together, these data indicate that the diglycine variant of cytohesin-1 specifically recognizes $PI(3,4,5)P_3$ whereas the triglycine variant may bind both $PI(4,5)P_2$ with slightly higher affinity and $PI(3,4,5)P_3$ with lower affinity.

### 3.4.4 Phosphoinositide binding of CYTH1 regulates membrane ruffling and cell migration

To test directly whether phosphoinositide recognition of cytohesin-1 is required for the formation of cell protrusions and HGF-dependent cell migration, we compared CYTH1 KO cells expressing WT diglycine GFP-CYTH1 or the R280C mutant. In response to HGF, cells expressing the diglycine GFP-CYTH1 (R280C) had a reduced capacity to form peripheral actin ruffles in response to HGF when compared to cells expressing a WT diglycine GFP-CYTH1 (Figure 3.4A,C). The velocity and maximum displacement of the leading edge was also reduced in cells expressing the R280C diglycine mutant when compared to WT GFP-CYTH1 (23% and 39% of WT respectively) (Figure 3.4B,D,E). Consistent with this, HGF-dependent cell migration was reduced in cells expressing the diglycine GFP-CYTH1 (R280C) when compared to WT (47% of control) (Figure 3.4F) supporting that phosphoinositide engagement by diglycine cytohesin-1 is required for HGF-dependent cell migration.

To establish if phosphoinositide binding was required for the constitutive membrane ruffling induced by overexpression of the triglycine variant, CYTH1 KO cells expressing WT triglycine GFP-CYTH1 or R281C (equivalent to diglycine R280C) were examined. Cells expressing the R281C mutant showed significantly less peripheral actin ruffles compared to WT

(Figure 3.4G, I). When comparing the membrane dynamics of these cells, cells expressing triglycine GFP-CYTH1 R281C demonstrated reduced maximum displacement when compared to WT (33.5% of WT trigylcyine) (Figure 3.4H, J, K). Hence, phosphoinositide recognition is a required step for both diglycine cytohesin-1 dependent membrane ruffling in response to HGF and constitutive membrane ruffling promoted by overexpression of the triglycine Cytohesin-1.

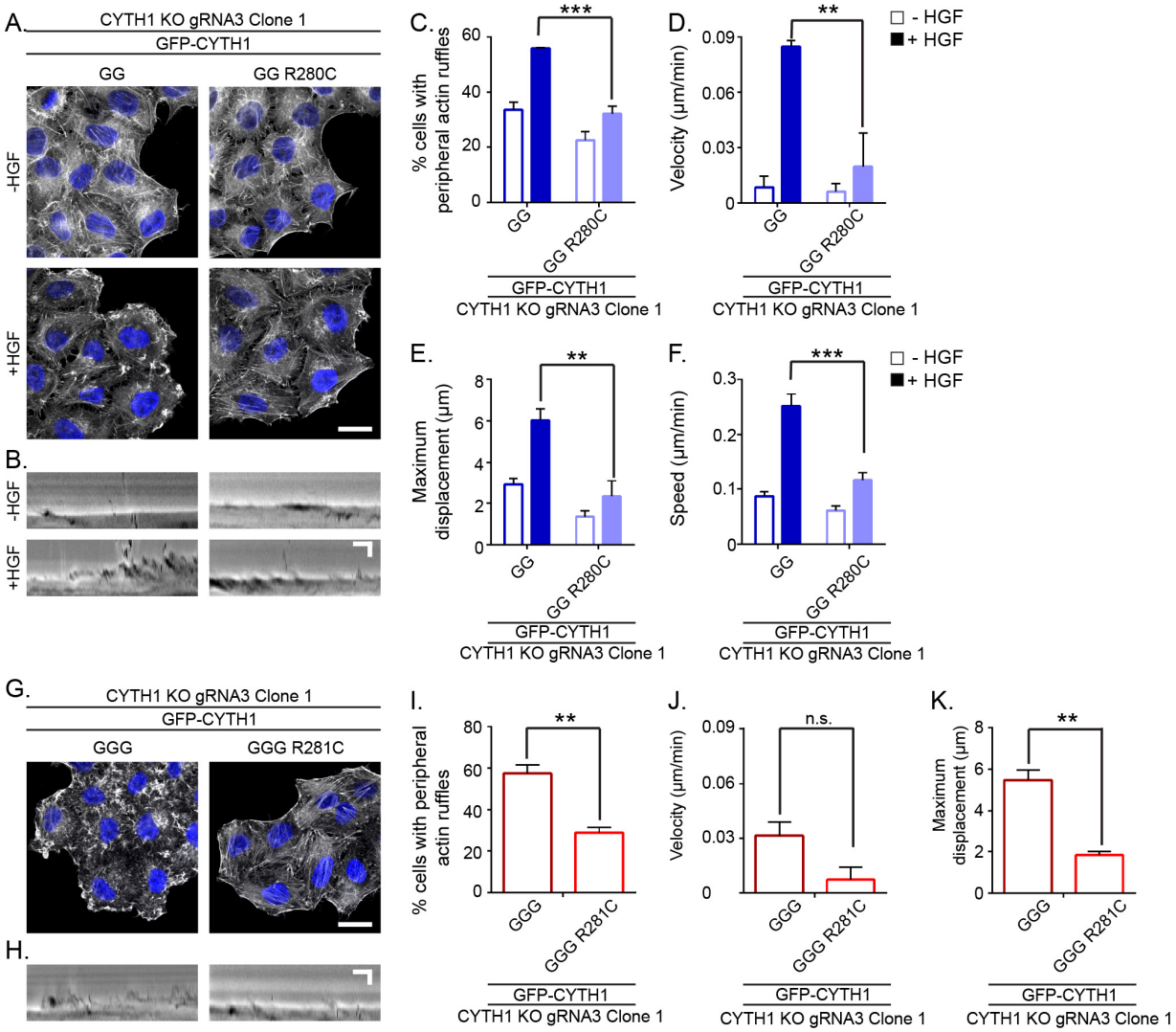



**Figure 3.4 Cytohesin-1 phosphinositide binding is required for membrane ruffling and HGF-dependent cell migration.** (A) Confocal images of cells counter-stained with phalloidin (F-actin) and DAPI. -/+ HGF. (B) Kymographs were generated from linescans of the cells' leading edge imaged between 15 and 60 mins after HGF treatment. (C) Quantification of experiments (n=3) shown in (A). (D & E) Quantification of experiments (n=3) shown in (B). (F) Random cell migration of diglycine eGFP-CYTH1 or R280C ovexpressing cells treated -/+ HGF (n=3). (G) Confocal images of cells counter-stained with phalloidin (F-actin) and DAPI. -/+ HGF (H) Kymographs were generated from linescans of the cells' leading edge imaged between 15 and 60 mins after HGF treatment. (I) Quantification of experiments (n=3) shown in (G). (J & K)

Quantification of experiments (n=3) shown in (H). Scale bar, 20μm. Error bars indicate standard error of the mean. ** $p<0.01$ *** $p<0.001$.

### 3.4.5 Selective membrane recruitment of Cytohesin-1 splice variants

The abundance of $PI(4,5)P_2$ at the plasma membrane is approximately two orders of magnitude higher than $PI(3,4,5)P_3$ (Stephens et al., 1991). However, HGF stimulation activates PI3K and enhances recruitment of $PI(3,4,5)P_3$ binding proteins (Maroun et al., 1999a). Hence to test if the diglycine variant of cytohesin-1 is specifically recruited to the plasma membrane upon PI3K activation we imaged membrane bound cytohesin-1 by partially permeabilizing cells stably expressing GFP-CYTH1 splice variants (Supplemental Figure 3.2D) with 0.05% saponin and allowing for cytosolic GFP-CYTH1 to dissipate. In response to HGF, diglycine GFP-CYTH1 localized to the plasma membrane within 3 mins and recruitment was stable for up to 60 mins (Figure 3.5A). We also observed that diglycine GFP-CYTH1 was polarized towards the leading edge of the cell up HGF stimulation. Importantly, mutation of the phosphoinositide binding pocket (R280C) abrogates recruitment of diglycine GFP-CYTH1 to the leading edge (Figure 3.5B). Together these data are consistent with an HGF-dependent rapid recruitment of PI3K to a Met signalling complex at the plasma membrane and generation of $PI(3,4,5)P_3$ at the leading edge of migrating cells (Abella et al., 2010b; Frigault et al., 2008; Maroun et al., 1999a; 1999b; Parachoniak et al., 2011).

In contrast, the triglycine GFP-CYTH1 variant is constitutively associated with the plasma membrane and is observed throughout the cell perimeter (Figure 3.5B). Recruitment was not further enhanced by HGF treatment supporting a distinct mechanism of membrane recruitment. We previously found that the triglycine variant of cytohesin-1 could bind $PI(3,4,5)P_3$ and $PI(4,5)P_2$ headgroups with low μM affinities. Therefore, we tested whether phosphoinositide recognition was required for membrane recruitment. In contrast to triglycine GFP-CYTH1 expressing cells, GFP-CYTH1 (R281C) was absent from the periphery of the cell (Figure 3.5C), showing that membrane recruitment of triglycine cytohesin-1 is still phosphoinositide dependent but independent of HGF.

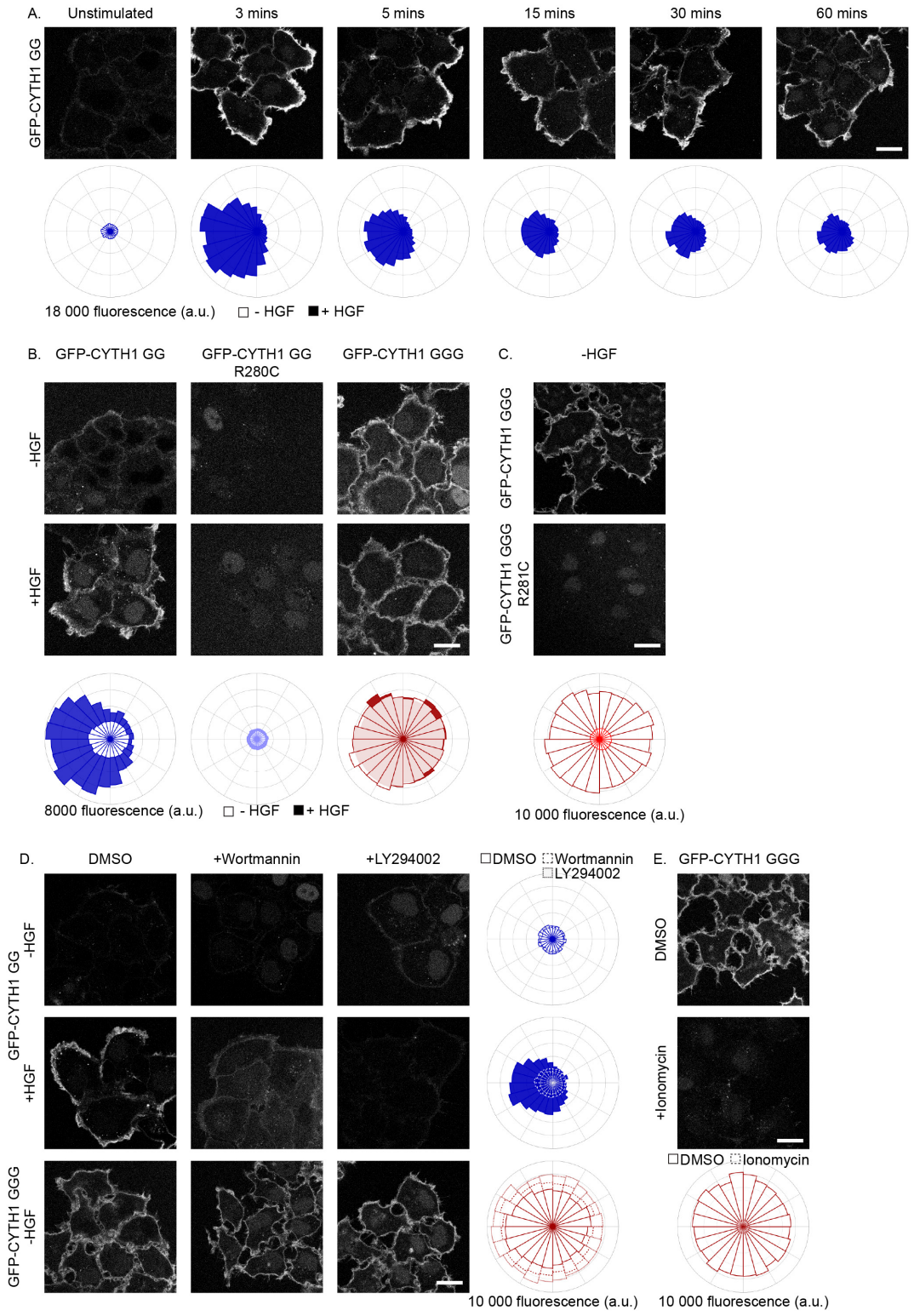



**Figure 3.5 Cytohesin-1 localization is defined by G272.** (A) HeLa cells stably expressing eGFP-tagged diglycine CYTH1 were either untreated or treated with HGF or EGF for the indicated time points, permeabilized with ice-cold 0.05% saponin in PIPES buffer and imaged by confocal microscopy (B)

**Figure 3.5 (continued)** HeLa cells stably expressing eGFP-tagged variants of CYTH1 were either untreated or treated with HGF for 15 mins, permeabilized with ice-cold 0.05% saponin in PIPES buffer and imaged by confocal microscopy. (C & D) Cells were prepared as in (A) except they were pretreated with the indicated inhibitors for 30 mins prior to HGF stimulation. (E) (B) HeLa cells stably expressing eGFP-tagged variants of CYTH1 were either untreated or treated with HGF for 15 mins, permeabilized with ice-cold 0.05% saponin in PIPES buffer and imaged by confocal microscopy. Scale bar, 20μm.

To test whether plasma membrane recruitment of cytohesin-1 was dependent on PI3K activity, cells expressing the diglycine or triglycine GFP-CYTH1 were pretreated with pan-PI3K inhibitors Wortmannin and LY294002 and localization of GFP-CYTH1 was assessed. Pretreatment with both inhibitors abrogated HGF-dependent membrane recruitment of diglycine GFP-CYTH1 (Figure 3.5D). In contrast, the localization of the triglycine GFP-CYTH1 to the plasma membrane was not significantly altered following treatment of cells with PI3K inhibitors (Figure 3.5D). Together these data support that the diglycine cytohesin-1 is specifically recruited to the plasma membrane following generation of by $PI(3,4,5)P_3$, whereas the triglycine requires a distinct signal.

Finally, we pretreated cells with ionomycin to reduce plasma membrane levels of $PI(4,5)P_2$ and assesed the localization of the triglycine GFP-CYTH1 (Botelho et al., 2000). Ionomycin pretreatment abolished peripheral localization of triglycine GFP-CYTH1 (Figure 5E). Together these data support that cytohesin-1 splice variants are differentially recruited to the plasma membrane *in vivo*. Whereas the diglycine variant binds $PI(3,4,5)P_3$ generated downstream from growth factor signalling, the triglycine variant is constitutively recruited to the plasma membrane in a $PI(4,5)P_2$ dependent manner. Therefore, the phosphoinositide binding specificity of microexon containing splice variants of CYTH1 defines the context for membrane ruffling and cell migration.

## 3.5 Discussion

Initiation of cellular signalling through activation of receptor tyrosine kinases is well recognized as a key event in cellular mitogenic or morphogenic response to growth factors. However, the cooperating molecular determinants of signal localization are still poorly understood. Here, we identify a role for microexon splicing of cytohesin-1 in regulating cell migration in response to Met RTK signalling. We demonstrate that a diglycine but not triglycine microexon

splice variant of Cytohesin-1 is required for Met RTK-dependent actin rearrangement, membrane ruffling and cell migration using multiple experimental strategies. We demonstrate that while both diglycine and triglycine microexon derived isoforms of cytohesin-1 are functionally active, the specific recognition of $PI(3,4,5)P_3$ by diglycine cytohesin-1 mediates polarized recruitment to lamellipodia as well as to the developing leading edge and is required for cell migration in response to activation of the Met RTK. In contrast, whereas the triglycine cytohesin-1 isoform is constitutively recruited to the plasma membrane in a $PI(4,5)P_2$ dependent manner, and although it promotes membrane ruffling and rearrangement of the actin cytoskeleton this activity is dispensable for cell migration in response to Met activation. Our data demonstrate that the phosphoinositide binding specificity of microexon containing splice variants of CYTH1 specifies their subcellular localisation and function in Met RTK signalling, regulating cell migration and raises the importance of developing functional understanding of microexons in health and disease.

In addition to cytohesin-1, our initial screen identified functions for two members of the BRAG/IQSEC family of Arf GEFs. These proteins possess a calmodulin-binding IQ motif, as well as a Sec7 and PH domain. IQSEC1 directly interacts with phosphorylated EGFR to promote invasion of breast cancer cells, as well VEGFR2 to regulate binding to its co-receptor Nrp1 (Morishige et al., 2007; Zhu et al., 2017). We noticed an increase in cell migration upon IQSEC1 silencing, consistent with IQSEC1 promoting β1 integrin endocytosis through Arf5 (Moravec et al., 2012). Silencing IQSEC2 also altered HeLa cell morphology independent of HGF stimulation. IQSEC2 loss of function mutations have been found in patients with X-linked intellectual disability and silencing IQSEC2 alters gross morphology of neurons (Hinze et al., 2017; Shoubridge et al., 2010). Therefore, these two proteins may have a functional role beyond growth factor signalling.

Cytohesin family members have been implicated in multiple processes that involve membrane ruffling, including, bacterial invasion (Humphreys et al., 2016); phagocytosis and migration in Dictyostelium discodium (Müller et al., 2013); adhesion of lymphoid cells (azreq and Bourgoin, 2011); and kidney repair following acute injury (Reviriego-Mendoza and Santy, 2015). We and others have shown that isoforms of cytohesin family members interact selectively with $PI(4,5)P_2$ or $PI(3,4,5)P_3$ (Cronin et al., 2004; Klarlund et al., 2000). These data suggest that cytohesin family members may act downstream of several receptor tyrosine kinases or other signals that regulate

$PI(4,5)P_2$ or $PI(3,4,5)P_3$ membrane levels. Indeed, cytohesin-1 was shown to be transiently recruited to the plasma membrane upon EGF stimulation and this was dependent on PI3K activity and the PH domain of Cytohesin-1, (Venkateswarlu et al., 1999). In endothelial cells, cytohesin-3 but not 2 was proposed to act downstream from Met to promote integrin recycling and angiogenesis (Hongu et al., 2015). A distinction between microexon splice variants was not addressed in either study. In HeLa cells silencing of cytohesin-2 or 3 differentially affects β1 integrin trafficking and cell adhesion (Oh and Santy, 2010). This is presumably due to differential expression of cytohesin-2 and 3 splice variants since overexpression of diglycine cytohesin-2 or 3 reduced cell adhesion, whereas triglycine cytohesin-2 and 3 increased cell adhesion (Oh and Santy, 2012). Therefore, understanding the functional impact of microexon splice variants of cytohesin family members will be essential to interpreting their role in different biological processes.

Our study established diglycine cytohesin-1 as a molecular link between Met activation, PI3K signalling, Arf6 and Met biology. HGF-dependent recruitment of diglycine cytohesin-1 to the leading edge mirrors activation of PI3K supporting our model that cytohesin-1 is an important PI3K effector (Maroun et al., 1999a). Our previous findings have also shown that Met engages with the Arf6 effector GGA3 to mediate recycling through the endolysosomal network (Parachoniak et al., 2011). However, CYTH1 KO does not affect Met stability suggesting that Met could interact with Arf6 at multiple steps of the endolysosomal network (data not shown). The Gab1 scaffold is the major determinant for recruitment of the p85 adapter protein and PI3K activation following HGF stimulation of Met (Maroun et al., 1999a). Gab1 localizes to the Met RTK and is stabilised through an interaction with $PI(3,4,5)P_3$ rich membrane domains through its PH domain and acts to amplify PI3K signalling in these microdomains following HGF stimulation (Abella et al., 2010a; 2010b; Maroun et al., 1999b; Paliouras et al., 2009). While the role for PI3K in cell migration has been examined extensively, many studies have focused on the role for the lipid product $PI(3,4,5)P_3$ on activation of the serine/threonine kinase Akt (Fruman et al., 2017). It is now understood that pathways other than Akt may predominantly modulate cell migration and other PI3K dependent biological responses are not well understood (Lien et al., 2017). Arf6 also acts upstream from the Rac1 GEF, DOCK 180, to regulate cell migration (Koubek and Santy, 2017; Santy et al., 2005). $PI(3,4,5)P_3$ is also recognized by multiple GEFs, including DOCK180, Vav2 and P-REX1, that activate Rac to promote cell migration (Côté et al., 2005; Graziano et al.,

2017; Ménard et al., 2014). Therefore, understanding how multiple signals coordinately feed into Rac activation to promote cell migration will be important for further study.

While Arf proteins are downstream substrates for Sec7 domain containing GEFs, Arf proteins along with phospholipids may also act upstream of cytohesins to promote their recruitment to the membrane (Cohen et al., 2007; Karandur et al., 2017; Malaby et al., 2013; Stalder et al., 2011). Therefore, positive feedback loops may exist that promote further recruitment of Cytohesin family members (Stalder and Antonny, 2013). Indeed, Arf proteins directly recruit PI4P5K to generate additional $PI(4,5)P_2$ at the plasma membrane which could in turn recruit additional triglycine Cytohesin-1. Arf6 also relieves an autoinhibitory interaction between the Sec7 and PH domains of Cytohesin-1 to promote membrane recruitment of Cytohesin-1 in vitro and overexpression of dominant active Arf6 enhances Cytohesin-2 membrane recruitment in vivo. However, we observed efficient recruitment of GFP-Cytohesin-1 E157K mutants to the plasma membrane that phenocopied their WT counterparts (data not shown). While Arf proteins may cooperate with phosphoinositides to relieve autoinhibition and stimulate exchange activity (Malaby et al., 2018), GEF activity of cytohesin-1 is not required for its recruitment to the plasma membrane.

Alternative splicing diversifies the number of possible transcripts from a single gene. Indeed, it is believed that ~95% of multiexon genes undergo alternative splicing (Pan et al., 2008). While significant effort has been put into establishing the regulatory mechanisms of alternative splicing, the functional significance of many of these events remain unknown. Microexons have been reported in both plants and metazoan. A single nucleotide microexon in the Arabidopsis thaliana gene APC11 is essential to maintain the reading frame (Guo and Liu, 2015). Two neuronal splice variants of the non-receptor tyrosine kinase Src contain micrexon insertions in the SH3 domain (Brugge et al., 1985; Pyper and Bolen, 1990). These splice variants are highly autophosphorylated compared to c-Src and N-2 Src loss was observed in tumour tissue of a small cohort of symptomatic neuroblastoma patients (Keenan et al., 2015; Matsunaga et al., 1993). Comprehensive identification of microexons has recently been performed (Irimia et al., 2014; Li et al., 2015b). These were found to be important for neurological function and are altered in individuals with autism spectrum disorders (Irimia et al., 2014; Quesnel-Vallières et al., 2016; 2015). Data from brain tissue indicated that triglycine, $PI(4,5)P_2$ binding, cytohesin-1 was the

predominant isoform, however recent analysis of RNA sequencing data from a variety of tissues indicates that "percent spliced in" values for the cytohesin-1 microexon can vary from 25% in liver and epithelial cells (predominantly diglycine); to >95% in muscle and white blood cells (predominantly triglycine) (Irimia et al., 2014; Ogasawara et al., 2000). Thus, implicating differential regulation of microexon splicing in different tissues. These values may reflect a need for constitutive versus growth factor dependent activation of Arf proteins. To our knowledge, our study is the first to characterize a functional difference between cytohesin-1 splice variants. This points to a key role for these proteins and their splicing in normal development and disease.

Our results establish cytohesin-1 as a molecular link between the phosphoinositides $PI(4,5)P_2$ and $PI(3,4,5)P_3$, Arf6 and the actin cytoskeleton. Localized signalling from receptor tyrosine kinases, such as Met, has emerged as a determinant of morphogenic stimuli that promotes rearrangement of the actin cytoskeleton and directed cell migration. Here we have established a function for the evolutionarily conserved alternatively spliced microexon in cytohesin-1. The in vivo relevance of microexons is only beginning to be understood and may have wide ranging implications from normal development, neurological disease and cancer.

## 3.6 Acknowledgments

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## 3.7 Experimental Procedures

### Experimental Models

HeLa and 293T cell lines were cultured under standard conditions at 37°C and 5% $CO_2$ in 10% Fetal Bovine Serum. CYTH1 KO clonal lines were generated using the lentiCRISPRv2 system(Sanjana et al., 2014). Briefly, phosphorylated and annealed CYTH1 specific guide RNAs

were cloned into the lentiCRISPRv2 vector using BsmBI restriction sites. Lentiviral particles were produced by Lipo2000 transfection of 293T cells with eGFP-CYTH1, psPAX2 and pMD2.G vectors. Filtered supernatant was then used to infect HeLa cells and 24 hours after infection cells were selected in puromycin for 2 days. Clonal populations were established by limiting dilution and screened for Cytohesin-1 expression by western blot. To establish stable cell lines expressing eGFP-CYTH1 diglycine and triglycine isoforms, as well as mutants, triglycine eGFP-CYTH1 was first PCR amplified and subcloned into pLVX-IRES-Hyg vector. NEB Q5 site directed mutagenesis was used to generate PAM motif silent mutations, the diglycine isoform, as well as Cytohesin-1 mutants. Lentiviral particles were produced by Lipo2000 transfection of eGFP-CYTH1, psPAX2 and pMD2.G vectors into 293T lines. Filtered supernatant containing lentiviral particles were then concentrated by adding 1 volume PEG8000 to 3 volumes of supernatant, overnight incubation at 4°C, centrifuging at 2750 xg for 30 mins at 4°C and resuspending the pellet in DMEM. HeLa clonal population 1 expressing lentiCRISPRv2 gRNA3 was infected and 2 days later stable cell lines were selected and cultured in 10% FBS in DMEM and 600 μg/mL Hygromycin.

**Live cell imaging**

Images were captured with a Axiovert 200 M inverted microscope (Carl Zeiss, Inc.), LD A-Plan 20×/0.3 Ph1 objective lens, AxioCam HRM (Carl Zeiss, Inc.) and digital camera; all contained within a transparent environment chamber Climabox (Carl Zeiss, Inc.) maintained at 5% (v/v) $CO_2$ at 37°C. The microscope was driven by AXIOVISION LE software (Carl Zeiss, Inc.).

**Cell Migration**

Wells of a 24-well dish were coated with 25 μg/mL collagen for 1 hour at 37°C and washed 2x with PBS. 7500 cells were plated in collagen coated wells and, where indicated, immediately transfected with 20nM siRNA using HiPerfect as per manufacturer's instructions. Assays were performed 24-48 hours after plating. Media was aspirated and replaced with growth media or growth media containing 0.5nM HGF. The dish was then transferred to a Axiovert 200 M inverted microscope. The motorized stage advanced to pre-programmed locations and images were captured every 15 minutes for 24 hours. Three independent fields of view were then captured per condition and 10 cells were tracked per field of view. Tracks between 16 and 24 hours post

stimulation were used for quantification. Cells were tracked using the track points application in MetaMorph (Molecular Devices).

**Kymograph analysis**

24 hours after plating 7500 cells on collagen coated 24 well dish, cells were rinsed twice with 0.02% FBS in DMEM and 0.9mL 0.02% FBS in DMEM was added. Cells were replaced in incubator overnight. The following morning, 0.1mL 0.02% FBS in DMEM or 0.5nM HGF in 0.02% FBS in DMEM was added to each well. To analyze membrane dynamics the dish was transferred to a Axiovert 200 M inverted microscope. The motorized stage advanced to pre-programmed locations and 15 minutes after HGF addition, images were captured every 15 secs for 1 hour. Images generated between 15 and 60 mins after HGF stimulation were used to generated kymographs using the kymograph function in MetaMorph.

**Analysis of actin rearrangement**

For analysis of the actin cytoskeleton, cells were prepared the same as for kymograph analysis except 1 hour after HGF addition, the cells were fixed with 4% PFA. Cover slips were then processed and counter stained with Alexa546 labelled Phalloidin and DAPI. Images were acquired using a 63X, 1.4 NA immersion objective on a LSM 800 laser scanning confocal microscope (ZEISS). Ten independent fields of view were chosen per condition and these were then manually scored for ruffling. >100 cells per condition were scored.

**PH domain purification**

All recombinant proteins were expressed using Rosetta-2(DE3) E. coli cells (EMD Biosciences) and purified using Glutathione Sepharose 4B resin (GE Healthcare). Bacterial cultures were grown at 37°C to an OD600 of 0.7-08 and then induced using 1mM IPTG and incubated overnight at 25°C. The cells were harvested using a JLA-10.1 rotor at 6000 rcf. The cell pellets were resuspended in Buffer A (50 mM Na/K phosphate buffer; pH 8.0, 150 mM NaCl, 0.1% β-mercaptoethanol, 1x protease inhibitor cocktail (Roche)). 200 μg/mL Lysozyme and 10 units/mL DNAse1 was added to the cells, and after 30 mins incubation on ice, samples were lysed by sonication, followed by addition of 0.5% Triton X-100. Lysates were then cleared by centrifugation at 4 °C (JA-20, 35 mins at 35000 rcf) and using a 0.45uM filter. Glutathione sepharose resin was added to the soluble fraction and incubated at 4°C for 1 hours, then washed

3x with Buffer A. GST-tagged PH domains were then eluted with 50mM reduced glutathione in buffer A and 50 units of Precission Protease (GE Healthcare) was added to the supernatant and the sample was dialyzed using a 3500 MWCO membrane (Spectrum labs) against Buffer B (25 mM Tris, 100 mM NaCl, 1 mM DTT, 1mM EDTA, pH 7.3) overnight at 4°C. The sample was then incubated with Glutathion sepharose resin for 1 hour to remove cleaved GST, followed by separation of the resin and concentration of the sample using an Amicon 10K unit (Millipore). The concentrated sample was then applied to a Sephadex S75 column (GE Healthacare) using MOPS (100 mM NaCl, pH 7.5) as the running buffer. Sample purity was assessed by SDS PAGE and the purest fractions were pooled for further experiments (Supplemental Figure 3.3). The concentration of the sample was calculated using BioRad Bradford Protein Assay.

**Isothermal Titration Calorimetry (ITC)**

All ITC experiments were performed on a MicroCal microcalorimetry system (GE Healthcare). PH domains, PIP liposomes (Echelon Bioscience), and inositol phosphates (Echelon Biosciences) were prepared in 1 x MOPS. To measure binding kinetics to liposososmes, the reaction cell contained 17.5 μM of PI(3,4,5)P3 containing liposomes and was titrated with 155 μM of recombinant CYTH1. To measure binding kinetics to inositol phosphates, the reaction cell was filled with 25 μM CYTH recombinant protein and the sample was titrated with either Ins(1,3,4,5)P4 or Ins(1,4,5)P3 (Echelon Biosciences). All experiments were performed at 20°C, the reaction cell contained 320 μL of sample, and 70 μL of the titrant in the syringe which was set between 19-38 injections injections at 2.5-1.25 μL per injection. The binding isotherm was fitted with a model that uses a single set of independent sites to determine the stoichiometry and thermodynamic binding constant.

**Modeling of CYTH1 PH domain**

The three dimensional protein structural model of the diglycine CYTH1 PH domain was generated using the methods as described on Swiss-Model (https://swissmodel.expasy.org/) ((Arnold et al., 2006)). After target and template selection by the software, the final CYTH1 model was built against the structure of the PH domain from Cytohesin-3 (PDB: 2R09; 2R0D). The ligand present in the template structure was transferred by homology to the model. Additional analysis of the binding pocket with the I(1,4,5)P3 and I(1,3,4,5)P4 ligands was performed by overlaying the model structure to the PH domains from ARNO (PDB: 1U27; 1U29).

**Imaging subcellular localization of eGFP-Cytohesin-1**

Assays were performed 48 hours after plating 7.5 x $10^4$ cells in a ibidi glass bottom dish (81158). Media was aspirated and cells were rinsed twice with 0.02% FBS in DMEM and 1.35mL 0.02% FBS in DMEM was added. Cells were replaced in the incubator for 2-3 hours. A bolus of 5nM HGF was added to each plate ($C_f$ = 0.5nM). After the indicated time points media was aspirated and ice cold 0.05% saponin in piperazine-N, N'-bis(2-ethanesulfonic acid) (PIPES) buffer (80mM PIPES KOH pH 7.0, 5mM EGTA, 1mM $MgCl_2$) was added. Cells were imaged immediately after the cytosolic fraction had dissipated (~2-5mins). When cells were treated with DMSO or inhibitor, this was added 20 mins prior to HGF stimulation (Wortmannin and LY294002) or 10 mins prior to permeabilization (ionomycin). Images were acquired using a 63X, 1.4 NA immersion objective on a LSM 800 laser scanning confocal microscope (ZEISS).

Using MetaMorph, a linescan with a width of 10 pixels was then manually drawn along the perimeter of the cell, starting from the innermost point of the cell relative to the colony. Linescans were normalized and divided into 24 subsections. The mean fluorescence of subsections was averaged across cells and plotted using the polar histogram function in MATLAB.

**Biochemical Assays**

HeLa cells were lysed in Triton X-100-glycerol-Hepes (TGH) lysis buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 1% Triton X-100, 10% Glycerol, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium vanadate, 10 µg/mL aprotinin and 10 µg/mL leupeptin). Equal amounts of protein were aliquoted, SDS sample buffer was added and boiled for 5 min. Samples were then resolved by SDS-PAGE and transferred to Immobilon-FL PVDF transfer membranes. Membranes were blocked with LI-COR blocking buffer (LI-COR Biosciences), incubated with primary antibodies followed by incubation with infrared conjugated secondary antibodies prior to detection on the Odyssey IR Imaging System (LI-COR Biosciences).

**qRT-PCR**

Total RNA was isolated using QIAGEN AllPrep DNA/RNA mini kit as per manufacturer's instructions. 1000 ng total RNA was used for QIAGEN OneStep RT-PCR kit as per manufacturer's instructions and data collected and analyzed using a Roche LightCycler 480. Data was normalized to GAPDH.

## QUANTIFICATION AND STATISTICAL ANALYSIS

Quantitative data are presented as the means ± standard error of the mean (SEM). Statistical significance was assessed using two-tailed Student's t-test or two-way ANOVA where indicated in the figure legend. p values and the number of experiments (n) used for quantification and statistical analysis are indicated in the corresponding figure legend.

## KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---|---|---|
| Antibodies | | |
| Mouse monoclonal Cytohesin 1 (2E11) | Thermo-Pierce | MA1-060 |
| Rabbit polyclonal GAPDH (FL-335) | Santa Cruz | sc-25778 |
| Rabbit polyclonal Anti-GFP | Life Technologies | A6455 |
| Bacterial and Virus Strains | | |
| BL21 | | |
| Chemicals, Peptides, and Recombinant Proteins | | |
| Hepatocyte Growth Factor | Genentech | N/A |
| Inositol 1,4,5-triphosphate (Ins(1,4,5)P3) | Echelon | Q-0145 |
| Inositol 1,3,4,5-tetrakisphosphate (Ins(1,3,4,5)P4) | Echelon | Q-1345 |
| PI(3,4,5)P3 PolyPIPosomes | Echelon | Y-P039 |
| Wortmannin | LC Laboratories | W-2990 |
| LY294002 | Selleckchem | S1105 |
| Ionomycin calcium salt from Streptomyces conglobatus | Sigma Aldrich | I0634 |
| HiPerfect Transfection Reagent | QIAGEN | 301707 |
| Lipo2000 | ThermoFisher | 11668019 |
| Alexa Fluor 546 Phalloidin | Molecular Probes | A22283 |
| Puromycin dihydrochloride | Sigma | P7255 |
| Hygromycin B | ThermoFisher | 10687010 |
| Critical commercial Assays | | |
| Q5 Site Directed Mutagenesis Kit | NEB | E0554S |
| AllPrep DNA/RNA Mini Kit | QIAGEN | 80204 |
| One Step RT-PCR Kit | QIAGEN | 210212 |
| Experimental Models: Cell Lines | | |
| Human: HeLa | | |

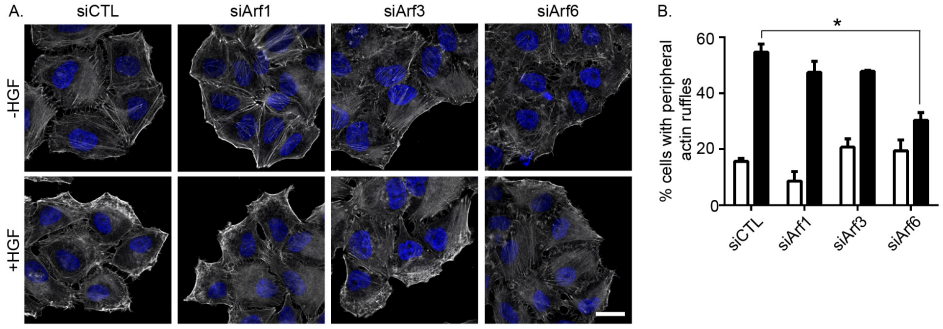
| | | |
|---|---|---|
| Human: HeLa LentiCRISPRv2 empty Clone 6 | This paper | N/A |
| Human: HeLa LentiCRISPRv2 empty Clone 8 | This paper | N/A |
| Human: HeLa LentiCRISPRv2 empty Clone 12 | This paper | N/A |
| Human: HeLa CYTH1 KO gRNA1 Clone 3 | This paper | N/A |
| Human: HeLa CYTH1 KO gRNA1 Clone 5 | This paper | N/A |
| Human: HeLa CYTH1 KO gRNA1 Clone 12 | This paper | N/A |
| Human: HeLa CYTH1 KO gRNA3 Clone 1 | This paper | N/A |
| Human: HeLa CYTH1 KO gRNA3 Clone 6 | This paper | N/A |
| Human: HeLa CYTH1 KO gRNA3 Clone 12 | This paper | N/A |
| Human: HeLa CYTH1 KO + pLVX empty vector | This paper | N/A |
| Human: HeLa CYTH1 KO + eGFP-CYTH1 diglycine | This paper | N/A |
| Human: HeLa CYTH1 KO + eGFP-CYTH1 diglycine (E157K) | This paper | N/A |
| Human: HeLa CYTH1 KO + eGFP-CYTH1 diglycine (R280C) | This paper | N/A |
| Human: HeLa CYTH1 KO + eGFP-CYTH1 triglycine | This paper | N/A |
| Human: HeLa CYTH1 KO + eGFP-CYTH1 triglycine (E157K) | This paper | N/A |
| Human: HeLa CYTH1 KO + eGFP-CYTH1 triglycine (R281C) | This paper | N/A |
| Oligonucleotides | | |
| AllStars Neg. Control siRNA | QIAGEN | 1027281 |
| siGENOME Human CYTH1 (9267) siRNA – SMARTpool | Dharmacon | M-011926-01 |
| siGENOME Human CYTH2 (9266) siRNA – SMARTpool | Dharmacon | M-011925-01 |
| siGENOME Human CYTH3 (9265) siRNA – SMARTpool | Dharmacon | M-019268-00 |
| siGENOME Human IQSEC1 (9922) siRNA – SMARTpool | Dharmacon | M-006458-01 |
| siGENOME Human IQSEC2 (23096) siRNA – SMARTpool | Dharmacon | M-024676-02 |
| siGENOME Human PSD3 (23362) siRNA – SMARTpool | Dharmacon | M-014030-01 |
| CYTH1 gRNA1_FWD: CACCGGAACATCCGACGGAGAAAAC | IDT | N/A |
| CYTH1 gRNA1_REV: AAACGTTTTCTCCGTCGGATGTTCC | IDT | N/A |
| CYTH1 gRNA3_FWD: CACCGGCAGCTCCTGTTTTCTCCGT | IDT | N/A |
| CYTH1 gRNA3_REV: AAACACGGAGAAAACAGGAGCTGCC | IDT | N/A |
| CYTH1_Xho1_eGFP_5'_FWD: AAAACTCGAGATGGTGAGCAAGGGCGAGGAGC | IDT | N/A |

| | | |
|---|---|---|
| CYTH1_Xba1_3'_REV: AAAATCTAGATCAGTGTCGCTTCGTGGAGG | IDT | N/A |
| CYTH1_BamH1-PH_257_FWD: AAAAGGATCCACGTTCTTCAACCCAGACCGAG | IDT | N/A |
| CYTH1_EcoR1-PH_381stop_ REV: TTTTGAATTCCTAGAAAGGGTCCCTGCTGATGGCTGC TTTAATGC | IDT | N/A |
| CYTH1_PH_BamH1_FWD: AAAAGGATCCCCCTTTAAAATCCCAGAAGAC | IDT | N/A |
| CYTH1_PH_EcoR1_REV: TTTTGAATTCTCAGTGTCGCTTCGTGGAGGA | IDT | N/A |
| CYTH1_CCSec7_del_FWD: ACTCACACTTTCTTCAATCCAG | IDT | N/A |
| CYTH1_CCSec7_del_REV: AGATCTGAGTCCGGACTTGTAC | IDT | N/A |
| CYTH1_gRNA3_PAM_SDM_FWD: TGGAGAACATTCGACGGAGAAA | IDT | N/A |
| CYTH1_gRNA3_PAM_SDM_REV: GTTCTTGACGCTCCTCTGCTG | IDT | N/A |
| CYTH1_3Gto2G_SDM_FWD: TGGCAGGGTAAAGACTTGGAAGAG | IDT | N/A |
| CYTH1_3Gto2G_SDM_REV: CCGAGTTTCAATAGCCAGCCTT | IDT | N/A |
| CYTH1_E157K_SDM_FWD: GCTACCCGGAAAGGCCCAGAA | IDT | N/A |
| CYTH1_E157K_SDM_REV: CGGAAGCTCCACAGGAACTG | IDT | N/A |
| CYTH1_R281C_SDM_FWD: TTGGAAGAGATGCTGGTTCATTC | IDT | N/A |
| CYTH1_R281C_GGG_REV: GTCTTTACCCTGCCACCTC | IDT | N/A |
| CYTH1_R280C_GG_REV: GTCTTTACCCTGCCACCG | IDT | N/A |
| CYTH1_qRTPCR_FWD: AAATCCCAGAAGACGACGGG | IDT | N/A |
| CYTH1_qRTPCR_REV: GTCTTTACCCTGCCACCTCC | IDT | N/A |
| CYTH2_qRTPCR_FWD: AACCTGTACGACAGCATCCG | IDT | N/A |
| CYTH2_qRTPCR_REV: CCGGTCCGGGTTGAAGAAG | IDT | N/A |
| CYTH3_qRTPCR_FWD: CGTGCCTGAAGACCTCTCAT | IDT | N/A |
| CYTH3_qRTPCR_REV: TCGTTTTGCTCTCCTCTACGG | IDT | N/A |

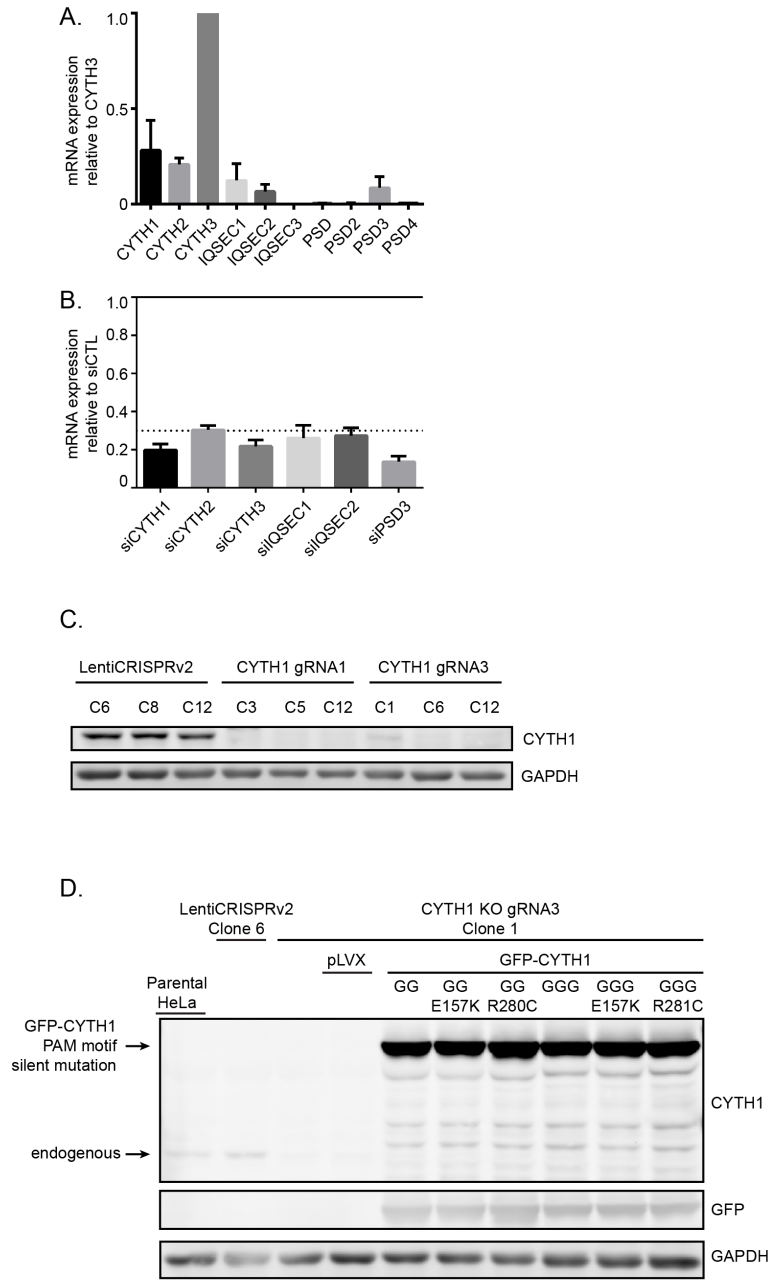
| | | |
|---|---|---|
| IQSEC1_qRTPCR_FWD: GCACAGGATAGAGTCGGAGC | IDT | N/A |
| IQSEC1_qRTPCR_REV: CCCGACTCCTTTTTGAGGCT | IDT | N/A |
| IQSEC2_qRTPCR_FWD: TCCAGTCCCATATCCGGGTT | IDT | N/A |
| IQSEC2_qRTPCR_REV: GAGGGCTGGGTTACAGACAC | IDT | N/A |
| IQSEC3_qRTPCR_FWD: CTACCACTGCGAGAACCCAG | IDT | N/A |
| IQSEC3_qRTPCR_REV: GATGCCCTTGTCGGGGTTTA | IDT | N/A |
| PSD_qRTPCR_FWD: GGCTGTACCGACTAGATGGC | IDT | N/A |
| PSD_qRTPCR_REV: AGCTTGGTCCAGAGTCATGC | IDT | N/A |
| PSD2_qRTPCR_FWD: ACCCTGATGACAGCACTTCG | IDT | N/A |
| PSD2_qRTPCR_REV: TTTTGCCAATGTTGTGGCCG | IDT | N/A |
| PSD3_qRTPCR_FWD: AGCGTGGCACATGAACAAAC | IDT | N/A |
| PSD3_qRTPCR_REV: CCTCGACCCTTCCCCTAGAA | IDT | N/A |
| PSD4_qRTPCR_FWD: TTGGAGGCCATGTTTGGGTC | IDT | N/A |
| PSD4_qRTPCR_REV: CACACTGACACACCTCCCTC | IDT | N/A |
| Recombinant DNA | | |
| LentiCRISPRv2 | Sanjana et al., 2014 | Addgene #52961 |
| pMD2.G | Didier Trono | Addgene #12259 |
| psPAX2 | Didier Trono | Addgene #12260 |
| pLVX-IRES-Hyg | Clontech | 632185 |
| pGEX6p.1 | GE Healthcare Life Science | 28-9546-48 |
| eGFP-CYTH1 (triglycine) | Audrey Claing | N/A |
| LentiCRISPRv2 – CYTH1 gRNA1 | This paper | N/A |
| LentiCRISPRv2 – CYTH1 gRNA3 | This paper | N/A |
| pLVX-IRES-Hyg eGFP-CYTH1 diglycine gRNA3 PAM mutant | This paper | N/A |
| pLVX-IRES-Hyg eGFP-CYTH1 diglycine (E157K) gRNA3 PAM mutant | This paper | N/A |
| pLVX-IRES-Hyg eGFP-CYTH1 diglycine (R280C) | This paper | N/A |
| pLVX-IRES-Hyg eGFP-CYTH1 triglycine gRNA3 PAM mutant | This paper | N/A |
| pLVX-IRES-Hyg eGFP-CYTH1 triglycine (E157K) gRNA3 PAM mutant | This paper | N/A |
| pLVX-IRES-Hyg eGFP-CYTH1 triglycine (R281C) gRNA3 PAM mutant | This paper | N/A |
| pGEX6p.1 CYTH1 diglcyine (amino acids 257-380) | This paper | N/A |

| | | |
|---|---|---|
| pGEX6p.1 CYTH1 diglycine (amino acids 257-380 R280C) | This paper | N/A |
| pGEX6p.1 CYTH1 diglycine (amino acids 243-397) | This paper | N/A |
| pGEX6p.1 CYTH1 triglycine (amino acids 257-381) | This paper | N/A |
| Software and Algorithms | | |
| MetaMorph Version7.7.7.0 | Molecular Devices | N/A |
| Zen Blue Edition | Carl Zeiss Microscopy | N/A |
| Prism Version 6.03 | GraphPad Software | N/A |

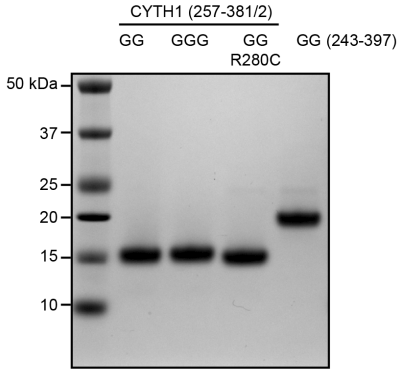
## 3.8 Supplemental Information




**Supplemental Figure 3.1 Arf6 regulates HGF dependent actin remodelling.** Arf6 regulates HGF dependent actin remodelling. (A) Confocal images of cells counter-stained with phalloidin (F-actin) and DAPI. -/+ HGF (B) Quantification of experiments (n=3) shown in (A). Scale bar, 20μm. Error bars indicate standard error of the mean. * $p<0.05$




**Supplemental Figure 3.2 Expression and depletion of Arf GEFs and Cytohesin-1 isoforms and mutants in HeLa cells.** (A) Relative levels of known Arf GEFs from HeLa cell lysates measured by qRT-PCR. (B) Relative levels of siRNA mediated depletion of Arf GEFs expressed in HeLa cells measured by qRT-PCR. (C) Western blot analysis of Cytohesin-1 protein levels in empty vector or CYTH1 gRNA expressing knockout clones generated using the CRISPR/Cas9 LentiCRISPRv2 system. (D) Western blot analysis of populations of expressing Cas9 resistant eGFP-CYTH1 isoforms and mutants were generated from CYTH1 knockout gRNA3 clone 1.




**Supplemental Figure 3.3 Purification of Cytohesin-1 PH domain variants.** Coomassie stained acrylamide gel of purified Cytohesin-1 PH domain variants.

# Discussion

# 4 General discussion

At the beginning of this thesis a role for integrin trafficking in cancer cell migration had clearly been established (Caswell et al., 2009). It was known that integrin receptors could internalize and recycle back to the plasma membrane and that this was important for cancer cell migration. However, the molecular determinants of integrin recycling were only beginning to be understood under specific circumstances such as p53 mutation or serum stimulation. In Chapter 2, I describe a novel interaction between GGA3 and SNX17. SNX17 has previously been implicated in β1 integrin trafficking through recognition of the distal NPXY motif by the SNX17 FERM domain (Böttcher et al., 2012; Steinberg et al., 2012). This recognition protects β1 integrin from degradation and sorts it into a Rab4 positive recycling pathway. We show that silencing GGA3 does not affect SNX17 levels, nor its association with endosomes, however it redistributes SNX17 to lysosomes. Our data supports a model whereby GGA3 regulation of α2β1 levels is mediated by SNX17. Therefore, GGA3, together with SNX17, selectively regulates integrin trafficking via Rab4 endosomes to promote cancer cell migration.

## 4.1 SNX17 selects cargo for Retriever dependent recycling

SNX proteins are associated with membrane tubulation and sorting of cargo in endosomes (Gallon and Cullen, 2015). SNX27 is the best characterized SNX family member and, together with the Retromer complex, regulates trafficking of several cargoes via recognition of a C-terminal PDZ-binding motif (Temkin et al., 2011). However, β1 integrin was a notable exception (Steinberg et al., 2012). β1 integrin lacks a PDZ binding motif that is required for recognition by SNX27 and silencing Retromer subunits does not affect α5β1 stability. SNX17 is another FERM domain containing SNX that is able to recognize β1 integrin through a distal NPXY motif to protect it from degradation and sort β1 into a Rab4 recycling compartment(Böttcher et al., 2012; Steinberg et al., 2012). These data suggest that SNX17 may function independently of Retromer to mediate cargo sorting and recycling. To this end, the Cullen laboratory have recently identified the Retriever complex as a Retromer-like complex that is composed of C16orf62 (Vps35L), DSCR3 (Vps26C), and Vps29 (McNally et al., 2017b). These three proteins structurally resemble Retromer (Vps35, Vps26 and Vps29), associate with the cargo adaptor SNX17 and are recruited to early endosomes via the WASH complex. Unlike Retromer, Retriever components and SNX17 are required for α5β1 integrin sorting. Silencing SNX17 reduces the abundance of many different cargo at the plasma membrane, including α2 integrin, APP, VLDLR and Met receptor (McNally et al., 2017b).

In this thesis, we showed that α2β1 integrin stability was dependent on GGA3 (Ratcliffe et al., 2016). Met RTK and the APP processing enzyme, BACE1, are also regulated by GGA3. Given the partial overlap between GGA3 and SNX17 dependent cargo, these data support two hypotheses.

1. Known GGA3 cargo may be regulated by the Retriever complex.
2. Known SNX17 or Retriever cargo could recycle via a GGA3 pathway.

The FERM domain of SNX17 can interact with both phosphorylated and unphosphorylated NPXY motifs in a wide variety of receptors including RTKs, GPCRs and integrins (Ghai et al., 2013). These include known GGA3 cargo such as TrkA and EGFR (Li et al., 2015a; Puertollano and Bonifacino, 2004). Therefore, these specific cases suggest that SNX17 and GGA3 may act on the same cargo. It will be important to test the impact of GGA3 silencing on the localization of the Retriever complex and the relevance of Retriever in APP or Met RTK trafficking.

The Retriever complex interacts with the WASH complex (McNally et al., 2017b). WASH localizes to endosomes and activates the Arp2/3 complex to promote actin polymerization (Derivery et al., 2009). Actin polymerization drives endosome fission and inhibition of WASH induces elongated tubules on endosomes. Actin subdomains also contribute to cargo sorting in endosomes (Puthenveedu et al., 2010). On sorting endosomes GGA3 associates with clathrin light chain or "gyrating clathrin" (Parachoniak et al., 2011). Clathrin light chain also regulates β1 recycling (Majeed et al., 2014). Therefore, GGA3 and Retriever may represent a hub for endosomal trafficking and link endosomal clathrin and actin.

## 4.2 Integrin receptor trafficking regulates cancer cell migration

In chapter 2, we observe that GGA3 regulates α2 and β1 integrin levels in both HeLa and MDA-MB-231 cancer cell lines (Ratcliffe et al., 2016). However, α5 integrin levels are affected in HeLa but not MDA-MB-231 cells suggesting that α5 integrin recycling may recycle via another pathway in MDA-MB-231 cells. In MDA-MB-231 cells, α5 integrin recycling and cell invasion is regulated by RCP and Rab25. MDA-MB-231 cells express the DNA-binding domain p53 mutant (R280K), whereas HeLa cells express wild-type p53. Overexpression of mutant p53 is sufficient to promote α5 integrin recycling through an RCP-dependent pathway (Muller et al., 2009). Therefore, differential regulation of α5 integrin in MDA-MB-231 and HeLa cells may be due to the p53 status in these lines. Indeed, when we compared levels of α5 integrin in cell lines derived

from the MMTV-Met$^{mt}$;Trp53fl/+;Cre breast cancer mouse model compared to MMTV-Met$^{mt}$ derived cell lines we found much higher α5 levels in the MMTV-Met$^{mt}$;Trp53fl/+;Cre cells.

## 4.3 Arf cycling promotes integrin trafficking and cell migration

Clearly, Arf6 plays a central role in β1 integrin trafficking. Indeed, we show that silencing Arf6 but not Arf1 or Arf3 impairs cell spreading. In line with previously published data, silencing Arf6 causes perinuclear accumulation of internalized β1 (Figure 2.7D). Both exchange factors and activating proteins have been implicated in β1 trafficking, however with somewhat counter-intuitive results.

GGA3 is an Arf6 effector that specifically binds to GTP loaded Arf6. Regulation of integrin trafficking, adhesion sites, cell spreading and cell migration depends on the GGA3 Arf binding site. Whereas overexpression of GFP-tagged WT GGA3 can rescue the effects of silencing endogenous GGA3, a GGA3 mutant (N194A) that is uncoupled from Arf proteins cannot. Our data support a model where Arf6 activation and effector recruitment is required for integrin recycling. In line with this, silencing the Arf GEF cytohesin-2 impairs integrin recycling and cell spreading (Oh and Santy, 2010). Overexpression of the Arf GEF, PSD, enhances Arf6 dependent retrograde transport in axons (Eva et al., 2012). Therefore, enhanced Arf6 activation promotes cell migration though increased integrin recycling.

Silencing the Arf6 GAP, ACAP1, increases active Arf6. However, ACAP1 silencing also reduces β1 recycling (Li et al., 2005a; 2007a). Overexpression of ACAP1, reduces Arf6-GTP levels and promotes integrin recycling (Eva et al., 2012). These data support a model whereby Arf6-GDP promotes integrin trafficking. How do we reconcile that both Arf6-GTP and GDP promote integrin trafficking? A clue comes from the Arf6 mutants that lock Arf6 in a constitutively active (Q67L) or inactive (T27N) conformation. GGA3 interacts with Arf6 Q67L but not T27N, suggesting that this mutant would promote integrin trafficking whereas the T27N would block it. However, overexpression of both mutants leads to accumulation of internalized β1 in cytosolic vesicles (Brown et al., 2001; Powelka et al., 2004). While Arf6 Q67L is able to recruit known Arf6 effectors, it is unable to hydrolyze GTP and therefore is unable to complete a full cycle of GTP binding, hydrolysis and GDP release. Silencing Arf6 GEFs and GAPs would also impair GTP flux through Arf6 whereas overexpression of GEFs and GAPs would promote it. To support this hypothesis, overexpression of a hyperactive Arf6 mutant (T157A) that enhances active Arf6 by

promoting cycling, increases integrin recycling and cell migration (Morgan et al., 2013). Therefore, current data supports a model that requires Arf6 cycling promotes integrin recycling and cancer cell migration.

## 4.4 Arf GEFs and PH domains have multiple interactions sites with the membrane

In Chapter 3 we demonstrate that membrane recruitment of Cytohesin-1 is mediated by the PH domain and specific recognition of phosphoinositide headgroups drives membrane binding. Mutation of a conserved arginine residue (R280C or R281C) abrogates phosphoinositide binding and membrane recruitment *in vivo.* These mutants are unable to rescue membrane ruffling in *CYTH1* KO cells, highlighting a critical role for phosphoinositide binding. While phosphoinositide binding is a key step in Cytohesin-1 membrane recruitment and the key difference between microexon-containing splice variants, additional determinants of Cytohesin-1 membrane binding have also been studied. Below, I discuss these and how they relate to Cytohesin-1 microexon splicing.

### 4.4.1 β1/ β2 loop regulates membrane binding

The dynamin PH domain specifically recognizes $PI(4,5)P_2$, however with low affinity (Salim et al., 1996; Zheng et al., 1996). The dynamin PH domain must dimerize in order to be recruited to $PI(4,5)P_2$ on membrane suggesting that higher order oligomerization and additional determinants beyond phosphoinositide binding in cells is required for its function (Klein et al., 1998). The β1/ β2 loop of most PH domains contain a KXn(K/R)XR motif that mediates non-specific interactions with the inner leaflet of the plasma membrane (Isakoff et al., 1998). Membrane penetration of the β1/ β2 PH domain loop is also required for dynamin-mediated scission (Ramachandran et al., 2009). Mutation of Ile533 to Cys in this loop impairs membrane binding and CCP constrictions *in vivo*. The Cytohesin-1 β1/ β2 loop has a hydrophobic Val residue flanked by basic Arg and Lys residues. It is conceivable that this Val residue inserts into the membrane while the basic residues interact with the negatively charged inner leaflet of the plasma membrane. This may be more or less important for the di- or tri-glycine variants of Cytohesin-1 as the extra glycine at the base of the β1/ β2 loop might provide more flexibility or push the V274 (triglycine - V275) closer to the membrane. Molecular dynamic simulations of diglycine Cytohesin-3 and triglycine cytohesin-2 with a phospholipid bilayer reveal that both PH domains

make multiple contacts with phosphoinositide headgroups as well as the membrane (Yamamoto et al., 2016). The β1/ β2 loop also forms contacts with the membrane bilayer. However, in this study, the Val residue in the β1/ β2 of diglycine cytohesin-3 did not penetrate below the average position of phospholipid headgroups of the bilayer. In contrast, Lai et al. and Lumb et al. both observed membrane penetration of diglycine cytohesin-3 Val278 in molecular dynamic simulations (Lai et al., 2013; Lumb et al., 2011). This discrepancy may be due to transient perturbations of the membrane by positive interactions with the flanking basic residues that would be masked upon averaging the position of the lipid bilayer (Lai et al., 2013). The impact of microexon splicing and a third glycine residue at the base of the β1/ β2 loop on bilayer penetration has not been directly investigated by molecular dynamic simulation or experimentally.

### 4.4.2 Coupling cytohesin-1 membrane recruitment to Arf activation

Indirect recruitment of cytohesin proteins to the plasma membrane has also been described. While Arf6-GDP is recognized by the Sec7 domain, Arf6-GTP can interact with Cytohesin-1 and 3 PH domains and C-terminal helices (DiNitto et al., 2007). Overexpression of Arf6 promotes plasma membrane association of full length cytohesin-2 and cytohesin-3 (Cohen et al., 2007). The Cytohesin-2 PH domain was both necessary and sufficient for this interaction. It is possible that overexpressed Arf6 activates PI4P5K, elevates $PIP_2$ levels and this in turn promotes membrane recruitment of Cytohesin. However, a mutation that retains phosphoinositide binding but impairs cell spreading (K340L) is not recruited to the membrane upon Arf6 overexpression. To explain this, structural studies from the Lambright laboratory have shown that cytohesin proteins can adopt an autoinhibited state. The PH domain and C-terminal helix folds over the Sec7 domain and occludes a docking site for the switch regions of Arf6-GDP in the Sec7 domain (DiNitto et al., 2007). Autoinhibition can be relieved by membrane-bound Arf6-GTP binding to the PH domain and C-terminal helix (Malaby et al., 2013). Cytohesin binding to membrane-bound Arf6-GTP couples membrane recruitment to activation. Based on these data, a positive feedback loop has been proposed to promote Arf6 activation. This model would depend on cytohesin activity to promote further recruitment of cytohesin molecules. However, when we tested membrane recruitment of cytohesin-1 E157K, neither the diglycine nor the triglycine splice variant was distinguishable from WT. While we have not ruled out initial recruitment to the membrane by

steady state levels of Arf6-GTP, structural models suggest that Arf6-GTP may stabilize an active state and not membrane recruitment *per se* (Malaby et al., 2018).

### 4.4.3 The cytohesin polybasic sequence regulates lipid binding

Immediately following the PH domain in all cytohesin family members is a short C-terminal helix followed by a polybasic sequence. While sequences of all cytohesin family members are polybasic, they are distinct. Each polybasic sequence is predicted to have a net charge of ~$6^+$ at pH 7.4. Whereas the Cytohesin-3 polybasic region is predominantly Arg residues, the cytohesin-2 polybasic region is the longest and is Lys rich. The cytohesin-2 polybasic region has seven basic residues; however, one net charge is cancelled out by the presence of a Glu residue. Phosphorylation of a lone Ser residue in the cytohesin-2 polybasic region regulates its ability to promote GDP exchange of Arf6 on $PIP_2$ and $PIP_3$ containing liposomes *in vitro* (Santy et al., 1999). Mutation of this Ser to Glu reduces cytohesin-2 liposome binding and reduces membrane association *in vivo*. Whereas the PH domain contributes to phosphoinositide recognition, the polybasic sequence interacts non-specifically with negatively charged phospholipids such as phosphatidylserine (Macia et al., 2000). These data support a model whereby an electrostatic switch regulates Cytohesin-2 membrane recruitment.

The cytohesin-1 polybasic sequence increases the affinity of the cytohesin-1 PH domain for a $PIP_3$ containing lipid monolayer by ~30-fold (Nagel et al., 1998). Deletion of the polybasic sequence also reduces membrane localization in Jurkat cells. Addition of the cytohesin-1 polybasic sequence can support membrane association of the $PI(4,5)P_2$ binding PH domain of βARK, suggesting that it is promoting non-specific association with the negatively charged inner leaflet. The Cytohesin-1 polybasic sequence is intermediate in length compared to the cytohesin-2 and 3 polybasic sequences and also has 2 Ser residues, followed a Thr residue. Phosphorylation of the Thr and the second Ser residue have been detected by mass spectrometry (PhosphositePlus). Phosphorylation of cytohesin-1 is observed upon treatment of with phorbol ester, a compound that activates PKC (Dierks et al., 2001). These sites are phosphorylated by PKCδ *in vitro* and Ala mutants of these sites impair Jurkat cell adhesion. Intriguingly they do not affect subcellular localization or liposome binding. However, it is not immediately clear which isoform is being used in studies of cytohesin family PH domains and the polybasic region. Perhaps the increased affinity of diglycine cytohesin PH domains is sufficient to drive membrane recruitment, whereas the

polybasic sequence must cooperate with the triglycine PH domain to interact with negatively charged phospholipids $PIP_2$ and phosphatidylserine.

Our study is the first to examine membrane association of specific cytohesin-1 isoforms *in vivo*. We find that diglycine cytohesin-1 is recruited to the membrane in response to growth factor signalling and in a PI3K dependent manner, whereas the triglycine variant is constitutively at the membrane. Specific membrane recruitment is presumably driven by the different relative abundance of $PI(4,5)P_2$ and $PI(3,4,5)P_3$ at the plasma membrane at resting or upon HGF treatment. However, we have not ruled out a contribution of the polybasic sequence to these processes. Indeed, cationic surface probes of 6+ or above, localize specifically to the membrane and associate with phosphatidylserine suggesting that charge is an important factor in membrane association (Yeung et al., 2008). Protein clustering driven by polybasic sequences into plasma membrane nanodomains have been reported for K-Ras and Rac1 (Remorino et al., 2017; Zhou et al., 2017). The K-Ras polybasic sequence regulates lipid content of K-Ras phosphatidylserine-rich nanoclusters at the plasma membrane (Zhou et al., 2017). Mutation of 6 Lys residues to Arg maintains the net charge of the K-Ras polybasic sequence, however mutant K-Ras nanoclusters are enriched in $PI(4,5)P_2$ and depleted of phosphatidylserine. K-Ras nanoclusters with single point mutants of individual Lys residues also have distinct lipid composition compared to WT. This has important consequences for cell signalling since single point mutants of the polybasic sequence have distinct phosphorylation profiles. Intriguingly, Rac1 nanoclusters at the leading edge of the cell are enriched in $PI(3,4,5)P_3$ and the Rac1 polybasic-CAAX motif was sufficient to drive nanoclustering (Remorino et al., 2017). Together these data suggest that polybasic regions provide additional lipid specificity and the cytohesin-1 polybasic region might modulate Cytohesin-1 signalling in plasma membrane microdomains.

## 4.5 Endosomal trafficking regulates $PIP_3$ signalling

Localization of $PI(3,4,5)P_3$ is an evolutionarily conserved signal for cell polarization and cell migration. $PI(3,4,5)P_3$ is localized at the front of migrating *Dictyostelium discodium* and mammalian neutrophils. In this thesis, we show that diglycine cytohesin-1 localizes to the leading edge of cancer cells in response to growth factor stimulation. Diglycine cytohesin-1 is required for cancer cell migration in response to HGF and for generation of a stable lamellipodia. Therefore, we propose that diglycine cytohesin-1 is an important $PI(3,4,5)P_3$ effector and polarized generation

of $PI(3,4,5)P_3$ is essential for cytohesin-1 dependent cell migration. Triglycine cytohesin-1 specifically binds $PI(4,5)P_2$ and is constitutively recruited to the plasma membrane. In contrast to diglycine cytohesin-1, membrane bound triglycine cytohesin-1 localizes throughout the periphery of the cell. We propose that, in part due to its peripheral localization, triglycine cytohesin-1 is unable to promote stable lamellipodia formation and define a leading edge. This highlights a role for a stable $PI(3,4,5)P_3$ gradient in cancer cell migration.

A stable $PI(3,4,5)P_3$ gradient is generated, in part, by localized activation of PI3K. In response to HGF, diglycine GFP-CYTH1 is recruited to the leading edge for up to 60 minutes. This is in stark contrast to EGF stimulation. We and others have found that cytohesin-1 is transiently recruited to the plasma in response to EGF (Figure 4.1A) (Venkateswarlu et al., 1999). Recruitment peaks at approximately 3 minutes post stimulation and declines to background levels by 15 minutes (Venkateswarlu et al., 1999). This time course closely mirrors $PIP_3$ levels upon EGF stimulation (Malek et al., 2017; Maroun et al., 2003). While EGFR and Met both internalize upon ligand binding, EGFR is degraded more rapidly. This may be due to differential capacity of Met and EGFR to engage GGA3 and recycle back to the plasma membrane (Parachoniak et al., 2011; Puertollano and Bonifacino, 2004). Supporting this hypothesis, we found that GGA3 silencing shortens diglycine GFP-CYTH1 recruitment to the leading edge upon HGF stimulation (Figure 4.1B). Membrane bound diglycine GFP-CYTH1 is no longer detectable 15 minutes after stimulation. These data support a model where Met recycling prolongs PI3K signalling at the leading edge and promotes diglycine GFP-CYTH1 recruitment.

Global dephosphorylation of $PI(3,4,5)P_3$ by PTEN is required to balance local activation of PI3K to generate a $PI(3,4,5)P_3$ gradient. Silencing PTEN causes defects in cancer cell migration. To test whether PTEN affects diglycine GFP-CYTH1 membrane recruitment, we silenced PTEN and stimulated cells with HGF. If PTEN was regulating $PI(3,4,5)P_3$ levels at the plasma membrane levels we predicted that diglycine GFP-CYTH1 would localize to the entire periphery of the cell in response to HGF and would appear similar to triglycine GFP-CYTH1. Surprisingly, we observed GFP puncta throughout the cytosol upon HGF stimulation in PTEN silenced cells (Figure 4.1C). These structures resembled endosomes and would suggest that PTEN regulates $PI(3,4,5)P_3$ levels on endosomes. PTEN has recently been shown to specifically recognizes vesicular PI(3)P in mammalian cells through the C2 domain (Naguib et al., 2015). The C2 domain colocalizes with the PI(3)P binding Hrs-FYVE domain membrane recruitment to cytoplasmic vesicles. The identity

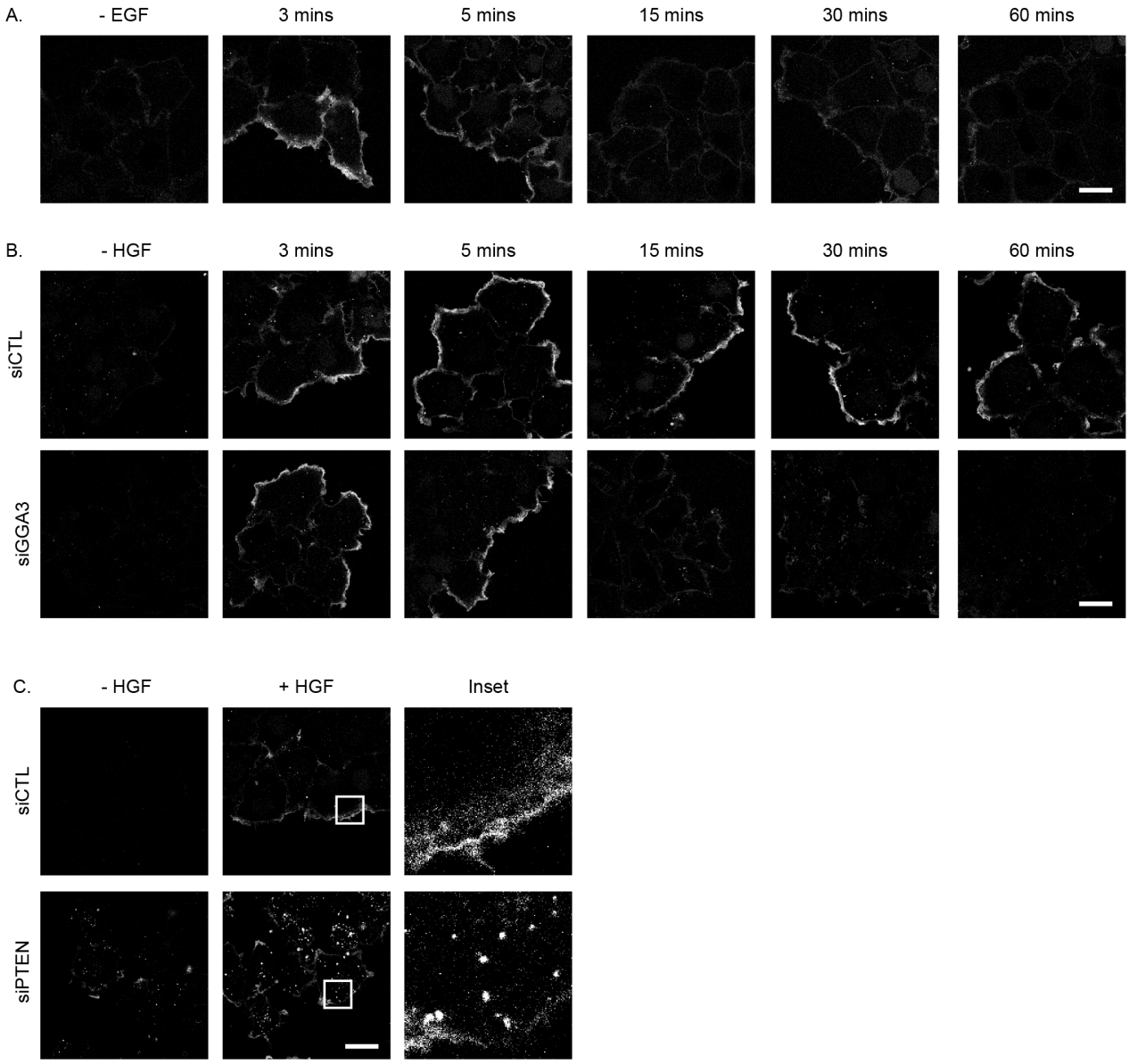



**Figure 4.1 Determinants of diglcyine GFP-CYTH1 localization.** HeLa cells stably expressing eGFP-tagged diglycine CYTH1 were either untreated or treated as indicated for the indicated time points, permeabilized with ice-cold 0.05% saponin in PIPES buffer and imaged by confocal microscopy (A) Cells were treated with 10 ng/mL EGF. (B) HeLa cells were treated with control or GGA3 siRNA for 72 hours and then treated with HGF. (C) HeLa cells were treated with control or PTEN siRNA for 48 hours and then treated with HGF for 15 mins

of these vesicles has not been formally determined. However, given PTEN binding to PI(3)P and colocalization with internalized EGF, it is highly likely that these represent a population of early endosomes (Naguib et al., 2015). Therefore, PTEN has unappreciated role in regulating $PI(3,4,5)P_3$ on endosomes and the biological significance in cancer cell migration is unknown.

## 4.6 Thinking beyond Akt downstream of PI3K in cancer

Breast cancer is a disease that affects 1 in 8 Canadian women and 1 in 31 women will die from the disease (Canadian Cancer Society, 2017). Advances in genomic and proteomic technologies have driven profiling efforts of thousands of breast cancer patients and identification of subgroups with distinct molecular characteristics and outcomes. This has led to molecular classification of breast cancer subtypes based on gene expression profiling (Perou et al., 2000). These subtypes include luminal A, luminal B, Her2-enriched and basal-like. Stratification of patients into different molecular subtypes permits identification of patients that are likely to benefit from targeted therapy (Sorlie et al., 2001). Basal-like breast cancers, which have the poorest prognosis, currently lack targeted therapy. 70% of women with breast cancer have mutations in the PI3K pathway and mutations in the PI3K pathway have been identified in all the subtypes of breast cancer (Cancer Genome Atlas Network, 2012; Lopez-Knowles et al., 2010).

The PIK3CA gene encodes for the α isoform of the p110 subunit of PI3K. PIK3CA is frequently mutated in a variety of common cancers including breast, colon, endometrium and prostate (Thorpe et al., 2015). 80% of PIK3CA mutations occur in one of three sites. Mutant p110α has enhanced lipid kinase activity, can transform cells and are tumourigenic *in vivo* (Engelman et al., 2008; Isakoff et al., 2005; Kang et al., 2005; Zhao et al., 2005). Despite high frequency of PIK3CA mutations in luminal A breast cancer, activation of downstream effectors including Akt and mTOR is not enhanced in these tumours (Cancer Genome Atlas Network, 2012). The apparent discordance between PIK3CA activity and Akt pathway activation in luminal A breast cancer suggests that other $PIP_3$ effectors could be important in those affected by PIK3CA mutations.

Enhanced Akt and mTOR phosphorylation is observed in a subset of basal-like patients with loss of either phosphatase PTEN or INPP4B (Cancer Genome Atlas Network, 2012). PTEN and INPP4B negatively regulate Akt signalling by dephosphorylating the 3' phosphate from $PI(3,4,5)P_3$ or the 4' phosphate from $PI(3,4)P_2$ respectively. These data support that $PIP_3$ is

abundant and available to activate downstream signalling in this subset of basal-like patients. Basal-like breast cancer tumours lose molecular and morphological characteristics of epithelial cells and acquire a mesenchymal-like morphology. These are associated with changes in the actin cytoskeleton, increased capacity to migrate and metastasize. Overexpression of Met correlates with poor outcome and the basal-like subtype (Garcia et al., 2007; Ghoussoub et al., 1998; Ponzo and Park, 2014). In a mouse model of breast cancer driven by Met overexpression and p53 loss, small molecule inhibition of Met reverses the mesenchymal phenotype and reduces metastasis (Knight et al., 2013). In this model, Met inhibition is associated with an increased number of stress fibers and characteristics reminiscent of decreased cytohesin-1 activity. HGF and Met activation promotes phosphorylation and activation of Akt (Abella et al., 2005; Parachoniak et al., 2011). Many studies have correlated HGF-dependent Akt phosphorylation with cell survival and proliferation. However, in Met amplified cell lines that depend on Met signalling for proliferation, Akt inhibition has a marginal effect of cell proliferation (Lai et al., 2014). These data argue that while Met activation of Akt may be important under specific contexts, it is not an essential HGF-dependent PI3K effector. Therefore, it will be important to identify novel therapeutic targets downstream from PI3K in basal-like breast cancer.

## 4.7 Arf6 inhibition in cancer

Identification of oncogenic activating mutations in the small GTPase K-Ras prompted decades of research and efforts to therapeutically target Ras (Cox and Der, 2014; Malumbres and Barbacid; Reddy et al., 1982; Tabin et al., 1982; Taparowsky et al., 1982). Consequently, diseases associated with Ras mutations and activation have been described as “Rasopathies”. K-Ras mutants identified in cancer prevent GTP hydrolysis and lock Ras in an active conformation. K-Ras mutations are present in 12% of all patients in the TCGA dataset (Figure 4.2). However, in stark contrast to Ras, Arf mutations are relatively rare. While a clear role for Arf6 in models of cancer cell migration and metastasis have been established, the importance for Arf6 in human cancer is currently poorly understood. Scoring of Arf6 staining in a tumour microarray of breast cancer patients suggests that Arf6 expression correlates with tumour histological grade (Schlienger et al., 2016). Recently, a small molecule inhibitor of Arf6, NAV-2729, was identified (Yoo et al., 2016). NAV-2729 selectively inhibits Arf6 and reduces Arf6 signalling in uveal melanoma cell lines. Importantly, NAV-2729 also reduced tumour incidence and growth upon orthotopic

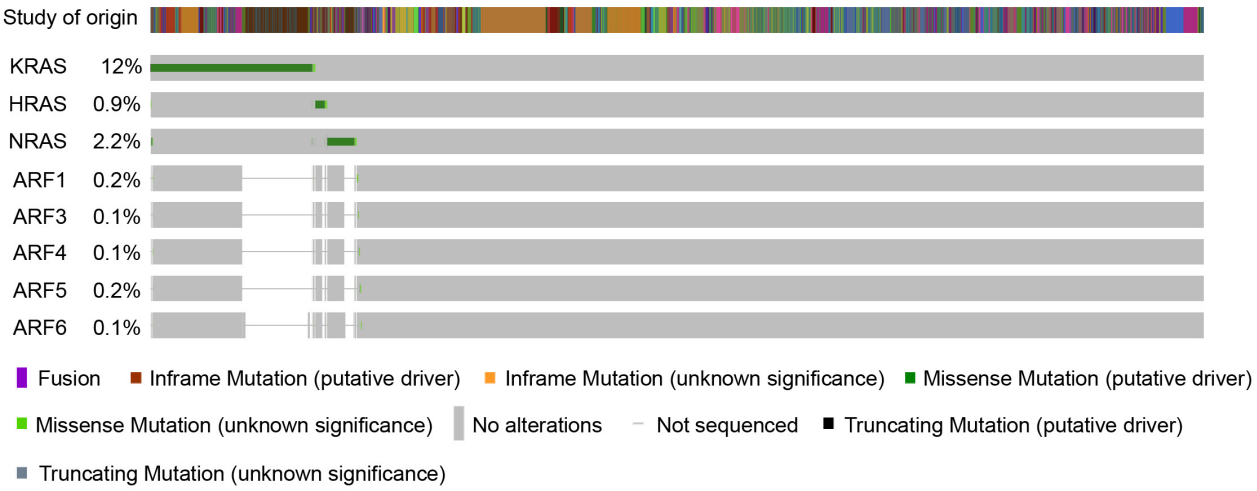



**Figure 4.2 Mutational frequency of the Ras and Arf family in TCGA studies.** All listed studies in the cBio Cancer Genomics Portal database were queried for "Mutation Only" alterations in KRAS, HRAS, NRAS, ARF1, ARF3, ARF4, ARF5 and ARF6. Database accessed on February 9th 2018.

injection of a uveal melanoma cell line. This opens the possibility for testing pharmacological inhibition of Arf6 in a variety of well-established models of cancer

SecinH3 is a small molecule inhibitor of cytohesin family members that inhibits the GEF activity of the Sec7 domain. It was identified in a chemical screen of compounds that displaced M69, an aptamer that binds and inhibits the Sec7 domain (Hafner et al., 2006). Our data would support targeting cytohesin family members, and specifically cytohesin-1, to inhibit cancer cell migration. We show that *CYTH1* KO reduced HGF-dependent actin rearrangement and cell migration. Diglycine Cytohesin-1 could rescue these phenotypes, however this depended on its GEF activity. A Sec7 domain mutant (E157K) of diglycine cytohesin-1 was unable to rescue HGF-dependent actin rearrangement and cell migration. Consistent with a role for Arf6 in cell migration, treatment of HeLa cells with SecinH3 inhibits cell spreading and integrin recycling, however this was found to be due to cytohesin-2 inhibition (Oh and Santy, 2010). PDGF-dependent lamellipodia formation and cell migration of SUM159 cells (basal-like breast cancer) is also inhibited by treatment with SecinH3 (Miao et al., 2012). Treatment of mice with SecinH3 inhibits Arf6 dependent processes including insulin signalling, tumour angiogenesis and HGF-dependent kidney recovery following acute kidney injury (Hafner et al., 2006; Hongu et al., 2015; Reviriego-Mendoza and Santy, 2015). Mice treated with SecinH3 appear healthy and did not exhibit weight

loss, suggesting that SecinH3 might be suitable for further studies (Hongu et al., 2015). Therefore, SecinH3 not only inhibits cancer cell migration but also processes in the patient that are associated with tumour progression.

## 4.8 A role for Cytohesin splice variants in bacterial invasion

#### 4.8.1.1 *Listeria Monocytogenes*

*L. monocytogenes* is a gram-positive bacterium that can infect many cell types including, enterocytes, hepatocytes, fibroblasts, epithelial cells and endothelial cells (Cossart and Lebreton, 2014). *L. monocytogenes* invasion of host cells is mediated by binding of the bacterial surface proteins internalin A (InlA) or internalin (InlB) to receptors on the host cell (Dramsi et al., 1995; Gaillard et al., 1991). Whereas InlA binds to E-Cadherin to mediate *L. monocytogenes* entry, InlB interacts directly with the Met receptor (Mengaud et al., 1996; Shen et al., 2000). Met is required for bacterial invasion of epithelial cells. InlB stimulates Met phosphorylation and recruitment of downstream signalling proteins, including PI3K and Gab1. Inhibition of PI3K impairs *L. monocytogenes* invasion (Ireton et al., 1996). Increased levels of $PI(3,4,5)P_3$ at sites of bacterial entry promotes recruitment of Rac1 and the Arp2/3 complex to promote actin rearrangement and polymerization (Bierne et al., 2001; 2005; Bosse et al., 2007). Indeed, there are many commonalities between HGF and InlB Met activation and study of one ligand should inform the other. ARAP2 promotes actin rearrangement during *L. monocytogenes* invasion and silencing ARAP2 reduces bacterial invasion (Gavicherla et al., 2010). ARAP2 is a Arf6 GAP that binds $PI(3,4,5)P_3$ and therefore may promote Arf6 cycling or inactivation during *L. monocytogenes* invasion. These data highlight a role for Arf6 downstream from *L. monocytogenes*. It is currently unknown whether any Arf6 GEFs regulate *L. monocytogenes* invasion, however, given our work on Cytohesin-1 downstream from Met, Cytohesin-1 is a strong candidate for investigation. More specifically, we would predict that the diglycine isoform of cytohesin-1 is required for *L. monocytogenes* invasion given the important role that PI3K plays in this process.

#### *4.8.1.2 Salmonella enterica* serovar Typhimurium

*S.* Typhimurium (Salmonella enterica serovar Typhimurium) is a gram-negative bacterium that invades the intestinal epithelium leading to gastroenteritis and enteric fever. *S.* Typhimurium invasion of epithelial cells depends on rearrangement of the actin cytoskeleton, stimulated by the

injection of bacterial proteins into the host cell (Truong et al., 2014). Bacterial proteins hijack the host cytoskeleton to promote membrane ruffling and uptake into a structure called the *Salmonella* containing vacuole. Membrane ruffling is stimulated by the bacterial protein SopE which acts as a Rac1 GEF (Humphreys et al., 2012). Both Arf1 and Arf6 have been implicated in this process, as well as cyothesin-2 (Humphreys et al., 2013). In this model, Arf6 activates cytohesin-2 through an interaction with the PH domain and C-terminal helix as described previously. Active cytohesin-2 then promotes GDP exchange of Arf1. Active Arf1, together with Rac1 drives actin polymerization and bacterial uptake. Whether other cytohesin family members are involved in this process or the specific isoform of cytohesin-2 that is important is unknown. However, *S.* Typhimurium invasion is not inhibited by Wortmannin, suggesting that this process is independent of Class I PI3K (Steele-Mortimer et al., 2002). Together, these data would support a role for triglycine cytohesin-2 in *S.* Typhimurium invasion.

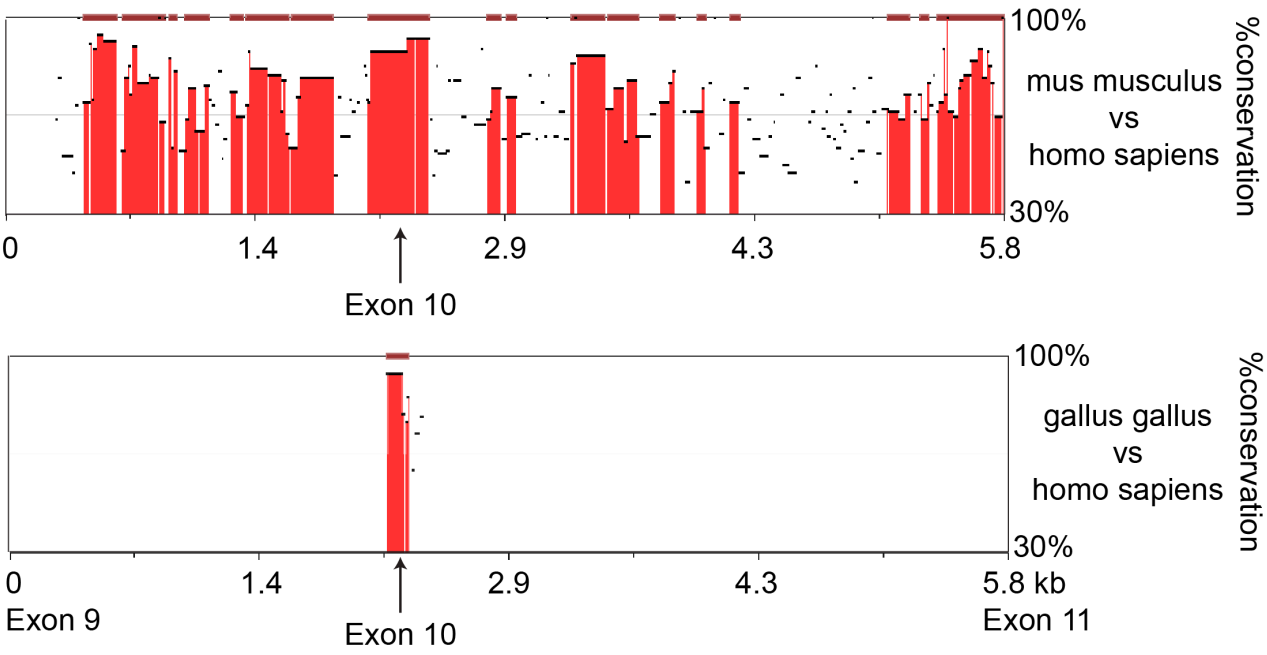



**Figure 4.3 Cytohesin-1 microexon is contained within an evolutionarily conserved sequence.** Percent conservation comparison of the human sequence between exons 9 and 11 of Cytohesin-1 and the homologous sequence in *Mus musculus* and *Gallus gallus*. Sequences were analyzed using the webtool at "mulan.dcode.org".

## 4.9 Microexons

The first microexon containing gene was identified in 1985 when two 5 nucleotide exons in the Drosophila Ubx gene were identified (Beachy et al., 1985). Individual examples of short microexons were later identified in the chicken Troponin T gene (Cooper and Ordahl, 1985), rat and mouse Ncam (Santoni et al., 1989; Small et al., 1988); and a brain specific isoform of the non-receptor tyrosine kinase Src (Brugge et al., 1985). Even a single nucleotide microexon has been identified in Arabidopsis Thaliana that maintains the reading frame of APC11 (Guo and Liu, 2015). However, only with recent large datasets of RNA sequencing data and improved methods to map short reads to the genome were microexons able to be comprehensively annotated and a formal definition generated (Irimia et al., 2014; Li et al., 2015b). Microexons are a recently described class of alternative splicing events that are $\leq 27$ nucleotides in size and enriched during neuronal differentiation. They are predominantly found in structured protein domains and in genes that regulate the cytoskeleton and membrane trafficking. The cytohesin-1 microexon is contained within a 5.9kb region between exons 9 and 11 (Figure 4.3). Comparison of the sequences between human, mouse and chicken reveal a highly conserved 90bp region surrounding exon 10. High sequence conservation suggests that cytohesin-1 microexon splicing may be recognized by specific splicing factors. However, splicing factors that bind directly to pre-mRNA surrounding microexons have not been identified.

## 4.10 Deregulation of microexons in disease

The splicing factors nsR100/SRRM4, PTPBP and RBFOX regulate microexon splicing, however the specific mechanism is unclear (Irimia et al., 2014; Li et al., 2015b). Silencing of these splicing factors alter the percent spliced in values of many microexons. However, despite higher conservation of the 100 base pairs immediately upstream of microexons compared to larger exons, a factor that directly binds to an upstream element that regulates microexon splicing has not been identified. Levels of nsR100/SRRM4 are dysregulated in a third of patients with autism spectrum disorder and mutations in nr100/SRRM4 lead to dysregulated microexon splicing as well as autism like characteristics in mice. Intriguingly, whereas neurons cultured from nsr100$^{\Delta 7\text{-}8/\Delta 7\text{-}8}$ mice displayed short neurites, this could be rescued by overexpression of a Unc13b splice variant that contained a 6 nucleotide microexon but not Unc13b lacking the microexon (Quesnel-Vallières et

al., 2015). This highlights that microexons may be collectively regulated, however each microexon-containing protein may have a very specific and context dependent function.

The non-receptor tyrosine kinase, c-Src, regulates cell growth, cell migration and the actin cytoskeleton downstream from integrin receptors and RTKs. In addition to c-Src, the *SRC* gene also encodes for two microexon containing splice variants, termed N1- and N2-Src (Brugge et al., 1985; Pyper and Bolen, 1990). In a small cohort of children with neuroblastoma, loss of N2-Src was correlated with increased disease stage (Matsunaga et al., 1993). c-Src contains a N-terminal myristoylation sequence followed by an SH3 domain, a SH2 domain, a kinase domain and a C-terminal tail (Boggon and Eck, 2004). The C-terminal tail contains a tyrosine that, when phosphorylated, is recognized by the SH2 causing Src to adopt a autoinhibited conformation. The SH3 domain recognizes proline rich sequences with a PxxP motif. These are found on many Src substrates, including focal adhesion kinase. SH3 binding, together with the SH2 domain is thought to bring Src within close proximity of its substrates. N-Src splice variants are primarily expressed in neuronal tissue. Two microexons are located between exons 3 and 4 of c-Src. N1-Src contains a 6 amino acid insertion in the SH3 domain encoded by the first microexon. N2-Src has a 17 amino acid insertion encoded by splicing in of both microexon. Insertion of these microexons reduces or abolishes tested SH3 domain interactions of known c-Src binding partners (Dergai et al., 2010; Keenan et al., 2015). However, both N1-Src and N2-Src have increased activation *in vitro* and *in vivo* (Keenan et al., 2015). Therefore, presence of alternatively spliced microexons may reduce SH3 domain-mediated intramolecular interactions that keep Src in an autoinhibited state. However, despite increased activation, N1- and N2-Src had reduced capacity to phosphorylate synaptophysin, a known c-Src substrate. N1- and N2-Src may have different SH3 domain binding specificities compared to c-Src, however novel binding partners have not yet been identified. Given the functional importance of Src in cell migration and cancer biology, it will be important to investigate a role for Src splice variants in neuroblastoma with modern techniques and model systems.

Work on individual microexon containing proteins has given us insights in their significance, however the comprehensive characterization of microexons will clearly inform future studies.

## 4.11 A subset of PH domains contain microexons

Our data clearly demonstrate the functional importance of a microexon splice variant in the PH domain of cytohesin-1. There are hundreds of microexons in proteins that regulate membrane trafficking and the cytoskeleton. However, the functional significance of many of these remains largely unexplored. To determine whether other PH domains might have a similar regulation by alternative splicing, we cross-referenced a list of 206 PH domain containing proteins (HGNC) with 696 genes containing microexons (Irimia et al., 2014). We identified 32 proteins that were present in both lists and determined the location of the microexon within each of these proteins and the microexon position relative to the PH domain. We found that only cytohesin family members contained a 3-nucleotide exon that when spliced in, results in the addition of a glycine residue. However, four other proteins had microexons in their PH domains (Figure 4.4). Notably, two of these, ACAP2 and RASA2, contained microexons within the specificity determining regions of the PH domain. However, as of publication, whether the PH domains of either ACAP2 or RASA2 bind phosphoinositide headgroups is unknown.

Interestingly, ACAP2 is an Arf6 GAP. ACAP2 acts downstream from the small GTPase Rab35 to reduce Arf6-GTP levels, integrin trafficking and cell migration. Silencing Rab35 increases Arf6 activation, integrin recycling and cell migration (Allaire et al., 2013). We have found that Rab35 silencing induces rearrangement of the actin cytoskeleton, consistent with Arf6 activation, and enhances HGF-dependent cell migration. Whether ACAP2 silencing has the same effect on HGF-dependent cell migration is untested. If ACAP2 impacts cell migration it would be

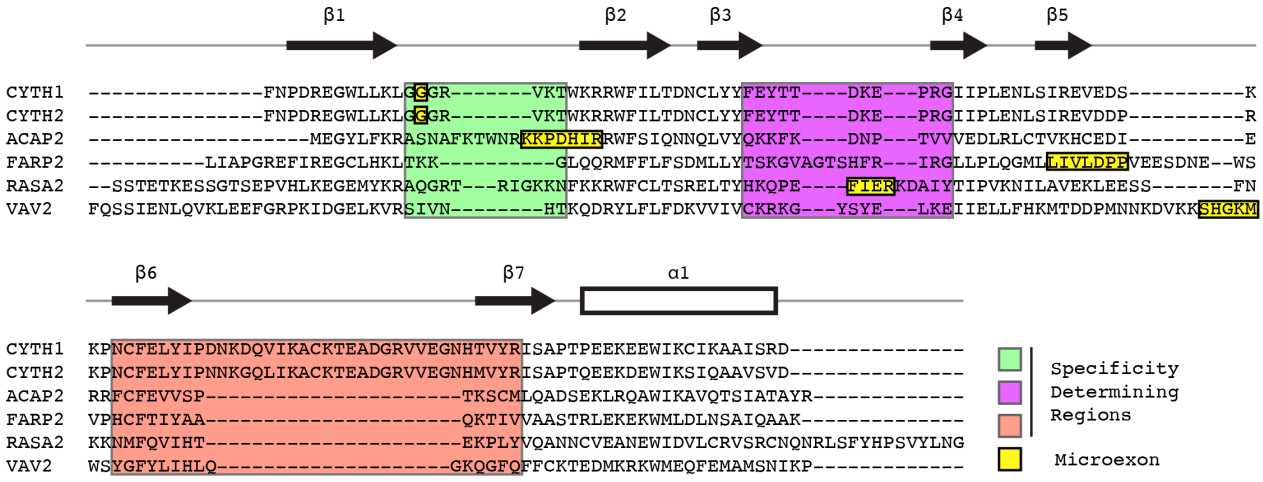



**Figure 4.4 Sequence alignment of microexon containing PH domains**

important to determine the specific splice variant this process. Therefore, microexon splicing might represent a mechanism to modulate the subcellular cycling of Arf6 through cytohesin-1 and ACAP2.

## 4.12 Summary

At the onset of this thesis, therapeutic agents targeting integrin receptors and the Met receptor tyrosine kinase were in clinical trials. Promising preclinical data suggested that integrins and Met would be strong candidates for drugs that affect both tumour growth and metastasis. Given that the majority deaths due to cancer are associated with metastasis, therapeutic treatment of metastasis represents a major goal for cancer treatment. However, several efforts to target integrins or Met failed to show clinical benefit. Despite these failures, integrins and the Met RTK represent important factors in cancer progression. Therefore, a deeper understanding of the biology associated with these molecules may reveal novel therapeutic opportunities or explain the negative outcomes of clinical trials.

In Chapter 2, I demonstrated that the Arf6 effector, GGA3, regulates a subset of integrin receptors. Integrin trafficking is regulated by several factors within the endolysosomal network. However, whether these represent truly independent pathways or function in series is unknown. I have extended previous studies that showed that SNX17 and Arf6 could both regulate β1 integrin trafficking. GGA3 forms a complex with each component of the endolysosomal machinery suggesting that both Arf6 and SNX17 act within the same endolysosomal compartment.

In chapter 3, I demonstrate a novel functional importance of alternative splicing of microexons in cancer cell migration. Limited studies of individual microexons have been conducted, however, the vast majority of microexon containing splice variants are uncharacterized. Most microexons are found in structured domains of proteins suggesting that they have functional importance. We have only begun to understand the functional role of microexons. Large sequencing efforts have permitted the comprehensive identification of alternative splicing events and their relative abundance in healthy tissue. This provides a basis for understanding how microexon splicing might be altered in diseases such as cancer.

Historically, targeting small GTPases has met with limited success. However, the functional importance of these enzymes in cancer and development cannot be ignored. Novel therapeutic agents targeting Arf6 and its exchange factors may provide clinical benefit to patients where

pathways regulated by Arf6 are upregulated. Given the pleiotropic role that Arf6 places in cancer cell migration, it will be important to test these inhibitors in contexts where integrin trafficking or RTK signaling is upregulated.

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