A Dissertation

entitled

The Role of Small GTPase RhoG in Focal Adhesion Dynamics and Contractility.

by

Ashtyn Elizabeth Zinn

Submitted to the Graduate Faculty as partial fulfillment of the requirements for the

Doctor of Philosophy Degree in Biology

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An Abstract of

The Role of RhoG in Focal Adhesion Dynamics and Cell Migration

by

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Cell migration is a critical physiological process that requires the careful cooperation of all cytoskeletal elements within the cell. A multitude of biological events such as embryogenesis, wound healing, tissue maintenance, and cancer metastasis rely upon the ability of the cell to effectively and efficiently migrate. Migration is comprised of four distinct steps: polarization (or reorientation of the cell in the intended direction of migration), protrusion, adhesion, and retraction or contractility. All of these steps are dictated by both internal and external cues, many of which are mechanical in nature. Here, we will primarily focus on the regulation of adhesion and contractility through a structure known as focal adhesions (FA).

FA are a complex of proteins formed to allow the cell physical contact between the cytoskeleton and the extracellular matrix (ECM). FA are dynamic structures involved in force transduction and the indirect regulation of the cytoskeleton, including actin and myosin II activity. FA form at the leading edge of cells to stabilize protrusions and disassemble at a later time to allow the cell to retract and progress forward. Many proteins

have been identified in the regulation of FA formation; however, the underlying mechanisms that regulate adhesion turnover remain poorly understood.

The small family of Rho GTPases are known to play a role in cell migration, including FA dynamics. Several Rho GTPases have been extensively studied in the context of cell migration; however, here we present data showing that the lesser studied RhoG, a Rho GTPase related to Rac, modulates FA dynamics and contractility. Using cell imaging techniques and automated quantification, we have demonstrated that when RhoG expression is silenced (KD), there is a distinct phenotype of increased FA within the cell and a greater number located centrally. Through live imaging, we have shown that this phenotype is the result of increased stability, and therefore longer FA lifetime, in RhoG KD cells. Using specific FA lifecycle markers, we have also shown that these adhesions are compositionally more mature.

During our study, it was also noted that FA within RhoG KD cells aligned in a striking manner within individual cells. Along with this, RhoG KD resulted in distinct differences in cytoskeletal structure, with increased number and thickness of contractile stress fibers (SFs). We believed that the cells were producing a greater amount of contractile force, also attributing to their smaller size and rounder shape. To our surprise, using both 2-dimensional (2D) traction force microscopy and 3-dimensional (3D) contractility assays, we found that RhoG KD cells exerted less force upon the substrate despite a FA and cytoskeletal phenotype that suggests the opposite.

It is well established that FA formation is dependent upon forces exerted by the actin cytoskeleton, however our data provides a novel role for RhoG in the disassembly of FA, a process that is regulated by microtubules (MTs). Using specific inhibitors of MT

dynamics, we found that RhoG KD cells showed a marked difference in their ability to recover after MT inhibition. Taken together, we have provided evidence for two novel roles of RhoG in MT-mediated FA disassembly and contractility.

This dissertation is dedicated to my children, Noelle and Kit. As I reflect, all of my best memories revolve around you. I was made better by you both and I love you infinitely.

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To my co-authors and collaborators: Dr. Yun Chen, Gabe Kreider-Letterman, Debonil Maity, Sahezeel Awadia, and Luis Cedeno-Rosario, thank you for all of your hard work and dedication to helping me complete my publication.

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List of Abbreviations

2D.....2-dimensional
3D.....3-dimensional

ADF .....Actin Depolymerizing Factor
Arp .....Actin-Related Protein

CMSC .....Cortical Microtubule Stabilizing Complex
CTRL .....Control

ECM.....Extracellular Matrix

FA .....Focal Adhesion
FAAI .....Focal Adhesion Alignment Index
FAAS .....Focal Adhesion Analysis Server
FAK .....Focal Adhesion Kinase
FRET.....Fluorescence Resonance Energy Transfer

GAP .....GTPase Activating Protein
GDI .....Rho Guanine Nucleotide Dissociation Inhibitor
GDP .....Guanine Diphosphate
GEF.....Guanine nucleotide Exchange Factor
GFP .....Green Fluorescence Protein
GTP.....Guanine Triphosphate

Ig .....Immunoglobulin

KD.....Knock Down

MAP.....Microtubule Associated Protein
MLC.....Myosin Light Chain
MLCK.....Myosin Light Chain Kinase
MT.....Microtubule
MTOC.....Microtubule Organizing Center

PAK .....p21 Activated Kinase

ROCK .....Rho Kinase

SF .....Stress Fiber
SFAI.....Stress Fiber Alignment Index

WASP .....Wiskott-Aldrich Syndrome Protein
WAVE.....Wasp-family Verprolin Homologous Protein

Chapter 1

Introduction

1.1 Cell migration

Due to the physiological environment in which cells exist, they must constantly produce forces to migrate in response to internal and external cues (Ridley, A., et al., 2003). Cell migration allows for essential biological functions, like wound healing, immune function, and embryogenesis (Ridley, A., et al., 2003; Webb, D., & Horowitz, R., 2003). Abnormalities in cell migration can lead to adverse effects such as mental retardation, cardiovascular disease, cancer metastasis, and chronic inflammatory disease (Ridley, A., et al., 2003; Webb, D., & Horowitz, R., 2003). The complexity of biological systems requires that cells migrate in different ways, depending on the type of cell and their specific function. For example, prokaryotic organisms commonly use flagella or cilia to produce propulsive forces, immune cells lacking complex cytoskeletons utilize amoeba-like migration to quickly reach sites of inflammation, some cells are known to migrate in sheets during embryogenesis or wound closure, and individual fibroblasts display the classical mode of cell migration using their sophisticated and intricate cytoskeleton (Ridley, A., et al., 2003).

While many of the same molecular mechanisms control aspects of these migration modes, they do exhibit fundamental differences. For example, amoeboid migration is achieved through a lack, or vast reduction, of cell adhesion to the surrounding substrate, achieved through an absence of adhesion-related proteins, allowing them to quickly move. Cells that migrate through the classical mode rely heavily on their ability to adhere to the substrate, forming stable contacts, and are therefore rich in adhesion-related proteins (Webb, D., & Horowitz, R., 2003). Distinct leading edges and retracting rears are observed in classical migration, as well as sheet migration. Interestingly, cells at the leading edge of the collective sheet exhibit protrusions and cells at the back exhibit retraction, both accomplished through actin polymerization and myosin-mediated contractility, just as it is in a single cell (Webb, D., & Horowitz, R., 2003). Furthermore, during amoeboid migration, the nucleus is positioned towards the front of the cell, whereas the nucleus moves towards the back in classical migration (Ridley, A., et al., 2003; Webb, D., & Horowitz, R., 2003). Here, we will focus on the classical model of cell migration, illustrated below in Figure 1-1, and its regulating factors.

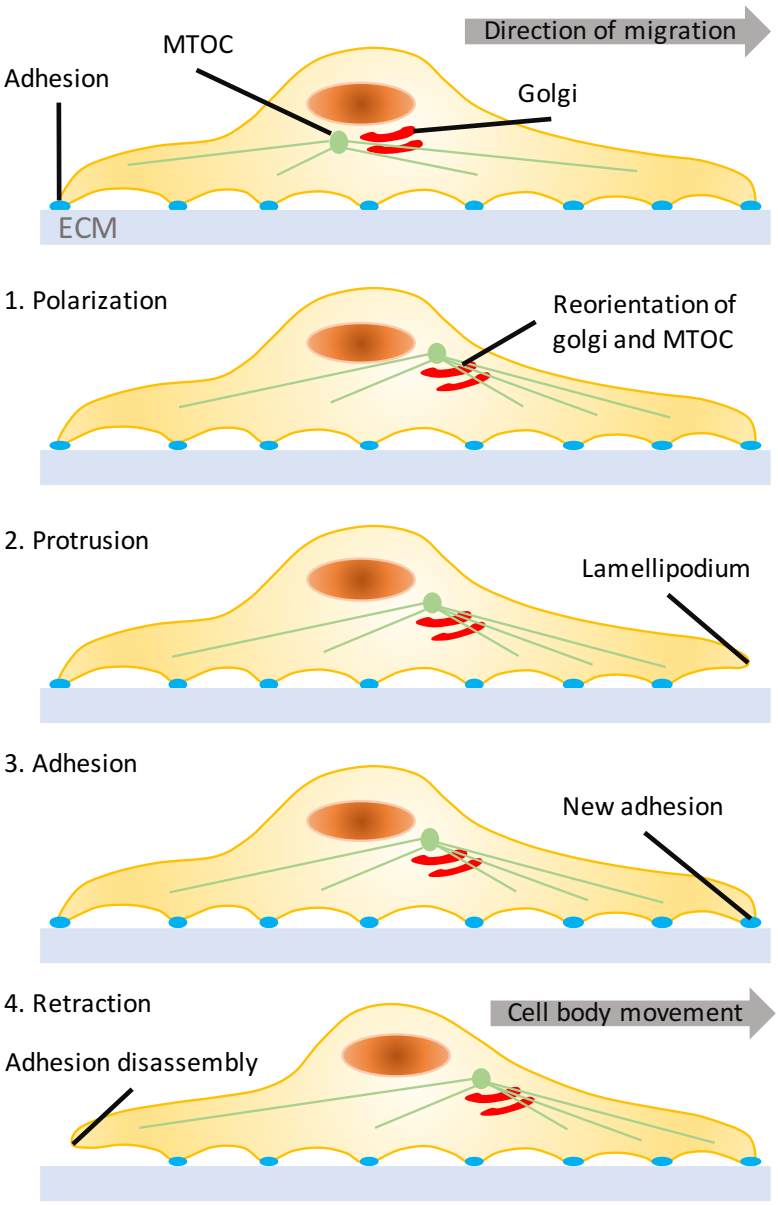

Figure 1-1. Classical cell migration. 1. During polarization the Golgi and MTOC are reoriented in the direction of migration to aid in vesicular transport of components. 2. A protrusion is formed at the leading edge of the cell, known as a lamellipodium. 3. New adhesions are formed at the leading edge to stabilize the lamellipodium. 4. Adhesions are disassembled at the cell rear to allow for retraction and translocation of the cell body

forward. (Modified with permission from *Molecular Cell Biology, 6th Edition; 17-38* under public domain ID:NBK45311.)

Classical cell migration, occurs through a series of steps, including polarization, protrusion, adhesion, and retraction, all requiring integration of cytoskeletal dynamics (Figure 1-1) (Ridley, A., et al., 2003). An imbalance of force, exerted through the actin cytoskeleton via myosin activity and traction points known as adhesions, is important in defining these regions (Burridge, K. & Guilluy, C., 2016). There must be a greater amount of protrusive force due to actin polymerization and adhesion at the leading edge to allow for forward progression, and less at the tail due to adhesion disassembly to allow for retraction and cell body translocation (Burridge, K. & Guilluy, C., 2016). Therefore, these steps are deeply intertwined, and when disrupted, abnormal cell activity can pursue (Burridge, K. & Guilluy, C., 2016). A cell must organize distinctive regions of molecular activity so that these activities are restricted to specific areas within the cell (Ridley, A., et al., 2003). When comparing the knowledge base of molecular mechanisms regulating the protrusive area of the cell versus the retracting region, it is clear that there has been a much greater focus on the leading edge.

1.1.1 Polarization

Polarization occurs in response to an internal or external cue, such as tension or a chemoattractant respectively, which then activates a series of mechano-sensitive proteins to induce actin polymerization, thus driving the formation of a protrusion (Ridley, A., 2011; Ridley, A., et al., 2003). During polarization, the microtubule-organizing center (MTOC), the site of MT nucleation, and Golgi apparatus are oriented towards the leading edge of the cell to assist in rapid vesicular transport and modification of proteins involved in migration

activities, respectively (Ridley, A., et al., 2003). Recruitment of these structures in the direction of migration is dependent upon both cytoskeletal elements: the Golgi apparatus is reoriented in an actin-dependent manner, whereas the MTOC is reoriented in a MT-dependent mechanism (Magdalena, J., et al., 2003). Additionally, the cell undergoes nuclear translocation, physical movement of the nucleus along with the cell body, a process driven by retrograde actin flow and myosin contractility (Gomes, E., et al., 2005). Repositioning of the nucleus aids in placement of the MTOC (Gomes, E., et al., 2005).

1.1.2 Microtubules

Exact positioning of the MTOC, and therefore MT, is especially important due the urgent need for transport of proteins to and from locations throughout the cell for the subsequent steps of migration. This is achieved through the packaging of proteins into small vesicles that travel along MT with the help of molecular motors, kinesin and dynein (Small, J., et al., 2002; Desai, A. & Mitchison, T., 1997). Similar to myosin and actin, kinesin and dynein bind to MT through head domains, "walking" along the MT unidirectionally whilst "carrying" the vesicles.

MT are polarized filaments, with characteristic fast-growing plus ends and slow-growing minus ends. They are able to keep up with the dynamics of a migrating cell by undergoing rapid events of growth and shrinkage, also known as dynamic instability (Small, J., et al., 2002; Desai, A. & Mitchison, T., 1997). MT are comprised of tubulin dimers, which are added upon the stabilization of the MT through binding of microtubule associated proteins (MAPs) (Desai, A. & Mitchison, T., 1997). MAPs not only stabilize the MT and promote polymerization, but they also reduce the rate of tubulin disassociation, commonly referred to as catastrophe (Desai, A. & Mitchinson, T., 1997). Several classes

of proteins have been implicated in MT catastrophe, including MT severing proteins and the kinesins (Desai, A. & Mitchinson, T., 1997). As stated previously, the regulation of MT dynamics is critical in every step of cell migration, from polarization all the way to retraction.

1.1.3 Protrusion

While there are different types of protrusions, here we will exclusively refer to lamellipodia due to the nature of cell types and mode of migration used in this study. Protrusion is carried out through actin polymerization. Actin monomers are added to quick-growing barbed facing the direction of protrusion, whereas slow-growing pointed ends are found further inwards, facing the cell body, and are subject to organized disassembly (Figure 1-2) (Ridley, A., 2011; Ridley, A., et al., 2003). The Wiskott-Aldrich syndrome protein (WASP)/Wasp-family verprolin homologous protein (WAVE) family of proteins, activated at the leading edge, activate the Actin-related protein (Arp)2/3 complex at barbed ends to induce polymerization, driving protrusion forward (Ridley, A., 2011; Ridley, A., et al., 2003). Capping proteins are responsible for halting actin polymerization, thus stopping protrusion formation, and the Actin-depolymerizing factor (ADF)/cofilin family of proteins disassemble older actin filaments at the pointed end so that monomers can be recycled, a common theme in cell migration (Ridley, A., 2011; Ridley, A., et al., 2003).

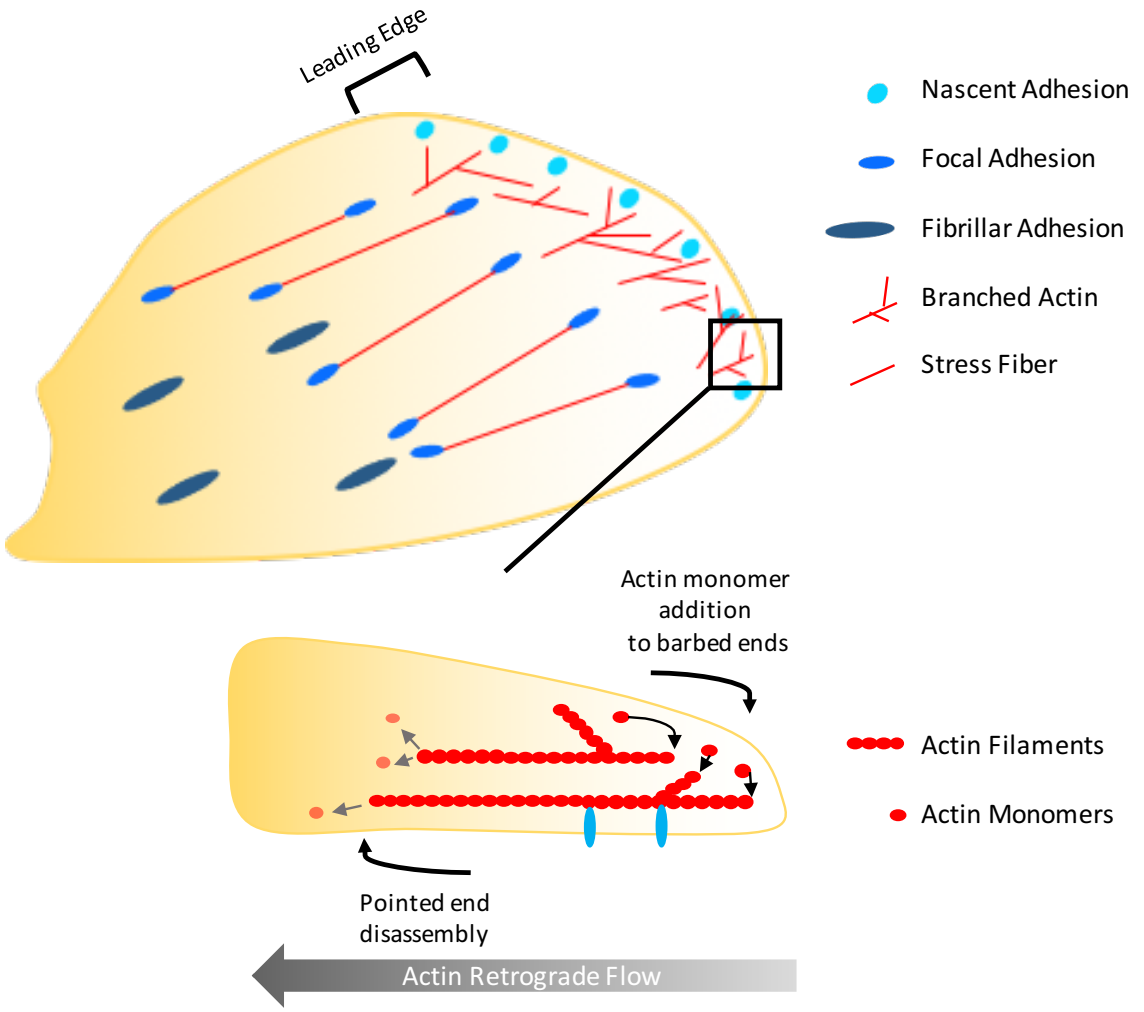

Figure 1-2. Actin polymerization in protrusion. Actin monomers are added to quick-growing barbed facing the direction of protrusion. Nearer to the cell body, slow-growing pointed ends are subject to organized disassembly. Actin polymerization drives the formation of protrusions, but force from the plasma membrane causes retrograde flow. Adhesions must anchor actin filaments to slow retrograde flow protrusion (Elosegui-Artola, A., et al., 2018; Case, L. & Waterman, C., 2015; Ridley, A., 2011) (Image modified with permission from Gardel, M., et al., 2010 with permission from CCC ISSN: 1530-8995).

However, it is important to note that the force produced by rapid actin polymerization at the leading edge is not enough to drive protrusion (Elosegui-Artola, A., et al., 2018; Case, L. & Waterman, C., 2015). Actin polymerizes through a Brownian ratchet mechanism, which is a mechanical property where a structure rotates, or actin monomers are added at one end in this case, but is prevented from rotation in the opposite direction through some type of anchor, adhesions in this case (Elosegui-Artola, A., et al., 2018; Case, L. & Waterman, C., 2015; Ait-Haddou, R. & Herzog, W., 2003). The polymerization of actin results in a very thin sheet that is not tethered to the ECM, pushing against the plasma membrane, but also results in movement of the actin network backwards in the direction ofs the cell body, a process known as actin retrograde flow (Elosegui-Artola, A., et al., 2018; Case, L. & Waterman, C., 2015; Alexandrova, A., et al., 2008). The opposing force from the plasma membrane and actin retrograde flow must be overcome in order to drive protrusion forward (Elosegui-Artola, A., et al., 2018; Case, L. & Waterman, C., 2015). This is achieved through the anchoring of actin filament by adhesions (Figure 1-2, bottom panel), discussed at length in Section 1.1.4.

1.1.3.1 The actin clutch hypothesis

Adhesions provide an anchor site for the actin cytoskeleton to the ECM (Geiger, B. & Yamada, K., 2011). The actin clutch hypothesis proposes focal adhesion (FA) proteins act as a mechanical clutch, allowing actin filaments at the leading edge to transmit force to the extracellular matrix (ECM) through integrins without direct contact, thus slowing down actin retrograde flow (Elosegui-Artola, A., et al., 2018; Case, L. & Waterman, C., 2015). The FA proteins are engaged, as a clutch, to anchor actin, allowing force to be maintained and withstand opposing force from the plasma membrane (Elosegui-Artola, A., et al., 2018;

Case, L. & Waterman, C., 2015). Actin polymerization can then continue, allowing for further protrusion (Elosegui-Artola, A., et al., 2018; Case, L. & Waterman, C., 2015). If actin is able to polymerize but is not anchored properly, then the cell will not be able to overcome actin retrograde flow, thus minimizing its protrusive ability (Elosegui-Artola, A., et al., 2018; Case, L. & Waterman, C., 2015).

1.1.4 Retraction

The other distinct region of molecular activity must define the tail of the cell, where retraction occurs to allow forward progression and translocation of the cell body. It is believed that active suppression of protrusion promoters, such as the Rho GTPase Rac, defines the tail region (Ridley, A., et al., 2003). It is also known that MT-mediated FA disassembly allows for the cell to detach from the ECM (Broussard, J., et al., 2008; Ezratty, E., et al., 2005; Kaverina, I., et al., 1998; Kaverina, I., et al., 1999). This is accomplished through a complex known as the cortical microtubule stabilizing complex (CMSC), which aids in targeting microtubules (MT) to FA to facilitate disassembly (Bouchet, B., et al., 2016). MT mediate the recycling of protein components through vesicular transport, either back to the leading edge, for the formation of new adhesions, or to other adhesive structures to carry out other function (Ridley, A., et al., 2003; Webb, D., et al., 2002).

Myosin-mediated contractility within the actin cytoskeleton causes retraction of the cell body forward (Ridley, A., et al., 2003). Non-muscle myosin acts in a similar manner to the classical actin-myosin contractility model in smooth muscle cells: myosin light chain kinase (MLCK) phosphorylates myosin II motor proteins activating them to slide actin filaments, causing contraction (Amano, M., et al., 1996; Chrzanowska-Wodnicka & Burridge, K., 1996; Kimura, K., et al., 1996). Actomyosin contractility is not only

important for the retracting rear though, it is also critical for the formation of adhesions (Burridge, K. & Guilluy, C., 2016).

1.1.5 Focal adhesions

FA serve as the physical link between the actin cytoskeleton of a migrating cell and the ECM through integrins (Geiger, B. & Yamada, K., 2011). They are traction points where the cell can exert or release force (Gardel, M., et al., 2010), tasks that are accomplished through their dynamic nature and maturation (Geiger, B. & Yamada, K., 2011). FA exhibit distinct lifetimes with assembly, stability, and disassembly stages (Parsons, J., et al., 2010) (Figure 1-3). They are formed at the leading edge for protrusion stabilization (Zaidel-Bar, et al., 2003). Their maturation is a force-dependent process during which time they appear to translocate inward due to cell progression over the adhesion (Ridley, A., et al., 2003). Eventually, they are disassembled at some point behind the leading edge (Ridley, A., et al., 2003). Disassembly also allows for the formation of new adhesions through the recycling of components back to the leading edge, or in some cases other types of adhesive structures such as invadopodia, discussed below (Block, M, et al., 2008).

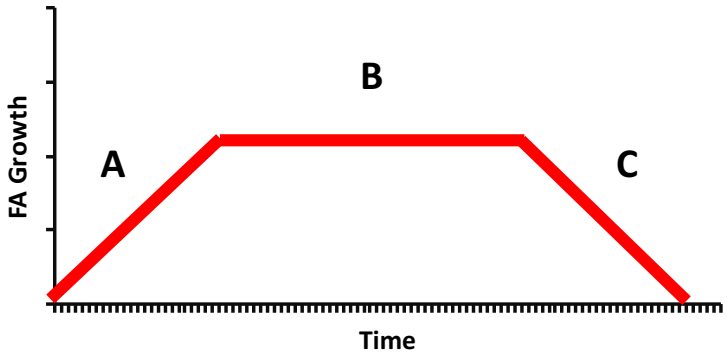

Figure 1-3. FA Lifetime. A graph representing the growth of a FA over time. (A) FA assembly occurs at the leading edge. (B) The FA may encounter a period of maturation and stability. (C) Eventually, the adhesion is disassembled at the retracting rear.

In a process known as maturation, various proteins are recruited to adhesions throughout their lifetime, allowing them to serve different functional purposes (Figure 1-4) (Geiger, B. & Yamada K., 2011; Webb, D., et al., 2002; Zamir, E. & Geiger, B., 2001). FA are able to exert and withstand different amounts of force depending on their maturation stage, contributing to the imbalance that allows for cell migration (Beningo, KA, et. al., 2001). In turn, recruitment of proteins to adhesions is based on force thresholds (Galbraith, C.G, et al., 2002). This is generally achieved through conformational changes of different components, leading to recruitment activities (Burridge, K. & Guilluy, C., 2016; Parsons, J., et al., 2010). The gradient of contractile forces and tension throughout the cell that adhesions experience throughout their lifetime is reflected in their composition from "toe" to "heel" (Wolfenson, H., et al., 2009); however, all adhesion maturation stages have several things in common, one being that they are linked to the ECM through heterodimeric integrins comprised of an alpha and a beta subunit (Geiger, B. & Yamada, K., 2011). Integrins engage with ECM ligands, providing a link for intracellular signaling through

their cytoplasmic tails (Geiger, B. & Yamada, K., 2011). Integrin subunits show specificity for ECM substrates and are characteristic of the maturation stages (Geiger, B. & Yamada, K., 2011).

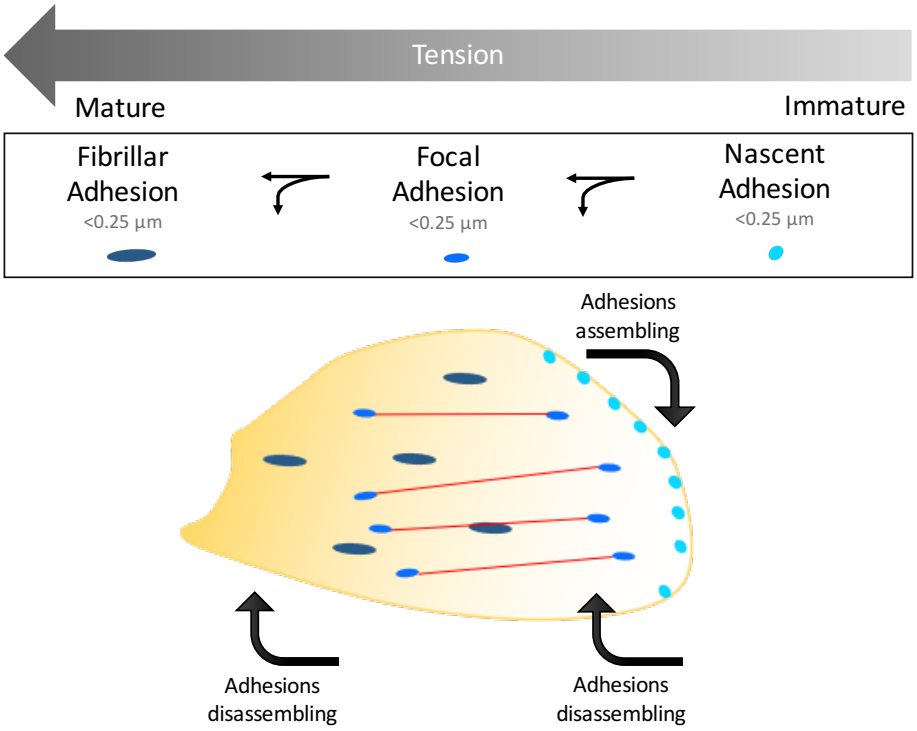

Figure 1-4. Focal adhesion maturation. Nascent adhesions are formed at the leading edge, maturing into focal adhesions (tethered to stress fibers), and finally fibrillar adhesions. Maturation is a force dependent process, and only a fraction of adhesions will mature, many are disassembled at some point behind the leading edge. (Image modified with permission from Gardel, M., et al., 2010 with permission from CCC ISSN: 1530-8995.)

Nascent adhesions are formed at the leading edge, beginning when actin filaments cluster in response to mechanical cues (Zaidel-Bar, R., et al., 2003). The tension-dependent nature of this event was previously demonstrated where intrinsic contractility was inhibited, and artificial external tension was applied resulting in SF and adhesion formation

despite the absence of internal contractility (Riveline, D., et al., 2001). The contraction of actin filaments causes αvβ3 integrin clustering at the plasma membrane (Zaidel-Bar, R., et al., 2004). Talin, vinculin, paxillin, and focal adhesion kinase (FAK) are amongst the first proteins observed in nascent adhesions (Zaidel-Bar, R., et al., 2004). FAK is activated during this time resulting in a cascade of events, including the suppression of FA turnover through inhibition of Rho activity, which builds the nascent adhesion (Ren, X., et al., 2000). Vinculin and talin, both mechanosensing proteins, bind directly to actin, providing a mechanical bridge between integrins and the cytoskeleton (Humphries, J., et al., 2007; Giannone, G., et al., 2003). These adhesions display concentrated areas of tyrosine phosphorylation and are very dynamic, quickly turning over or maturing, a necessary characteristic due to the changing morphology of cells at the leading edge (Zaidel-Bar, R., et al., 2004; Chrzanowka-Wodnicak, M. & Burridge, K., 1994).

As the cell continues to protrude, a fraction of the nascent adhesions matures into FA due to tension exerted by newly bundled actin SF (Ridley, A., 2011; Burridge, K. & Guilluy, C., 2016). FA become tethered to SF, providing the anchor site for actin polymerization (Zimerman, B., et al., 2004). They translocate behind the lamellipodia as the cell body moves forward over them (Zaidel-Bar, R., et al., 2004). These adhesions contain αvβ3 integrins, and are more stable than nascent adhesions, a necessary characteristic for their role in protrusion stabilization (Zaidel-Bar, R., et al., 2004). Additionally, in a mechano-sensitive process, zyxin is recruited to FA (Zaidel-Bar, R., et al., 2004).

The most mature adhesions, fibrillar adhesions, are characterized by their tensin content and marked length when compared to nascent adhesions or FA (Zaidel-Bar, R., et

al., 2004). Fibrillar adhesions are associated with the ECM substrate fibronectin, evident by the presence of fibronectin-specific α5β1 integrins (Zaidel-Bar, R., et al., 2004). Additionally, fibrillar adhesions play a role in ECM remodeling, creating structural platforms for the cell to migrate on (Katz, B., et al., 2000). A great deal of work has been put into determining the exact sequence in which proteins are recruited to adhesions, however there is very little known about the mechanisms of disassembly events.

However, MT targeting to FA is one mechanism that has been identified in FA disassembly (Broussard, J., et al., 2008; Ezratty, E., et al., 2005; Kaverina, I., et al., 1998; Kaverina, I., et al., 1999). This is accomplished through the previously mentioned CMSC, the site at which MT are captured near the FA to be disassembled (Noordstra, I. & Akhmanova, A., 2017). The CMSC serves as a physical link between the FA and MT (Noordstra, I. & Akhmanova, A., 2017). When the CMSC is disrupted, MT outgrowth is in turn disrupted, causing uncontrolled growth at the cell periphery, likely due to the lack of capturing at FA (Bouchet, B., et al., 2016). A defect in the CMSC could result in impaired FA disassembly, leading to accumulation of FA.

While it is important to characterize individual adhesion composition and characteristics, it is also worth noting global presentation of adhesions. For a cell to effectively migrate, it must coordinate its adhesion population in a manner that promotes productive migration (Parons, J., et al., 2010). A classic example is cells under shear stress, or fluid being passed over them in a manner similar to blood flow. Cells under these conditions align their cytoskeleton, and therefore adhesion population, to allow for sufficient adhesion to the blood vessel, and movement to a secondary site (Galbraith, C., et al., 1998). This is achieved through the coordinated molecular activity of cytoskeletal

regulating proteins, and maintenance of different adhesion populations within the individual cell (Galbraith, C., et al., 1998).

1.1.6 Invadopodia

As mentioned above, upon FA disassembly protein components can be recycled for use in structures other than nascent adhesion. One such area of reuse is in invadopodia, actin-rich adhesive structures that are used to degrade the ECM (Block, M., et al., 2008). invadopodia formation depends on the disassembly of FA, or other adhesion structures, and therefore it is believed that the balance between FA and invadopodia is important in regulating normal versus invasive cell migration (Block, M., et al., 2008). For this reason, it is important to understand the dynamics between FA and invadopodia.

Invadopodia are initially observed as small areas of actin and cortactin concentration, commonly referred to as puncta (Gimona, M., et al., 2008). Much like FA, other invadopodia-associated proteins are then recruited, such as paxillin and vinculin, also important components in FA, and proteinases, used in ECM degradation (Gimona, M., et al., 2008). Many proteins have been implicated in the regulation of both FA and invadopodia, specifically a family of proteins known as the Rho GTPases. We have recently shown that the lesser studied RhoG plays a role in invadopodia formation (Goicoechea, S., et al., 2017). Due to the intertwined nature of invadopodia and FA, this finding lead us to believe that RhoG may also regulate FA formation.

1.2 The Rho GTPases in cell migration.

The Rho family of small GTPases are heavily implicated in FA dynamics and cell migration through their regulation of the actin cytoskeleton and MT network (Tcherkezian, J. & Lamarche-Vane, N., 2007; Rossman, K. & Sondek, J., 2005). There are 20 Rho

GTPases in the human genome that function as molecular switches, maintaining either an active or inactive conformation (Tcherkezian, J. & Lamarche-Vane, N., 2007; Rossman, K. & Sondek, J., 2005). Rho guanine nucleotide exchange factors (GEFs) activate Rho GTPases by catalyzing the exchange of bound guanine diphosphate (GDP) for guanine triphosphate (GTP), whereas GTPase activating proteins (GAPs) stimulate intrinsic activity to hydrolyze bound GTP, resulting in GDP (Figure 1-5) (Tcherkezian, J. & Lamarche-Vane, N., 2007; Rossman, K. & Sondek, J., 2005). Once activated, Rho GTPases regulate downstream effectors to carry out various actions (Tcherkezian, J. & Lamarche-Vane, N., 2007; Rossman, K. & Sondek, J., 2005). Each Rho GTPase can be regulated by multiple GEFs and GAPs and can regulate multiple downstream effectors, resulting in a complicated network of activity (Tcherkezian, J. & Lamarche-Vane, N., 2007; Rossman, K. & Sondek, J., 2005). Additionally, Rho GTPases are regulated through post-translational prenylation at their C-termini, localizing them to the membrane (Lawson, C. & Ridley, A., 2018). Rho guanine nucleotide dissociation inhibitors (GDIs), bind to these modifications to dissociate Rho GTPases from the membrane (Garcia-Mata, R., et al., 2011). Several Rho GTPases have been thoroughly studied in the context of FA and cell migration, including Rho, Rac, and Cdc42.

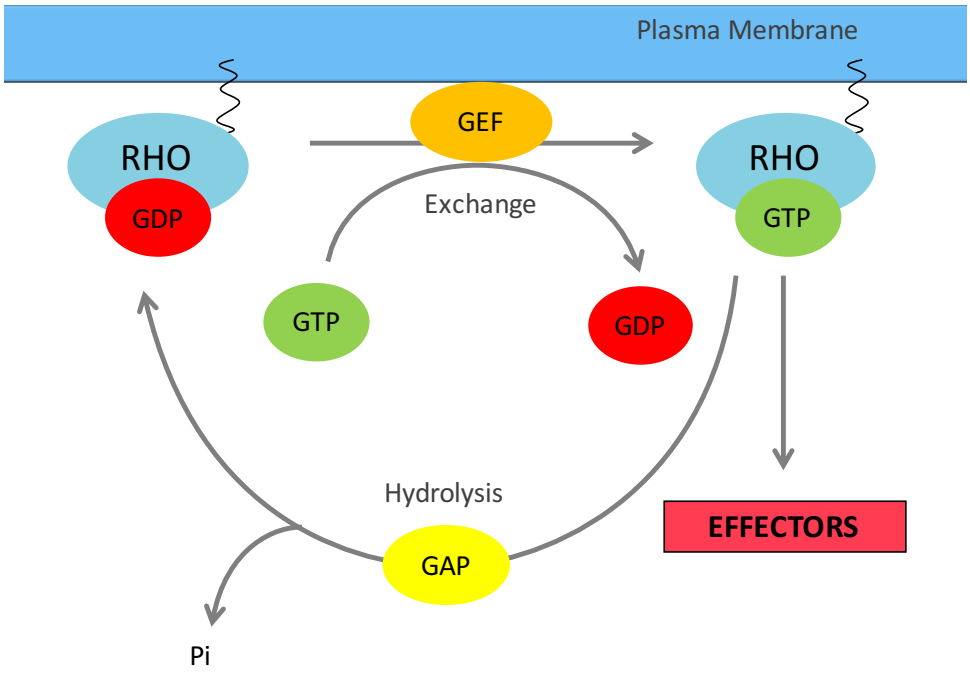

Figure 1-5. The regulation of RhoGTPases by GEFs and GAPs. RhoGEFs activate RhoGTPases by catalyzing the exchange of bound GDP for GTP, resulting in regulation of downstream effectors. RhoGAPs inactivate RhoGTPases by stimulating intrinsic GTPase activity to hydrolyze bound GTP to GDP.

Cdc42 is most well known for its role in polarization, helping the cell to establish specific areas of protrusion (Ridley, A., et al., 2003). Through downstream effectors, Cdc42 regulates localization of actin machinery, and orientation of the MTOC and the Golgi, all essential events for the establishment of polarity (Ridley, A., et al., 2003). Cdc42 has also been implicated in the formation of invadopodia through regulation of N-WASP and the Arp2/3 complex, promoting actin polymerization (Mader, C., et al., 2011).

Through a positive feedback loop at the leading edge, integrin signaling causes Rac activation, which drives actin polymerization, integrin clustering, and therefore the formation of protrusions (Ridley, A., 2015). Rac1 couples with p-21 activated kinase (PAK), inactivating cofilin, to promote actin polymerization (Ridley, A., 2015).

Additionally, PKCα activity localizes Rac1 activity to the leading edge, restricting areas of protrusion (Ridley, A., 2015). Several GEFs have been shown to regulate Rac in lamellipodia formation to affect downstream effectors such as those in the WAVE complex, mentioned previously (Ridley, A., 2015). On the other hand, RhoA activity is transiently suppressed at the leading edge, inhibiting contractility to allow for protrusion (Ridley, A., 2015).

However, Rho does play a role in the stabilization of lamellipodia through the maturation of FA (Ridley, A., 2015). Rho promotes contractility through its downstream effector Rho-kinase (ROCK), which increases MLC phosphorylation by inhibiting MLC phosphatase, thus promoting contractility (Ridley, A., 2015). The internal tension caused by Rho activity increases bond strength of adhesions, resulting in maturation (Ridley, A., 2015). This contractility is not only important in adhesion formation, maturation, and stabilization, but also in retraction of the cell rear.

1.2.1 RhoG

Here, we will focus on a member of the Rac-like subfamily, RhoG, which has a high percentage of sequence similarity to Rac and Cdc42. RhoG was first identified as an upregulated gene in response to serum stimulation (Vincent, S, et al., 1992). It is unique from Rac and Cdc42 in that it does not bind to CRIB, REM2, or POR-1 domain partners (Wennerberg, K., et al., 2002). There is relatively little known about the function of RhoG; however, RhoG has been identified in promotion of cancer cell migration and invasion (Hiramoto-Yamaki, N., et al., 2010; Fortin, S., et al., 2013; Kwiatkowska, A., et al., 2012). Despite there being evidence that RhoG plays a role in cell migration, the mechanism was

not characterized in detail (Hiramoto-Yamaki, N., et al., 2010; Meller, J., et al., 2008; Hiramoto, K., et al., 2006; Katoh, H., et al., 2006).

The activation of Rac through DOCK180 or DOCK4/ELMO is the most well-known function of RhoG. RhoG acts as an upstream activator of ELMO (Katoh & Negishi, 2003), which binds to DOCK180 or DOCK4, causing a conformational change and localization of the DOCK180 or DOCK4/ELMO complex to the membrane which acts as a Rac-specific GEF (Gumienny, T., et al, 2001; Brugnera, E., et al, 2002; Hiramoto, K., et al, 2006). The DOCK180 or DOCK4/ELMO/Rac complex localizes to lamellipodia and promotes migration (Grimsley, T., et. al., 2004); however, RhoG can promote lamellipodia formation in a Rac-independent manner (Samson, T., et al., 2010; Meller, J., et al., 2008; Wennerberg, K., et al., 2002; Gauthier-Rouviere, C., et al., 1998).

RhoG is broadly expressed in tissues throughout the human body (Fagerberg, L., et al., 2013). RhoG knockout has mild effects in mice, including increase in serum immunoglobulin G1 (IgG1) and IgG2, and an increase in humoral response to thymus-dependent antigengs (Vigorito, E., et al., 2004). Some differences in B and T cell proliferation were also observed, however, their development was unaffected (Vigorito, E., et al., 2004). While these findings are underwhelming, due to the number of Rho GTPases in the genome, and their sequence similarities, redundancy is a likely reason. Interestingly, deregulation of the GEFs and GAPs have been observed in cancerous states (Vigil, D., et al., 2010). This suggests that delineation of a pathway, including GEF and or GAP and the Rho GTPase, is important when considering Rho GTPases as therapeutic targets.

While no RhoG-specific GAPs have been identified, there are several known RhoG GEFs, including Ephexin4, Kalirin, PLEKHG6, P-Rex1, SGEF, TRIO, Vav1, Vav2, and

Vav3 (Damoulakis, G., et al., 2014; D'Angelo, R., et al., 2007; Ellerbroek, S., et al., 2004; May, V., et al., 2002; Bellanger, J., et al., 2000; Blangy, A., et al., 2000; Movilla, N. & Bustelo, X., 1999; Schuebel, K., et al., 1998). Of these GEFs, Ephexin4, SGEF, and TRIO have all been associated with regulating RhoG in the context of cell migration. Ephexin4 binds to and activates RhoG, causing downstream regulation of Rac1 through DOCK4, promoting cell migration (Hiramoto-Yamaki, N., et al., 2010). SGEF has been further characterized, finding that specific phosphorylation of SGEF at Y530 by Src blocks its interaction with RhoG, reducing cell migration (Okuyama, Y., et al., 2016). Lastly, the N-terminal GEF domain of TRIO activates RhoG, which regulates Rac1 and Cdc42 in cell migration (Blangy, A., et al., 2000). While RhoG is often associated with Rac activity, it was established early-on that downstream effects of RhoG are not solely dependent on Rac activity (Wennerberg, K., et al., 2002).

Others have shown that the MT network can mediate RhoG activity, an effect that was not seen with Rac1 or Cdc42 (Gauthier-Rouviere, C., et al., 1998). Additionally, the activation of RhoG by TRIO was MT-dependent (Gauthier-Rouviere, C., et al., 1998). While neither protein was shown to directly interact with MT, their localization to the cell periphery was lost when MT were disrupted (Gauthier-Rouviere, C., et al., 1998). It was suggested that this effect was due to a MT-dependent transport of TRIO and RhoG to the cell periphery (Blangy, A., et al., 2000). Further strengthening its connection to MT, RhoG was found to directly bind to kinectin, an anchor for kinesin, the MT motor protein (Vignal, E., et al., 2001). In subsequent chapters, we will further explore the role of RhoG in regulating FA dynamics through a MT-mediated pathway.

Chapter 2

The small GTPase RhoG regulates microtubule-mediated focal adhesion disassembly.

The following work has been previously published by Ashtyn Zinn, Silvia Goicoechea, Gabriel Kreider-Letterman, Debonil Maity, Sahezeel Awadia, Luis Cedeno-Rosario, Yun Chen, and Rafael Garcia Mata in *Scientific Reports* (2019).

2.1 Abstract

Focal adhesions (FA) are a complex network of proteins that allow the cell to form physical contacts with the extracellular matrix (eCM). FA assemble and disassemble in a dynamic process, orchestrated by a variety of cellular components. However, the underlying mechanisms that regulate adhesion turnover remain poorly understood. Here we show that RhoG, a Rho Gtpase related to Rac, modulates FA dynamics. When RhoG expression is silenced, FA are more stable and live longer, resulting in an increase in the number and size of adhesions, which are also more mature and brillar-like. Silencing RhoG also increases the number and thickness of stress fibers, which are sensitive to blebbistatin, suggesting contractility is increased. The molecular mechanism by which RhoG regulates adhesion turnover is yet to be characterized, but our results demonstrate that RhoG plays a role in the regulation of microtubule-mediated FA disassembly.

2.2 Introduction

Cell migration is a dynamic process involved in organogenesis, tissue maintenance, and cancer metastasis that depends on the ability of the cell to form physical contacts to the surrounding extracellular matrix (ECM)1. These contacts, known as focal adhesions (FA), are mechanosensitive structures that link the ECM to the actin cytoskeleton through integrin receptors2. The assembly and disassembly of FA drive cell migration through force transduction and the indirect regulation of actin polymerization and myosin II activity3. FA are formed at the leading edge, enabling the cell to adhere and stabilize protrusions, and must be disassembled behind the lamella or at the rear to allow the cell to detach, contract, and translocate forward1.

The composition of FA changes in response to internal and external mechanical tension, a process known as maturation2,4. FA formation begins at the front of the cell during initial protrusion5, and can be characterized by enriched areas of tyrosine-phosphorylation. These new adhesions, known as nascent adhesions, will continue to mature, growing in size, to become focal complexes which also contain vinculin6. Focal complexes stabilize the newly formed protrusions through their linkage to actin stress fibers (SF)7. Lastly, tensin-containing fibrillar adhesions aid the cell in ECM remodeling, providing a structural platform for migration8. The assembly and maturation of adhesions is a highly regulated process that has been well characterized. However, the molecular mechanisms that control FA disassembly are not well understood.

The Rho family of small GTPases plays a central role in the regulation of virtually every aspect of cell migration, including FA and stress fiber formation, lamellipodia dynamics, and actomyosin contractility9. Unfortunately, of the 20 members of the Rho

GTPase family, most studies have focused on the role of the three best-characterized ones, RhoA, Rac1 and Cdc42. However, there are other lesser studied Rho GTPases, such as RhoG, which also play a role in cell migration10,11,12,13. Recently, we found that RhoG plays an important role in the regulation of invadopodia turnover14. Invadopodia are actin-rich adhesive structures used by cancer cells to degrade the ECM, and are built using many of the same components as FA15. This finding led us to believe that RhoG may regulate non-invasive cell migration as well, through FA dynamics. We found that RhoG regulates FA turnover, specifically the lifetime and maturation of FA. Our results also show that microtubule-mediated FA disassembly is involved in the regulation of FA turnover by RhoG.

2.3 Results

2.3.1 RhoG regulates focal adhesion formation and cell morphology

To characterize the role of RhoG in FA, we used a previously established SUM159 breast cancer cell line in which RhoG expression was stably silenced (RhoG KD), and control cells expressing a non-targeting shRNA (CTRL)14. We also rescued the expression of RhoG in RhoG KD cells using an adenovirus encoding a shRNA resistant myc-tagged wild-type RhoG (Rescue) (Fig. 2-1c)14. We then plated CTRL, RhoG KD and Rescue cells on uncoated glass coverslips, or coated with either collagen or fibronectin, and stained them for vinculin as a marker for FA. Our results showed that RhoG KD cells appeared to be slightly smaller and rounder in shape than control cells, and had more FA, in particular at the center of the cells (Fig. 2-1a,b). These results were reproducible regardless of the substrate tested. The cell shape/size and FA phenotypes

were rescued by re-expressing myc-RhoG, indicating that the effects observed are due to the specific depletion of RhoG.

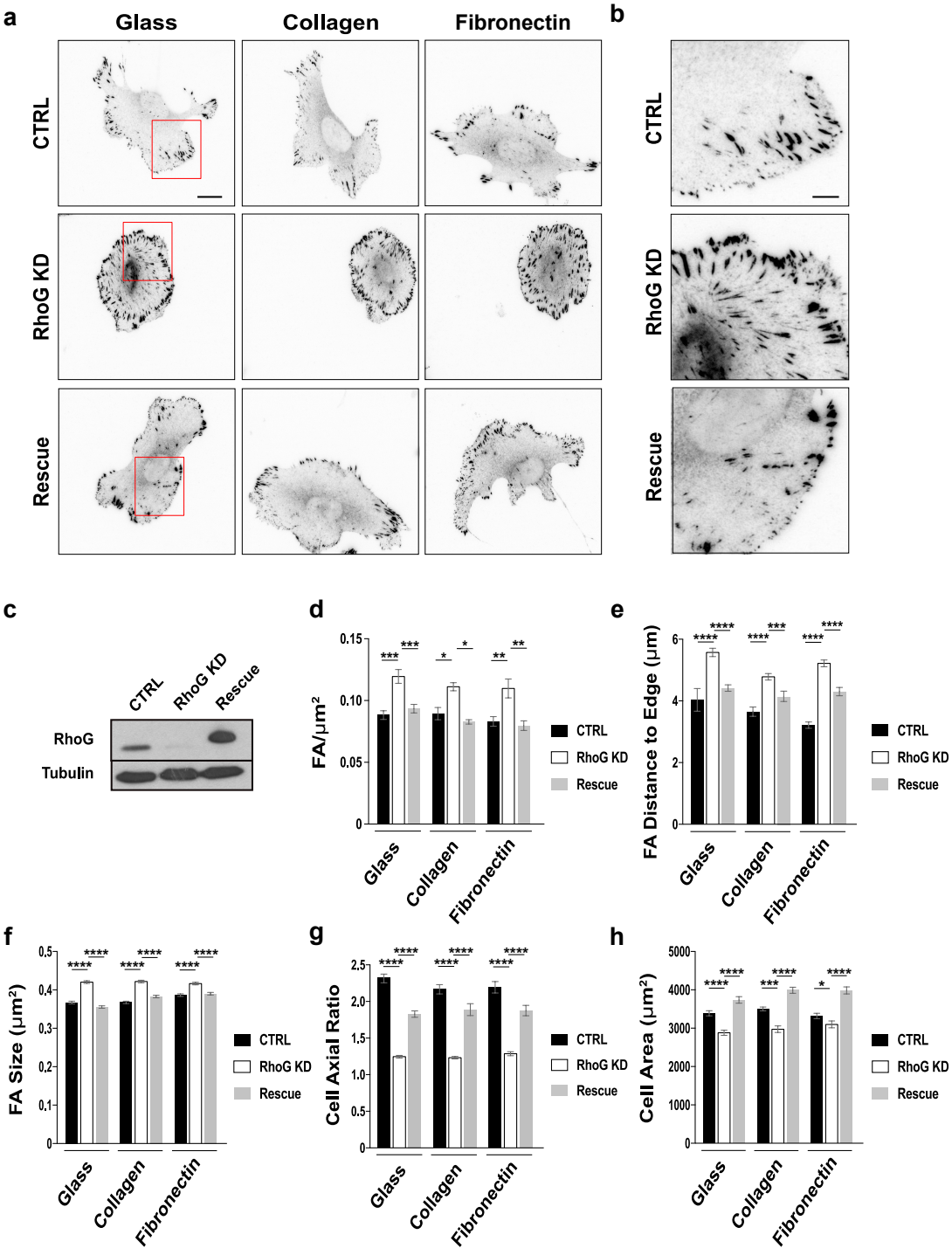

Figure 2-1 RhoG KD affects focal adhesion number and size, and cell morphology.

RhoG expression was stably silenced in SUM159 cells using lentiviral shRNA (KD) and rescued transiently in RhoG KD cells using shRNA-resistant myc-RhoG wt (Rescue). Control cells stably express a non-targeting shRNA (CTRL). (**a**) Cells were plated on either non-coated glass coverslips or coverslips coated with collagen or fibronectin. Cells were then fixed and stained for focal adhesions using anti-vinculin antibodies. Boxes indicate areas enlarged in panel b. Scale bars: 20 μm. (**b**) Enlarged areas from highlighted regions in panel a. Scale bars: 1 μm. (**c**) Lysates from CTRL, RhoG KD and Rescue cells were immunoblotted with anti-RhoG antibodies. Tubulin was used as a loading control. (**d**–**h**) Focal adhesion properties were analyzed on all substrates using the Focal Adhesion Analysis Server (FAAS)16. (**d**) Number of adhesions per μm2. (**e**) Distance between the centroid of the focal adhesion and the edge of the cell. (**f**) Adhesions size (μm2). (**g**) Cell axial ratio. (**h**) Cell area (μm2). Results are shown as mean ± SEM (error bars). All data are results of 4 independent experiments where 20 cells per experiment were analyzed ($n = 80$). *$p < 0.01$, **$p < 0.002$, ***$p < 0.001$, ****$p < 0.0001$.

To confirm these observations, we quantified the number, size, and other FA characteristics using ImageJ and The Focal Adhesion Analysis Server (FAAS), a MatLab based algorithm designed to identify and quantify FA characteristics16. Quantification confirmed that when RhoG expression was silenced, there was a significant increase in the number of FA (Fig. 2-1d), as well as a significant difference in their localization, being found more frequently in the center of the cell in RhoG KD cells (Fig. 2-1e). We also found that FA are significantly larger in RhoG KD cells (Fig. 2-1f). Re-expression of RhoG restored the number, localization and size of FA to normal levels (Fig. 2-1d,e). A similar phenotype was observed in human MRC5 fibroblasts upon the knockdown of

RhoG (Supplemental Fig. 2-10). We also measured the effect of silencing RhoG on cell morphology. We confirmed that RhoG KD cells were significantly rounder (Fig. 2-1g) and smaller (Fig. 2-1h). These changes in cell morphology may be directly related to the FA phenotype observed, which may compromise the ability of the cells to adhere and spread properly.

2.3.2 RhoG silencing increases FA lifetime

FAs form at the leading edge and translocate inward towards the center of the cell as they mature. Most adhesions are rapidly turned over at the edge of the cell right after they form and never progress to mature FA. The increased number of FA in RhoG KD cells suggests that RhoG may be playing a role in the regulation of FA turnover. To explore this possibility, we measured FA assembly and disassembly rates in CTRL and RhoG KD cells using time-lapse microscopy in cell expressing GFP-paxillin (Videos not included). Figure 2-2a shows sample adhesions tracked in CTRL (left panel) and RhoG KD (right panel) cells. Our results showed that even though there was a trend indicating faster assembly rates in RhoG KD cells, there was no significant difference in the rate of assembly or disassembly of FA when RhoG was silenced (Fig. 2-2b,c).

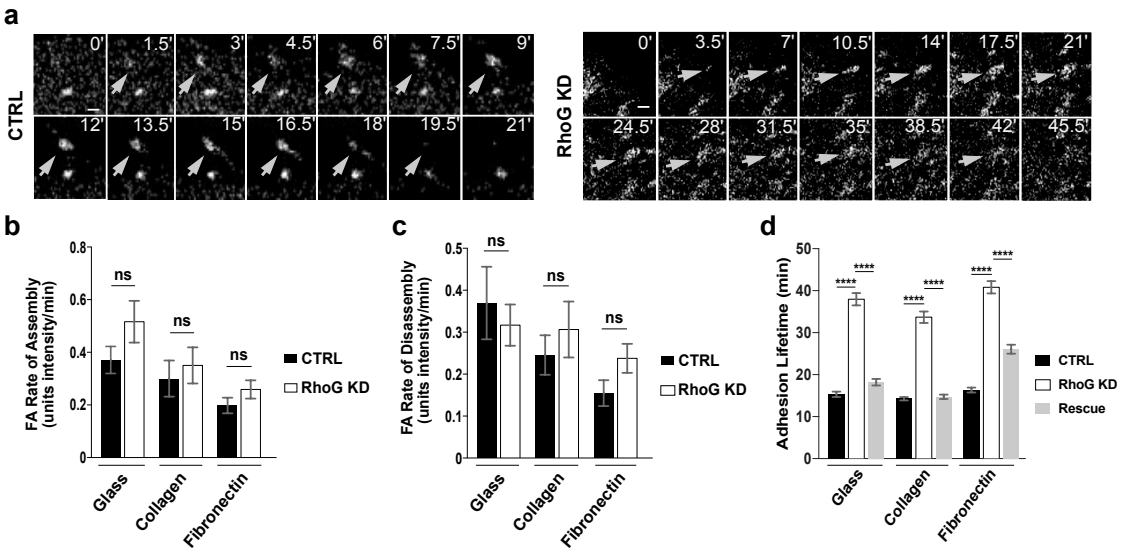

Figure 2-2 RhoG regulates adhesion lifetime, but not the rate of assembly and disassembly. CTRL and RhoG KD cells expressing GFP-paxillin were analyzed using time-lapse imaging every 10 seconds for approximately 40 minutes. (**a**) A representative adhesion tracked over the course of the indicated time (min), from appearance to disappearance, in CTRL (left panel) and RhoG KD (right panel) cells. Scale bars: 3 μm. (**b**,**c**) The rate of adhesion assembly/disassembly was calculated as described in the methods section. Data are results of at least 4 cells where at least 2 adhesions per cell were permitted into the data set, based on correlation criteria. $n \geq 17$. (**d**) For adhesion lifetime, time-lapse images were acquired every 10 seconds for up to 1.5 hours. Data are results of at least 6 cells where at least 13 adhesions per cell were analyzed. $n \geq 104$. All results are shown as mean ± SEM. ****$p < 0.0001$.

Previous work has defined three characteristic phases during FA turnover: the assembly and disassembly phases, which are typically linear, and a stationary/mature phase in between, where the intensity of the adhesion remains relatively stable16. A prolonged stationary phase may help explain the increase in FA number observed in

RhoG KD cells. If adhesions are more stable, FA would accumulate over time and translocate to the center of the cell as they mature. To test this hypothesis, we measured the lifetime of adhesions in extended movies (Videos not included) and found that adhesions lived significantly longer in RhoG KD cells (Fig. 2-2a,d). For example, in cells plated on glass, the average adhesion lifetime increased from 15.2+/−0.66 min in CTRL cells to 38.33+/−1.50 min in RhoG KD cells (Fig. 2-2d). We observed the same trend in cells plated on glass, collagen and fibronectin (Fig. 2-2d). Re-expression of RhoG rescued the FA lifetime to normal levels in all substrates. Our results show that the increased number of FA in RhoG KD cells may reflect an increase in their stability, as the FA live longer on average, and the assembly and disassembly rates are not significantly affected.

2.3.3 RhoG KD promotes FA maturation

As adhesions mature, they not only change in size and location, but also in protein composition, resulting in distinct populations of adhesions within a single cell that can be identified using specific markers6,17,18. The most mature population of adhesions, fibrillar adhesions, are characteristically elongated streaks found at the center of the cell that contain the protein tensin. Based on their increased number and altered location, we predicted that the FA in RhoG KD cells were probably more mature when compared to CTRL FA. To test this prediction, we transiently transfected CTRL and RhoG KD cells with mCherry-tensin (Fig. 2-3a, bottom panels), processed them for immunofluorescence and stained them for vinculin (Fig. 2-3a, top panels). Our results showed that when RhoG was silenced, the signal from mCherry-tensin in FA appeared to be stronger when compared to vinculin. To quantify this difference, we first measured the intensity profiles

of both vinculin and tensin along the distance of a single adhesion in CTRL (left panel) and RhoG KD (right panel) cells (Fig. 2-3a, inset). The results showed that in CTRL cells the intensity of vinculin along adhesions is higher than that of tensin (Fig. 2-3b, left panel). In contrast, the intensity of tensin was higher than that of vinculin in RhoG KD cells (Fig. 2-3b, right panel). Figure 3c confirms this result, showing the average vinculin/tensin ratio along multiple adhesions in different cells. To further confirm our findings, we calculated the average vinculin/tensin ratio using the intensity values from the whole adhesion, which also showed that, on average, the intensity of tensin was significantly higher than that of vinculin in RhoG KD FA (Fig. 2-3d).

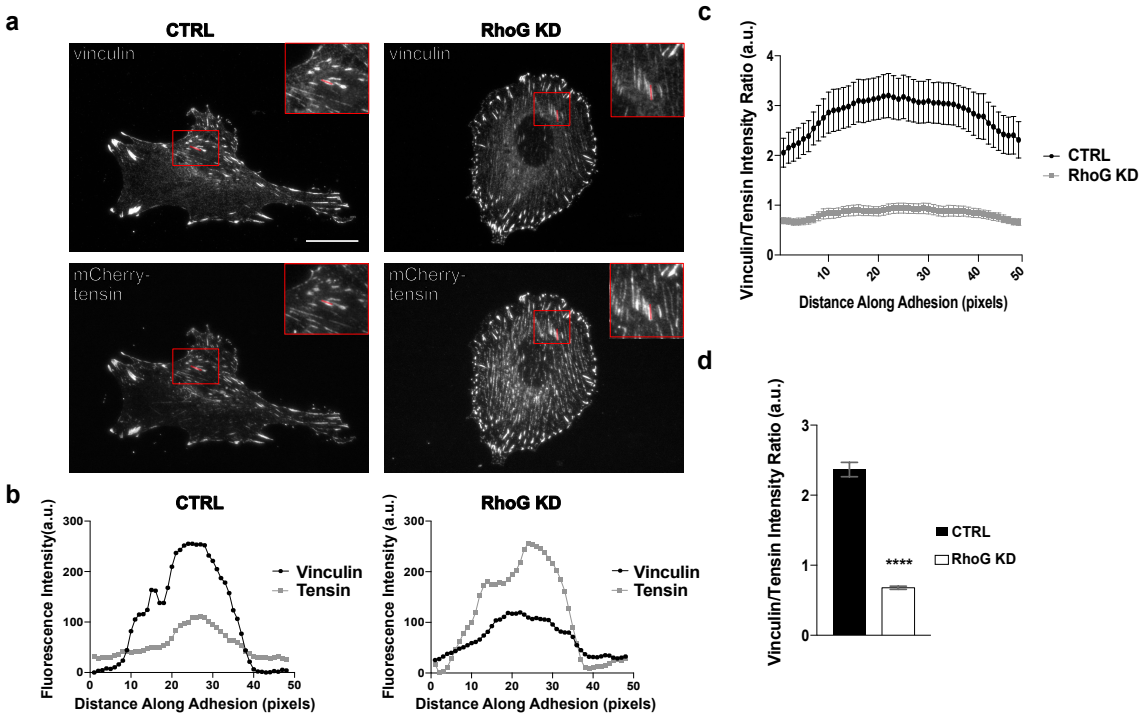

Figure 2-3 RhoG KD promotes focal adhesions maturation. (**a**) CTRL and RhoG KD cells expressing mCherry-Tensin (bottom panels) were fixed and stained with vinculin (top panels). Scale bars: 20 μm. (**b**) A line of 1-pixel width and 47 pixels length was drawn along the length of an adhesion and the intensity values along the line were plotted using ImageJ. Values for one representative adhesion, shown in zoomed insets in (**a**), are shown for both CTRL and RhoG KD cells. (**c**) Average ratio of vinculin/tensin intensity across an adhesion. (**d**) Average vinculin/tensin intensity ratio of a whole adhesion. ($p < 0.0001$). All results are shown as mean ± SEM. All data are results of 3 independent experiments where 5 cells and 10 adhesions per cell each were quantified. $n = 150$. ****$p < 0.0001$.

To examine if RhoG KD also affects the composition of nascent adhesions, we co-stained CTRL, RhoG KD, and Rescue cells for vinculin and tyrosine-phosphorylated paxillin (Supplemental Fig. 2-11a). These proteins are both known to be recruited to

adhesions found at the leading edge19,20. We saw no difference in the ratio of phospho-paxillin to vinculin along adhesions in RhoG KD cells compared to CTRL cells (Supplemental Fig. 2-11b).

Taken together, our results suggest that the increase in the lifetime of FA observed in RhoG KD cells, results in an increase in the number of mature FA.

2.3.4 RhoG plays a role in lamellipodia dynamics

The regulation of FA assembly and turnover can influence the dynamics of protrusion formation at the leading edge of the cell21. Our time-lapse movies suggested that depletion of RhoG had a negative effect in frequency and amplitude of protrusion (Videos not included). Quantitative analysis of protrusion dynamics using kymography showed that the distance and persistence of protrusions were significantly decreased in RhoG KD cells (Fig. 2-4b,c). Both parameters were rescued to normal levels upon RhoG re-expression (Fig. 2-4b,c). In contrast, there was no difference in the velocity of protrusion formation between CTRL, RhoG KD and Rescue cells (Fig. 2-4d). These results were consistent across all substrates tested. The exception was collagen, where both distance and persistence were increased slightly but were not significantly rescued by RhoG re-expression (Fig. 2-4b,c). Supporting these results, RhoG KD cells stained for lamellipodia, using an antibody against lamellipodin22, showed a significant decrease in the number and length of protrusions compared to CTRL and Rescue cells (Fig. 2-4e–g). Our data suggest that RhoG's regulation of FA may be affecting the ability of the cell to form and maintain stable lamellipodia.

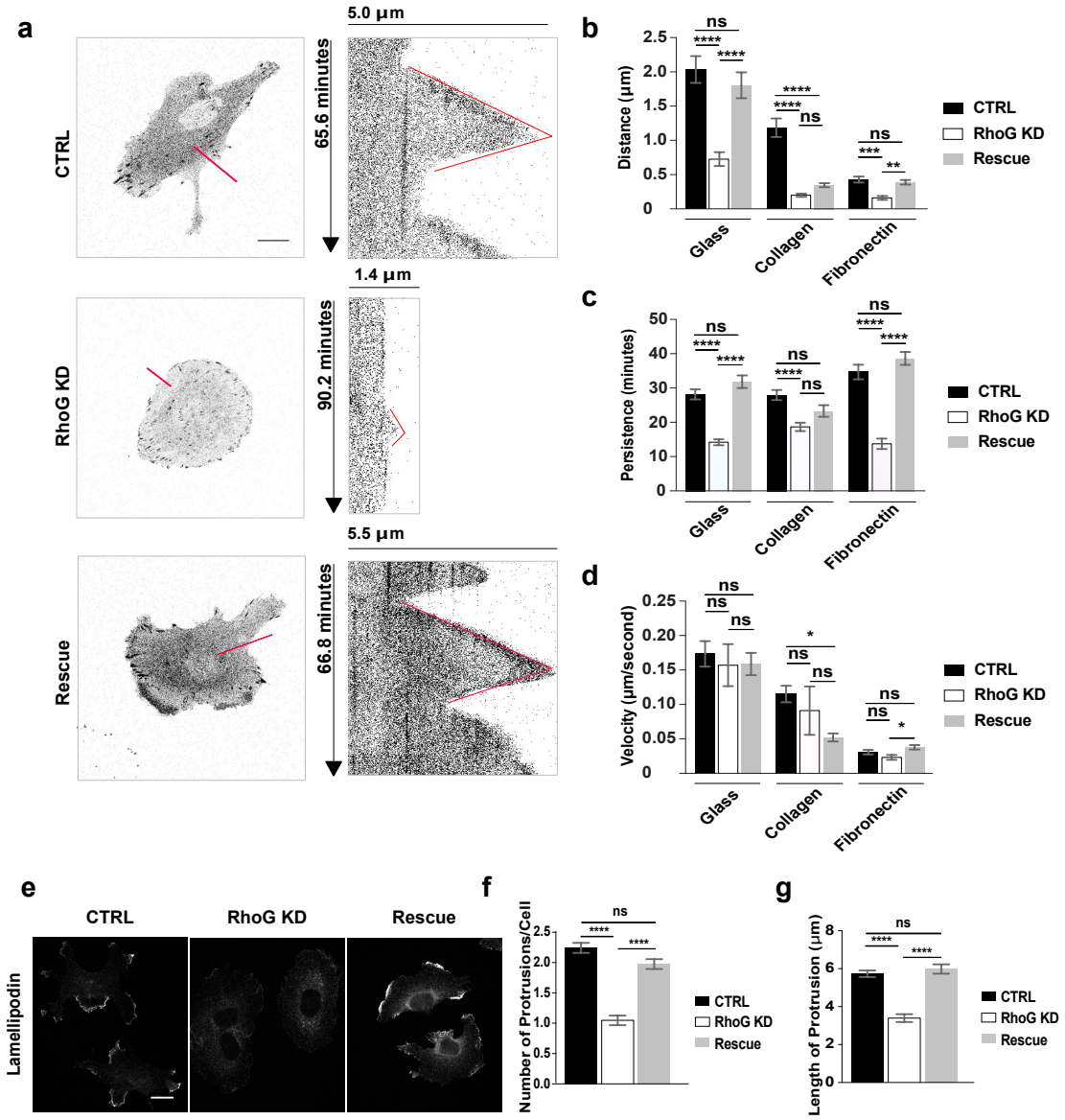

Figure 2-4 RhoG modulates protrusion dynamics and lamellipodia formation. CTRL, RhoG KD, and Rescue cells expressing GFP-paxillin were imaged every 10 seconds for up to 1.5 hours. (**a**) Representative kymographs constructed from red lines indicated on the left panel images for CTRL, RhoG KD, and Rescue cells. Scale bars: 10 µm. (**b**) Protrusion distance, (**c**) protrusion persistence, and (**d**) protrusion velocity were manually measured using ImageJ software. Protrusion data are results from 3 independent experiments where at least 2 cells were imaged and 3–6 kymographs per cell were

generated. n ≥ 35. (e) CTRL, RhoG KD, and Rescue cells were stained for lamellipodia using anti-lamellipodin antibody. Scale bars: 10 μm. (**f**) The number of lamellipodia was manually measured using ImageJ software. Lamellipodia data are the results of 3 independent experiments where at least 32 cells were counted. n ≥ 135. (**g**) The length of protrusions was manually measured using ImageJ software. Data are the results of 3 independent experiments where at least 34 protrusions were measured. n ≥ 155. All results are shown as mean ± SEM. *p < 0.01, ***p < 0.001, ****p < 0.0001.

2.3.5 RhoG regulates the alignment of focal adhesions and stress fibers

Silencing RhoG not only affected the composition and dynamics of individual adhesion, but also the pattern of the entire adhesion population within the cell. We noticed that in most RhoG KD cells, FA were aligned along an axis within individual cells (Fig. 2-5a). This axis was not the same for all cells, as individual cells developed their own pattern of alignment regardless of neighboring cells. To confirm that RhoG KD does affect adhesion alignment, we quantified global FA alignment, using a previously developed parameter, the focal adhesion alignment index (FAAI), which measures the deviation of adhesion angles from the most frequent or dominant angle observed in the whole cell23. The higher the FAAI, the greater is the global adhesion alignment within a single cell. We found that adhesion alignment was significantly increased in RhoG KD cells in all substrates tested (Fig. 2-5b–d). The increase in FA alignment was rescued by re-expressing RhoG in KD cells (Fig. 2-5b–d), except for Rescue cells on glass, where FAAI decreased slightly, but the difference was not significant from KD cells. This may be due to the axial ratio, or length, of adhesions in Rescue cells plated on glass. Since the FAAI requires that an adhesion's axial ratio be large enough to determine directionality,

if adhesions are more rounded, then the sample may be biased towards larger adhesions that are more aligned. Without the presence of substrate, we found that adhesions in Rescue cells are significantly smaller (Fig. 2-1f), supporting this notion.

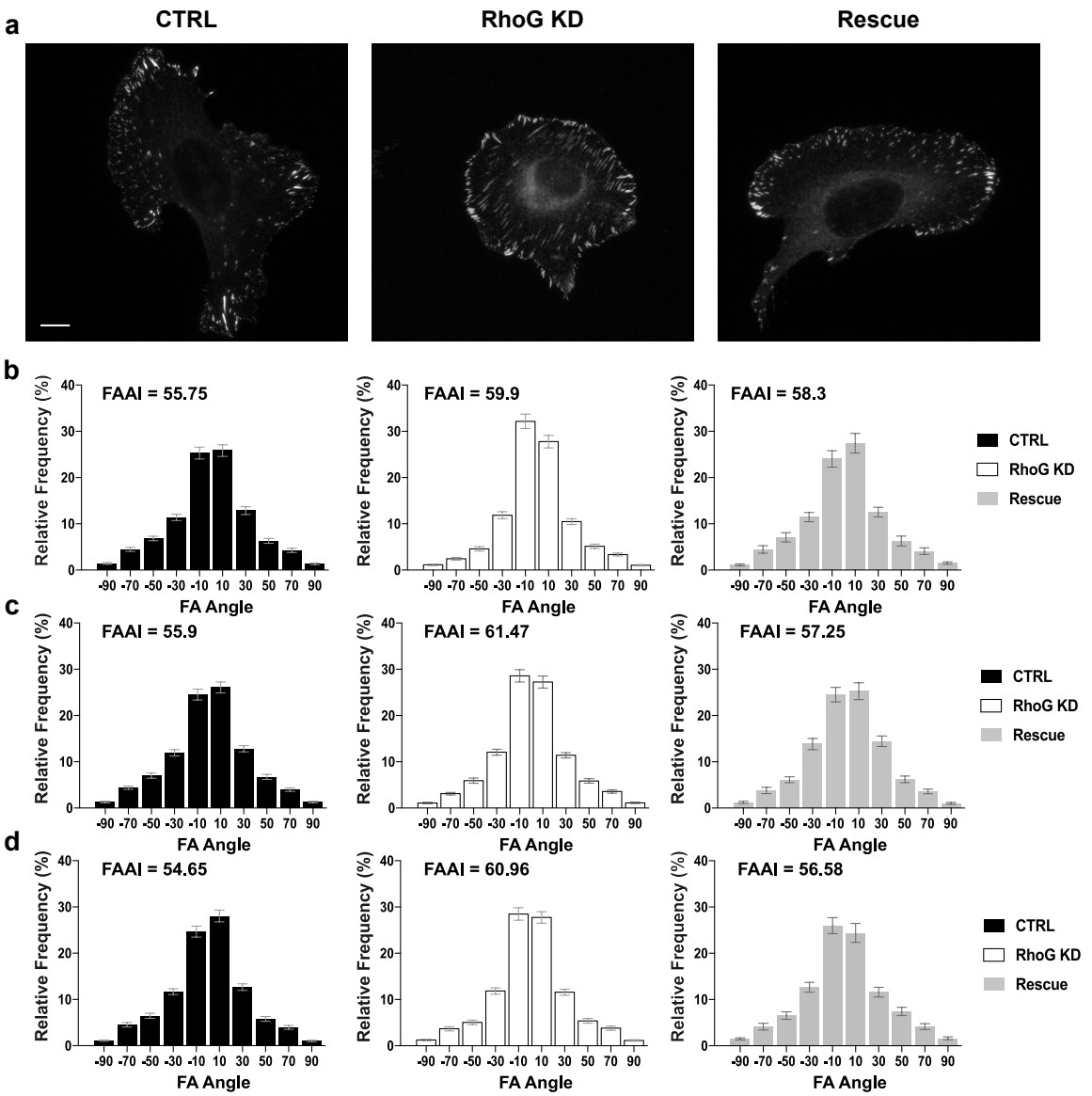

Figure 2-5 RhoG KD increases focal adhesions alignment. (**a**) Representative pictures of adhesion alignment in CTRL, RhoG KD, and Rescue cells plated on glass and stained for focal adhesions using anti-vinculin antibody. Scale bars: 10 μm. (**b**–**d**) Adhesion alignment was measured using the FAAS16, and then graphed as a frequency distribution. An index, termed the focal adhesion alignment index (FAAI) was calculated, as previously described by Wu *et al*.23, to measure the overall alignment of adhesions within individual cells (inset on all graphs). (**b**) The FAAI of CTRL, RhoG KD and

Rescue cells plated on glass. (**c**) The FAAI of CTRL, RhoG KD and Rescue cells plated on fibronectin. (**d**) The FAAI of CTRL, RhoG KD and Rescue cells plated on collagen. All results are shown as mean ± SEM. All data are results of 3 independent experiments where 20 cells per experiment were analyzed. n = 60.

Since most SF are attached at one or both ends to FA, we also evaluated the alignment of SF. We stained CTRL, RhoG KD, and Rescue cells for F-actin (Fig. 2-6a), and quantified actin alignment using CurveAlign, a software that can identify fibers and measure their alignment24 (Fig. 2-6b–d). We also adapted the FAAI approach to calculate a SF alignment index (SFAI) (Fig. 2-6b–d, inset on graphs). Our data showed an increase in SF alignment in RhoG KD cells, an effect that could be reversed by re-expression of RhoG (Fig. 2-6b–d). These results were observed on collagen and fibronectin as well. Together, these data suggest that RhoG-mediated regulation of FA dynamics also affects the global coordination of FA and the actin cytoskeleton within a cell.

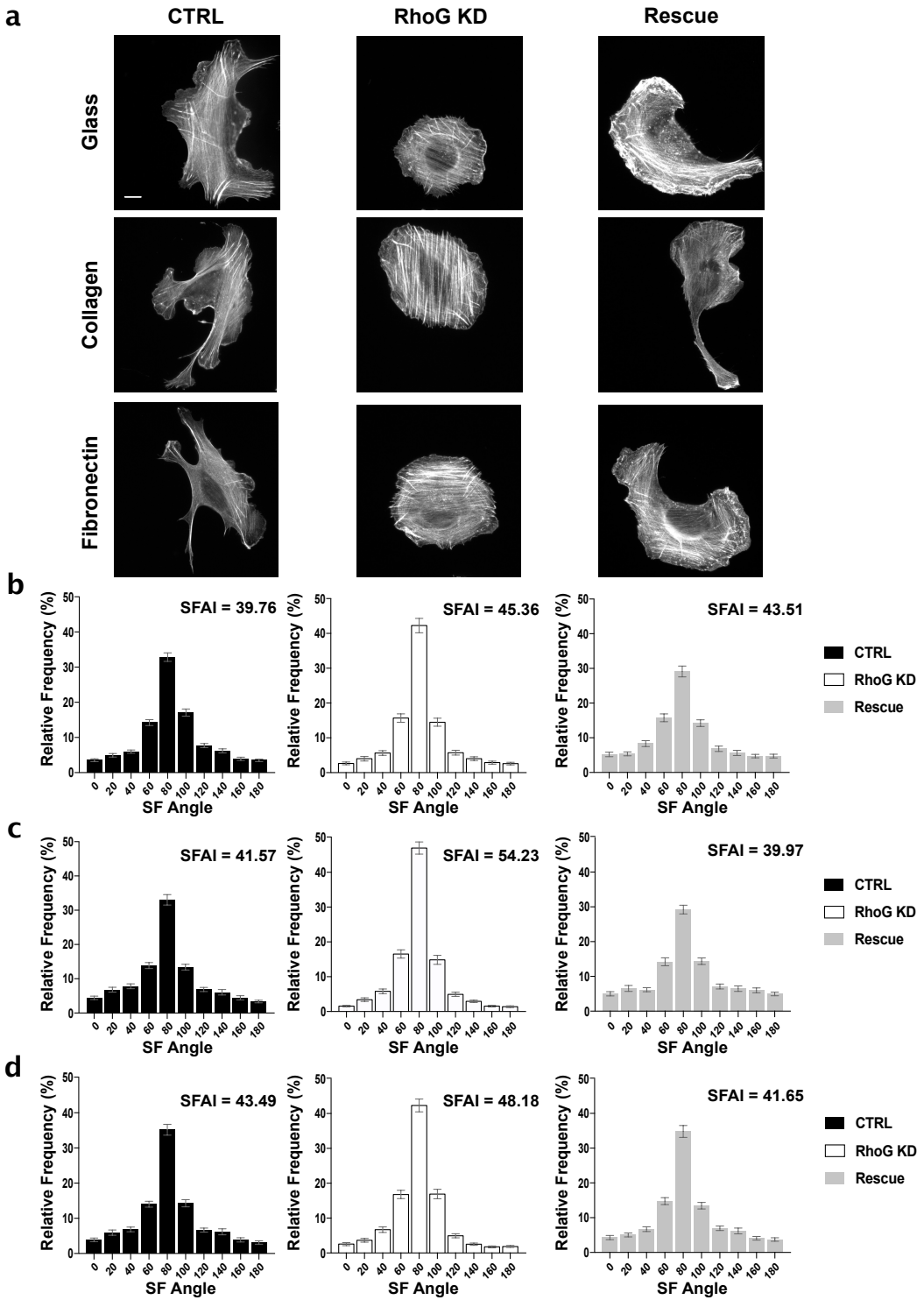

Figure 2-6 Silencing RhoG increases stress fibers alignment. (**a**) CTRL, RhoG KD, and Rescue cells were plated overnight on non-coated glass coverslips, or on coverslips

coated with collagen or fibronectin. Cells were then fixed and stained for F-actin using Alexa Fluor phalloidin. Scale bars: 10 μm. (**b**–**d**) Actin stress fiber alignment was measured using CurveAlign (V3.0 Beta 2) software. Angles from individual cells were graphed as a frequency distribution and then rotated so that 90 degrees was the dominant bin. SF alignment distributions of CTRL, RhoG KD, and Rescue cells on glass (**a**), collagen (**b**), and fibronectin (**c**) with the value for the stress fiber alignment index (SFAI) displayed at inset in each graph. All results are shown as mean ± SEM. All data are results of 3 independent experiments where 20 cells per experiment were analyzed. n = 60.

2.3.6 RhoG regulates actomyosin contractility

FA serve a mechanical role by forming a tether between the cytoskeleton and the ECM, thus acting as anchor sites for the cell to exert force onto the substrate25. The recruitment of mechanosensitive proteins, resulting in formation and growth of FA, and alignment of FA can be induced through the application of external force or activation of proteins involved in contractility pathways, such as RhoA26. Additionally, contractility plays a role in the polymerization and stabilization of actin filaments through activation of RhoA and the inhibition of cofilin, respectively26,27. We observed a phenotype with an increased number of FA and alignment of both FA and SF, which strongly suggested that cells were more contractile in the absence of RhoG. This was confirmed by treating CTRL and RhoG KD cells with contractility inhibitors. Both cell types displayed sensitivity to blebbistatin, a myosin II inhibitor, and Y27632, which inhibits ROCK, as indicated by the disassembly of SF and FA, suggesting that the SF are functional and contractile (Fig. 2-7a,b).

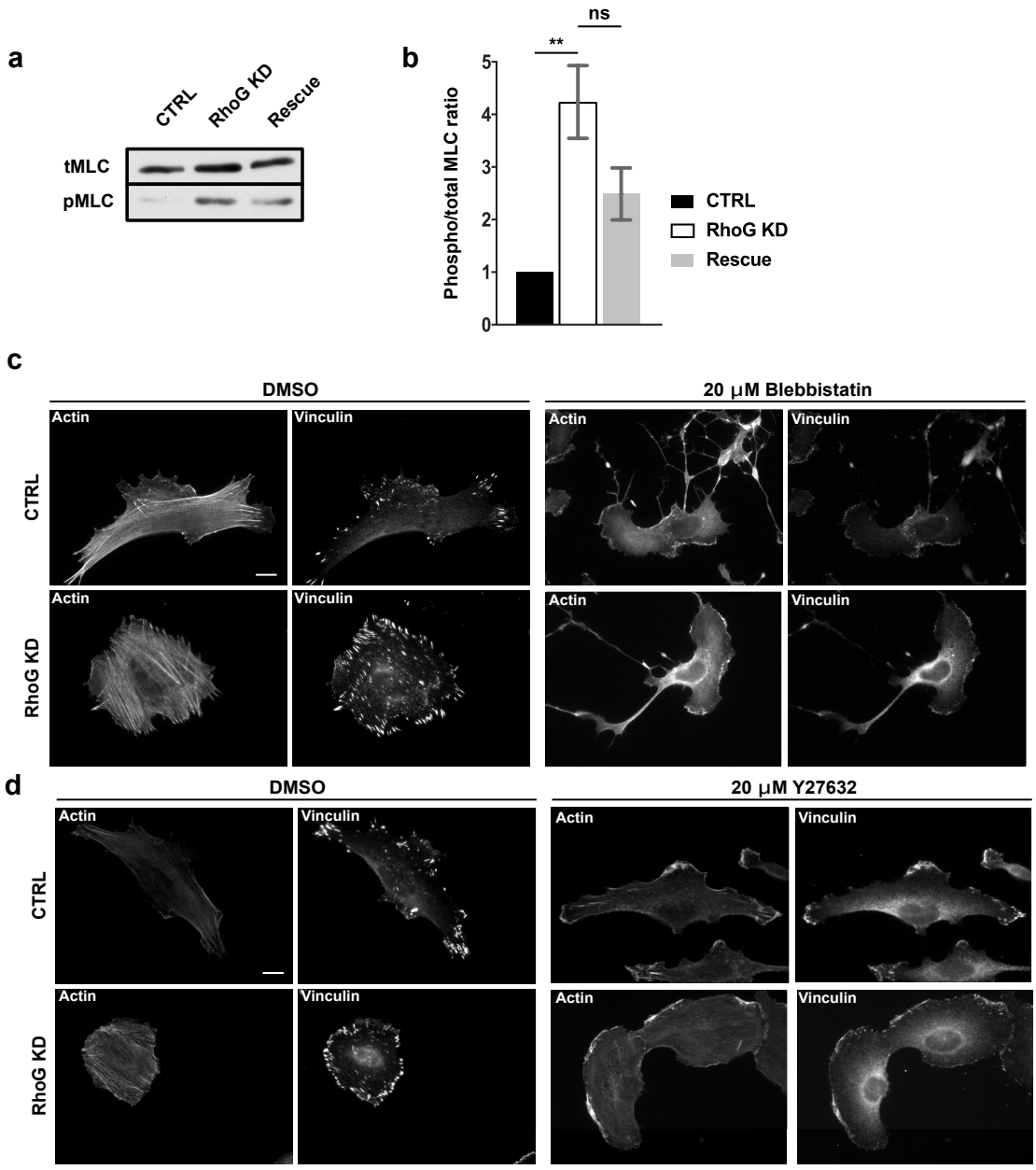

Figure 2-7 RhoG KD cells are responsive to inhibitors of contractility. (**a**) CTRL (top panels) and RhoG KD (bottom panels) cells were plated on glass coverslips overnight and then treated with DMSO or 20 μM blebbistatin for 10 min. Cells were then fixed and stained for vinculin and F-actin. Scale bars: 10 μm. (**b**) CTRL (top panels) and RhoG KD (bottom panels) cells were plated on glass coverslips overnight and then treated with DMSO or 100 μM Y-27632 for 10 min. Cells were then fixed and stained for vinculin

and F-actin. Scale bars: 10 μm. (**c**) CTRL, RhoG KD, and Rescue cells were blotted for total and phospho-MLC and quantified by densitometry (**d**). Data are the results of three independent experiments. n = 3. All results are shown as mean ± SEM., **p < 0.01.

An increase in contractility is usually associated with high levels of phosphorylated myosin regulatory light chain (MLC)28. The levels of phospho-MLC are regulated by the balance between the activity of myosin light chain kinase (MLCK) and MLC-phosphatase29,30. Based on our results, we expected to see an increase in MLC phosphorylation in the absence of RhoG. This was confirmed by measuring the ratio of phospho-MLC to total-MLC by western blot in CTRL, RhoG KD and Rescue cells (Fig. 2-7c,d). It is worth noting that we also observed an increase in the amount of total-MLC in RhoG KD cells (Fig. 2-7c,d), which shows that the expression levels of MLC are also regulated in response to changes in RhoG.

2.3.7 RhoG KD affects microtubule dynamics

The microtubule (MT) cytoskeleton has also been shown to play a role in the regulation of FA turnover31. MT can target adhesions located at the edge of the cell to promote their disassembly32,33,34. A defect in the capturing process could prolong adhesion life, leading to larger populations of mature adhesions, like those seen in RhoG KD cells. To test this hypothesis, we first stained CTRL and RhoG KD cells for α-tubulin to examine their MT distribution (Fig. 2-8a). Our results revealed a distinct difference in the MT pattern between CTRL and RhoG KD cells. In CTRL cells, MT irradiated from the center of the cell to the periphery, where each individual MT typically reached the edge of the cell at an angle perpendicular to the cell membrane. In contrast, MTs in RhoG KD cells curved when they reached the cell periphery and adopted an angle that was

often parallel to the cell edge (Fig. 2-8a). Quantification of MT linearity showed a significant decrease in RhoG KD cells, confirming that the MT are more curved in the absence of RhoG (Fig. 2-8b). In addition, the MT angle relative to the cell's edge shows a larger percentage of CTRL MT that are perpendicular to the cell edge, whereas in KD cells there is a higher frequency of MTs that align parallel to the cell membrane (Fig. 2-8c).

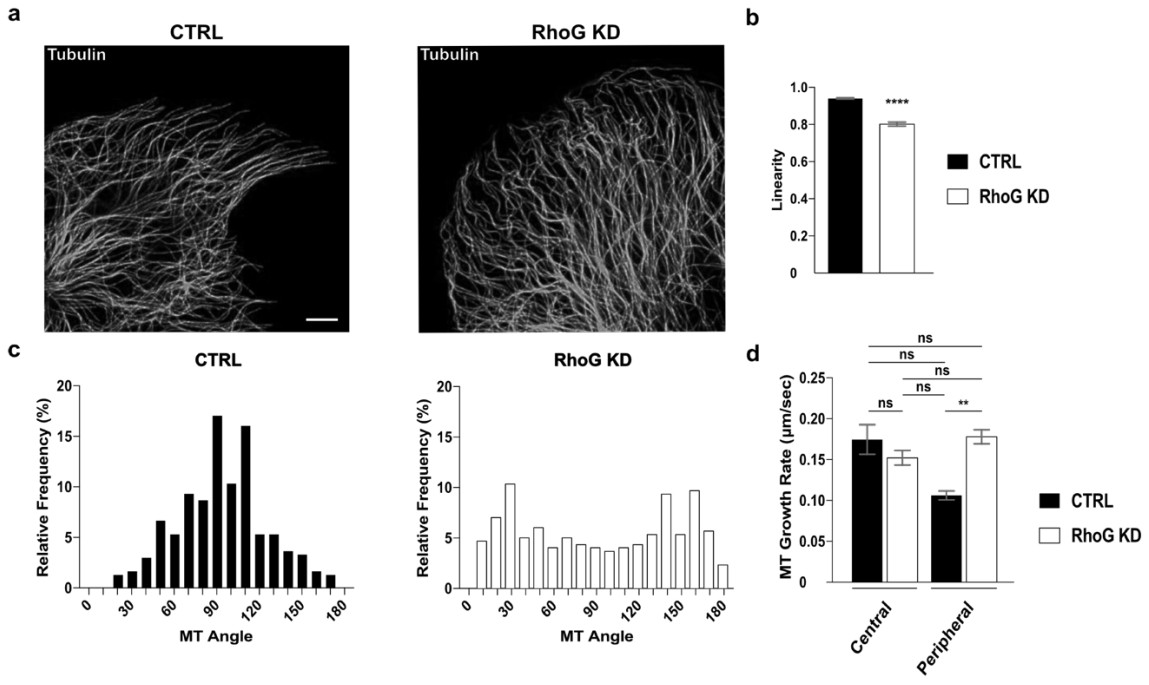

Figure 2-8 RhoG regulates MT outgrowth. (**a**) CTRL and RhoG KD cells were plated overnight on non-coated, glass coverslips. Cells were then fixed and stained for MT using anti-tubulin antibodies. Scale bars: 5 µm. (**b**) Linearity of MT in CTRL and RhoG KD cells, measured as the actual distance divided by the shortest distance between 2 points. Data are results from 3 independent experiments where 5 cells and 15 MT per cell were measured. n = 200. (**c**) The relative frequency of the angle of the MT relative to the cell edge in CTRL (left panel) and RhoG KD (right panel) cells. Results are from 3 independent experiments where 5 cells and 20 MT per cell were measured. n = 300. (**d**) CTRL and RhoG KD cells were transfected with EB3-mRFP. Cells were imaged every 3 seconds for approximately 8.5 minutes. Central or peripheral MT growth rate was manually measured for 5 MT in 12 cells using the MTrackJ plugin for ImageJ software. n = 60. All results are shown as mean ± SEM. ****$p < 0.0001$.

The angle of the MT relative to the cell edge can be indicative of their growth rate. In normal cells MT tend to grow faster at the center of the cell and slow down at the

edge due to the combined action of several factors, including targeting to FA35,36. In cells with abnormal MT targeting to FA, the MTs at the edge of the cell do not slow down, causing them to continue growing, eventually curving parallel to the edge of the cell36. We used live imaging to analyze the dynamics of the MT plus-end marker EB3-mRFP cells to examine MT growth rate in CTRL vs. RhoG KD cells (Videos not included). We found that there was no difference in the MT growth rate measured at the cell center. However, at the cell periphery, MT grew significantly faster in RhoG KD cells when compared to CTRL (Fig. 2-8d). These data suggest that RhoG regulates MT dynamics at the leading edge, which may impact MT-mediated FA disassembly. Thus, the absence of RhoG would lead to increased FA lifetimes, which would result in an increase in FA numbers that progress to mature FA at the center of the cell.

2.3.8 RhoG KD inhibits MT-mediated FA disassembly

As mentioned above, targeting of FAs by growing MTs coincides with their disassembly32,33,34. When cells are treated with nocodazole, the depolymerization of MTs induces the stabilization of FA37,38. Nocodazole washout stimulates the regrowth of MT after depolymerization, which induces a rapid disassembly of FA34. Given that RhoG KD affected both FA turnover and MT dynamics, we considered that RhoG might be playing a role in the regulation of MT-dependent FA disassembly. To test this possibility, we performed nocodazole washout experiments in CTRL and RhoG KD cells.

Our results showed, as expected, that FA size increased in both CTRL and RhoG KD cells treated with nocodazole (Fig. 2-9a). In CTRL cells, nocodazole washout promoted a rapid decrease in the number of FA. The maximum decrease in FA number was observed at 15 minutes in CTRL cells (Fig. 2-9a,b). FA number started to increase at

30 minutes and continued to increase steadily through the duration of the washout treatment. Importantly, after 120 min of washout the number of FA in CTRL cells was still significantly lower than in non-treated or nocodazole treated CTRL cells. In contrast, in RhoG KD cells FAs remained stable through the duration of the nocodazole washout. The decrease in FA number was significantly less pronounced than in CTRL cells, and the recovery was also faster, reaching non-treated or nocodazole treated levels after 30 min of washout (Fig. 2-9a,b). Quantification showed that in CTRL cells the number of FA decreased approximately 8-fold, whereas in RhoG KD cells the reduction was only 2.3-fold. MT staining showed that MT regrowth was not affected when RhoG was silenced (Supp. Fig. 2-123), suggesting that RhoG is required specifically for the process of FA disassembly after MT regrowth.

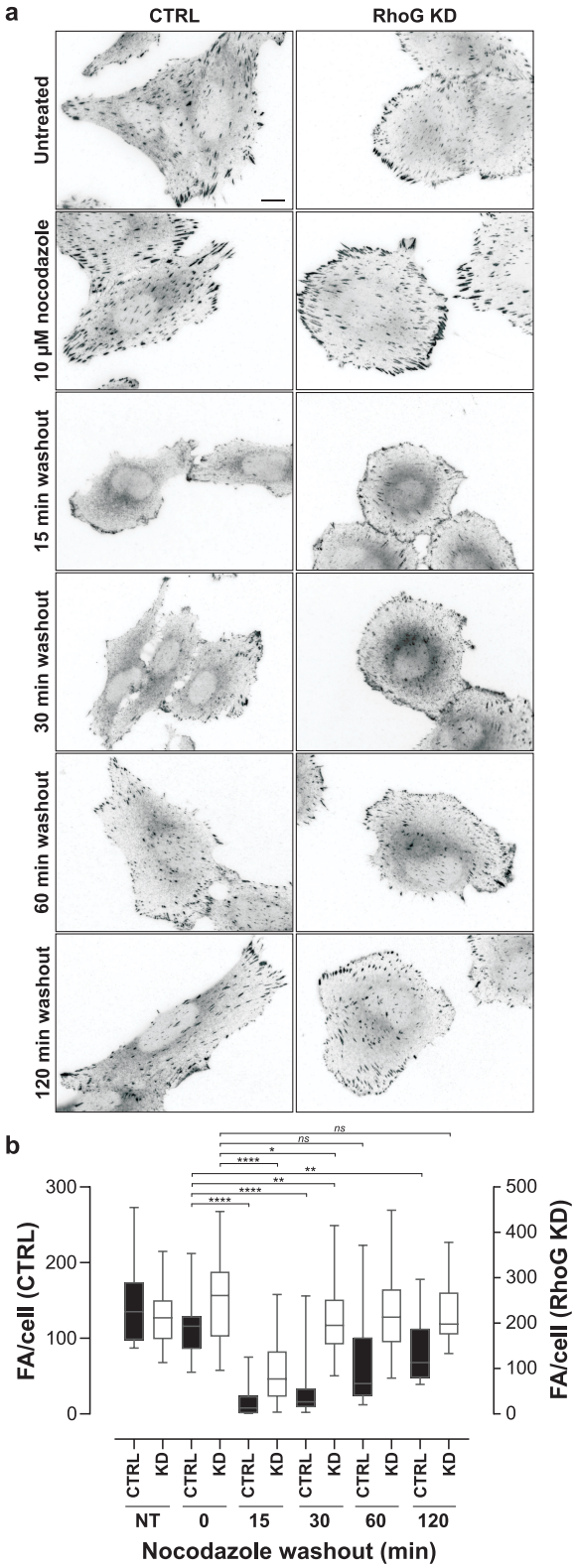

Figure 2-9 RhoG KD inhibits MT-mediated FA disassembly. (**a**) CTRL and RhoG

KD cells were starved overnight and then treated with nocodazole at 10 μM for 1 h. Following treatment cells were washed once with SFM and then incubated with SFM for the indicated times (nocodazole washout). After washout the cells were fixed and stained for vinculin. Scale bar: 10 μm. (**b**) The number of adhesions was calculated manually using Image J. Data are the results of 2 independent experiments where at least 25 cells were analyzed per condition. All results are shown as mean ± SEM. *$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$.

2.4 Discussion

RhoG has been previously shown to play a role during cell migration, but the mechanism has not been further explored10,11,12,13. Here we show that RhoG regulates the number, size, lifetime and maturation stage of FA, as well as SF contractility, suggesting that the role of RhoG in cell migration may be mediated through the regulation of FA turnover. Our results also demonstrate that RhoG controls FA turnover through the regulation of MT-mediated FA disassembly.

FA form at the leading edge and translocate towards the center of the cell as the cell body moves forward. Eventually, FA disassemble either behind the lamella or at the rear of the cell allowing the cell to move forward. As FA are disassembled, their components are recycled to new adhesions at the leading edge, completing the cycle1,39. Thus, the assembly and disassembly of FA must be precisely controlled for cells to migrate properly. Initially, we predicted that the adhesion phenotype in RhoG KD cells was the result of an increased rate of assembly and/or decreased disassembly, resulting in a net accumulation of FA at the center of the cell. However, we found no change in the assembly or disassembly rates of FA, but rather an increase in their lifetime.

The maturity of adhesions can be characterized by the diversity in protein composition at the different stages, which allows FA to carry out various functions that aid cell migration[2,39,40]. Due to the increase in number of FA and their location in RhoG KD cells, we hypothesized that RhoG was regulating maturation. We found that there was an increase in the amount of tensin in FA when RhoG was silenced, indicating more mature adhesions. Taken together, our results show that in the absence of RhoG, FA become more stable and live longer, leading to an increased number of mature FA found at the center of the cell. We have also shown that RhoG depletion impairs leading edge protrusion. The slower rate of FA turnover combined with altered lamellipodia dynamics are the most likely causes for observed defects in cell migration[10,11,12,13].

Contractile forces have long been the focus of studies elucidating the regulation of adhesion formation and the ability of the cell to respond to the environment[25]. Both MT and actin SF coordinate the regulation of contractility[41,42]. This dynamic interplay between cytoskeletal elements plays an important role in the balance between assembly and disassembly of FA[3]. We observed a phenotype where FA and SF were aligned along a common axis in individual RhoG KD cells. This was a particularly striking observation because events of comparable FA and SF alignment were observed where mechanical force was applied externally to the cell[43,44,45,46,47]. The phenotype adopted by RhoG KD cells, i.e. round cells, with more FA and thicker SF, suggested an increase in contractility. This was confirmed by their sensitivity to contractility inhibitors and increased levels of pMLC. Contractility of SF is especially important in cell migration because mechanical force held in the SF is exerted upon the ECM through FA, allowing the cell to progress forward.

The molecular mechanisms by which RhoG regulates these processes are unclear. However, our results suggest that MT-mediated FA disassembly may be playing a role in the regulation of FA lifetime by RhoG. MT target FA at the cell edge and induce their disassembly, in a process known as capturing[32,33,34]. This is accomplished through a recently identified complex of proteins that aids in MT capture near FA[48]. When MT do not efficiently capture at FA, their growth rates and angle relative to the cell edge are affected[35,36]. Here we show that the speed and directionality of MT outgrowth are affected in the absence of RhoG, which may be indicative of impaired capture and reduced number of disassembly events. This in turn would translate into increased number of FA over time, which would eventually mature and accumulate at the center of the cell. Nocodazole washout experiments also show that MT-dependent disassembly is impaired in the absence of RhoG, preventing or slowing down the disassembly of FA over time upon washout. We plan to further explore the possibility of RhoG's role in MT capture at FA, contributing to cell migration defects.

It is not clear whether RhoG localizes to FA and we have not been able to stain for endogenous RhoG with the currently available antibodies. Exogenously expressed RhoG localizes primarily to the cytosol, and at the perinuclear region where it targets to the Golgi through its association with RhoGDI3[49,50,51]. Local concentrations of RhoG have also been detected at the cell periphery, at places that may overlap with FA[13,49]. Interestingly, a proteomics study identified RhoG as one of the proteins that was reproducibly recovered in isolated FA, suggesting that a fraction of RhoG may be targeted to FA[52].

Our results suggest that a RhoG-specific GEF activates RhoG at the time of disassembly. We have previously identified SGEF and Trio as upstream regulators of RhoG signaling during invadopodia and circular dorsal ruffles disassembly14,53. However, our preliminary results using shRNAs targeting known RhoG-specific, including SGEF, Trio, Ephexin 4, PLEKHG6, Vav1-3, were inconclusive and suggests that none of them are involved in regulating RhoG in FA. This could be attributed to an incomplete KD, or to compensation by another GEF in response to the KD. Alternatively, RhoG may be regulated at FA by another yet to be characterized RhoG GEF (most Rho-GEFs have not been tested for RhoG specificity). We are continuing our efforts to identify the RhoG-GEF in FA.

In conclusion, we have identified a novel role for RhoG in cell migration, regulating the disassembly of FA in a process that involves MT. The molecular mechanism by which RhoG controls these processes is not known. The regulation of Rho GTPase activity relies on a complex system where multiple upstream GEFs and GAPs can regulate a single Rho GTPase, which can then activate a vast array of downstream effectors. The identification of the upstream regulators and the downstream effectors will be key to fully understand the role of RhoG during cell migration.

2.5 Materials and Methods

2.5.1 Reagents and antibodies

The following antibodies were used: mouse anti-RhoG (Santa Cruz, sc-1007), mouse anti-myc (9E10) (Santa Cruz, sc-40), mouse anti-vinculin (mouse) (Sigma, V9131), rabbit anti-vinculin (Thermo-Fisher, 700062); rabbit anti-phospho-paxillin (Y118) (Cell Signaling, 2541), rabbit anti-lamellipodin (Cell Signaling, 91138), rabbit

anti-Myosin Light Chain and rabbit anti-phospho-Myosin Light Chain (Ser 19) (Cell Signaling, 3671, 3672) mouse anti-alpha tubulin (Sigma, T9026), rabbit anti-tubulin (Abcam, ab18207); Alexa Fluor-488 and Alexa Fluor-594 anti-mouse IgG and anti-rabbit IgG conjugated secondary antibodies and Alexa Fluor-488 and Alexa Fluor-594 Phalloidin (Life Technologies). HRP-conjugated anti-mouse, anti-rabbit and anti-goat secondary antibodies (Jackson Immunoresearch). Hoechst 33342 (AnaSpec Inc., 83218).

Fibronectin (a gift from Keith Burridge, UNC-Chapel Hill, Chapel Hill, NC) and collagen type I (Thermo Fisher, A1048301) were used at indicated concentrations to coat coverslips. The contractility inhibitors (−)-blebbistatin (EMD Millipore) and Y27632 (LC Laboratories) were used as indicated. Nocodazole (Sigma) was used as indicated below.

2.5.2 cDNA constructs

mCherry-Tensin-C14 (a gift from Michael Davidson, Addgene plasmid #55143). mCherry-LifeAct (a gift from Jaap van Buul, Sanquin Institute, Amsterdam, Netherlands). GFP-EB3 (a gift from Kristen Verhey, University of Michigan, Ann Arbor, MI). Paxillin-GFP (a gift from Channing Der, UNC-Chapel Hill, Chapel Hill, NC) was subcloned into pAd/CMV/V5-DEST using Gateway recombination technology (Life Technologies). Virus particles were produced using the Virapower Adenoviral Expression System (Life Technologies). The shRNA resistant mycRhoG construct used for rescue experiments has been previously described14.

2.5.3 Lentiviral shRNA constructs and transduction

pLKO.1 lentiviral non-targeting shRNA control was from Sigma (SHC0161EA). pLKO.1 shRNA for human RhoG (#5 TRCN0000048022) were from Open Biosystems (Huntsville, AL). Lentiviruses were prepared at the Lenti-shRNA Core Facility,

University of North Carolina (Chapel Hill, NC). Cells were infected with lentivirus particles overnight. The following day, the infection media was removed and replaced with complete medium containing puromycin (2.5 μg/ml) (Sigma) to select for shRNA expressing cells and total cell lysates were subjected to Western blot analysis for protein expression as described. For some shRNAs, single cell colonies were isolated by serial dilution.

2.5.4 Cell lines

Human SUM159 cells were a gift from Dr. Carol Otey (UNC-Chapel Hill, Chapel Hill, NC) and were cultured in Ham's F12 media (Corning) supplemented with 10% fetal bovine serum (FBS, Rocky Mountain Biologicals), 0.5 μg/ml hydrocortisone (Sigma), and 2.5 μg/ml insulin (Life Technologies). Human MRC5 fibroblasts were purchased from ATCC and cultured in Dulbecco's modified Eagle's medium (DMEM, Corning) supplemented with 10% FBS. All cells were grown at 37 °C and 5% CO2. All experiments were conducted with early passage cells that were passaged no more than 15 times. Mycoplasma was tested regularly by staining with Hoechst 33342 (Anaspec).

2.5.5 Imaging and analysis

When indicated, acid washed coverslips were coated with 25 μg/ml (fixed imaging) or 50 μg/ml (live-imaging) fibronectin for 24 hours at 4 °C. Rat tail type I collagen coated coverslips were purchased from Neuvitro (Cat. GG-12-1.5-Collagen). Prior to plating cells, collagen and fibronectin coated coverslips were blocked with 1.5% BSA in PBS for 1.5 hours at 37 °C.

Immunofluorescence assays were performed as described previously54. Briefly, cells were fixed for 10 min with 3.7% paraformaldehyde, and quenched with 10 mM

ammonium chloride (for experiments involving fixed-imaging of MT, cells were fixed with −20 °C methanol or glutaraldehyde). Cells were then permeabilized with 0.1% Triton X-100 in PBS for 10 min. The coverslips were then washed with PBS and blocked in PBS, 2.5% goat serum (Sigma), 0.2% Tween 20 for 5 min followed by 5 min blocking in PBS, 0.4% fish skin gelatin (Sigma), and 0.2% Tween 20. Cells were incubated with primary antibody for 1 h at room temperature. Coverslips were then washed with PBS, 0.2% Tween 20 and incubated with Alexa Fluor 488 or 594 secondary antibodies for 45 min, washed as described above and mounted on glass slides in MOWIOL mounting solution. Images were acquired on an Olympus IX81 inverted microscope using a PlanApo N 60×/1.42 oil objective lens and a XM10 camera (Olympus).

Live imaging was performed for the indicated times in a Leica SP8 confocal microscopy using a PlApo CS2 N 63×/1.4 objective (Leica), equipped with an environmental chamber that controls temperature, CO2, and humidity (Tokai Hit). Images were processed using ImageJ software.

Images were threshold adjusted and then converted to binary prior to manually tracing edges for cell area measurements and perimeter coordinates, or for measuring the long axis and short axis for axial ratio. Focal adhesions characteristics were quantified using the Focal Adhesion Analysis Server (FAAS) (http://faas.bme.unc.edu)16.

Intensity profiles were measured by manually drawing a 1-pixel width line along the long axis of an adhesion using ImageJ software. Mean intensity value was measured at each pixel along the line. Adhesion perimeters were manually drawn in order to measure the total intensity of an adhesion.

2.5.6 Measuring Adhesion Dynamics

Cells were infected with adenovirus encoding GFP-paxillin for 24 hours. They were then plated in a glass-bottom MatTek plate (MatTek corporation) for an additional 24 hours. Images were acquired every 10 seconds for the indicated duration.

Adhesion assembly and disassembly was measured using a previously described method55. Briefly, adhesions were chosen manually using image stacks with an applied grid in ImageJ. Mean intensity value of adhesions were measured manually in ImageJ. Assembly was defined as the time between appearance to peak max intensity, and disassembly the time between peak max intensity to disappearance. An adhesion had to fully appear and disappear to be included in data. Background was subtracted using a randomly selected area from within the cell, absent of adhesion, at each frame. Max intensity values were graphed, and linear regression values and slope of the line were calculated in Microsoft Excel. In order for an adhesion to be included in the data set, a minimum linear regression value of 0.6 was required. The slope of the line represents the rate of assembly or disassembly. Adhesion lifetime was tracked manually, defined as the total time between appearance and disappearance.

2.5.7 Measuring protrusion dynamics

Images acquired for adhesion lifetime measurements were used to measure protrusion dynamics. Velocity, persistence, and distance were measured manually using ImageJ software, as previously described56. Briefly, a line of 1-pixel width was drawn perpendicular to a protrusion and a kymograph was generated using the KymographBuilder plugin for ImageJ. Measurements were then manually measured using ImageJ.

2.5.8 Measuring MT dynamics

Cells were transiently transfected with mCherry-EB3 for 24 hours. They were then plated in a glass-bottom Matek 35 mm plate for an additional 24 hours. Images were acquired every 2.95 seconds. MT velocity was manually measured using the ImageJ MTrackJ plugin. MT linearity, defined as the shortest distance between 2 points divided by the actual distance, and angle relative to the cell edge were manually measured using ImageJ software.

2.5.9 Nocodazole washout experiment

Nocodazole washout experiments were performed as previously described by Ezratty and colleagues34. Briefly, CTRL and RhoG KD SUM159 cells were starved overnight in serum-free media (SFM). The cells were then treated with nocodazole at 10 μM in SFM for 1 h. Following treatment cells were washed once with SFM and the incubated with SFM for the indicated times (nocodazole washout). After washout the cells were fixed and processed for immunofluorescence as described in Methods.

2.5.10 Statistical Analysis

Statistical analysis was performed using GraphPad Prism 7. One-way ANOVA was used to compare multiple condition assays, and unpaired t-test to compare independent groups.

2.6 Acknowledgements

We thank Kristen Verhey (University of Michigan) for GFP-EB3 and for technical support, Jaap van Buul (Sanquin Research Institute) for mCherry-LifeAct, and Channing Der (UNC-Chapel Hill) for human GFP-paxillin. R.G.M. was supported by the National

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2.7 Author Contributions

A.Z., S.M.G. and R.G.M. designed, performed and analyzed all experiments. S.A. designed, cloned and produced the pAdeno-paxillin virus. D.M. and Y.C. designed, performed and analyzed the contractility experiments. G.K.L. designed, performed and analyzed the nocodazole washout experiments. L.C.R. performed experiments related to measurement of pMLC. A.Z. and R.G.M. wrote the manuscript. All authors provided detailed comments.

2.8 Additional Information

The authors declare that they have no competing interests.

2.9 Supplemental Material

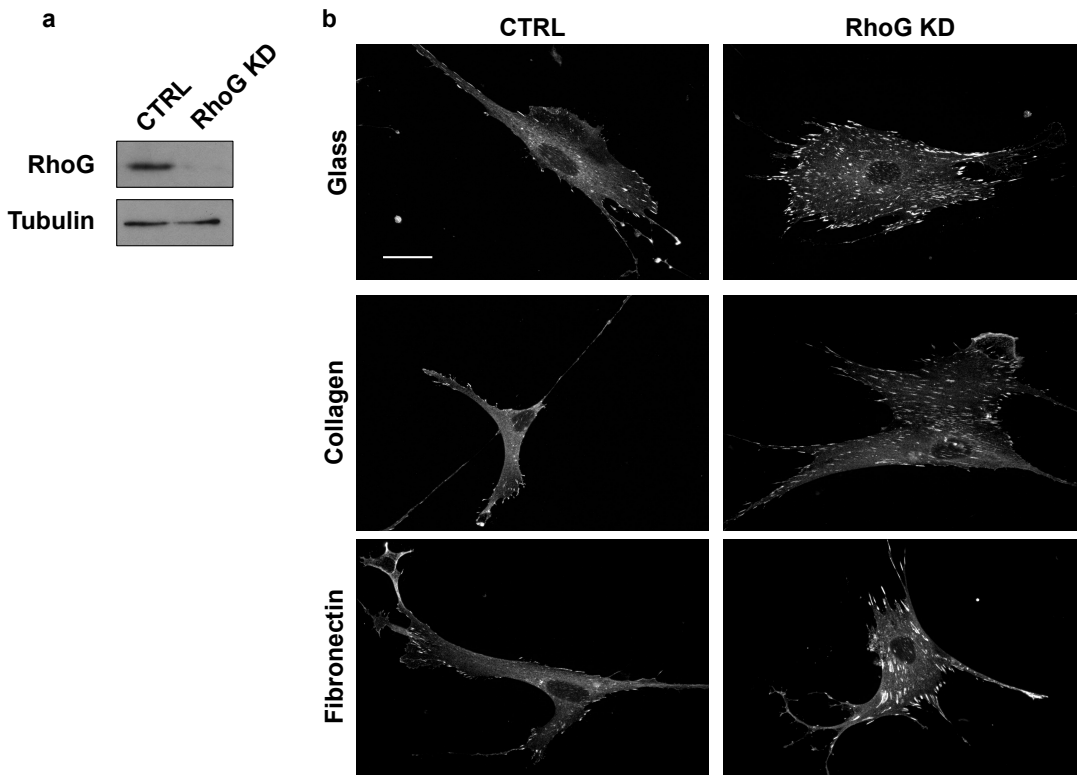

Figure 2-10 RhoG regulates FA in MRC5 fibroblasts. (a) RhoG expression was stably silenced in MRC5 fibroblasts using lentiviral encoding shRNA (RhoG KD). CTRL cells express a non-targeting shRNA. (b) CTRL and RhoG KD cells were plated on either non-coated coverslips, or coated with collagen or fibronectin, and stained for FA using anti-vinculin antibodies. Scale bar: 20 μm.

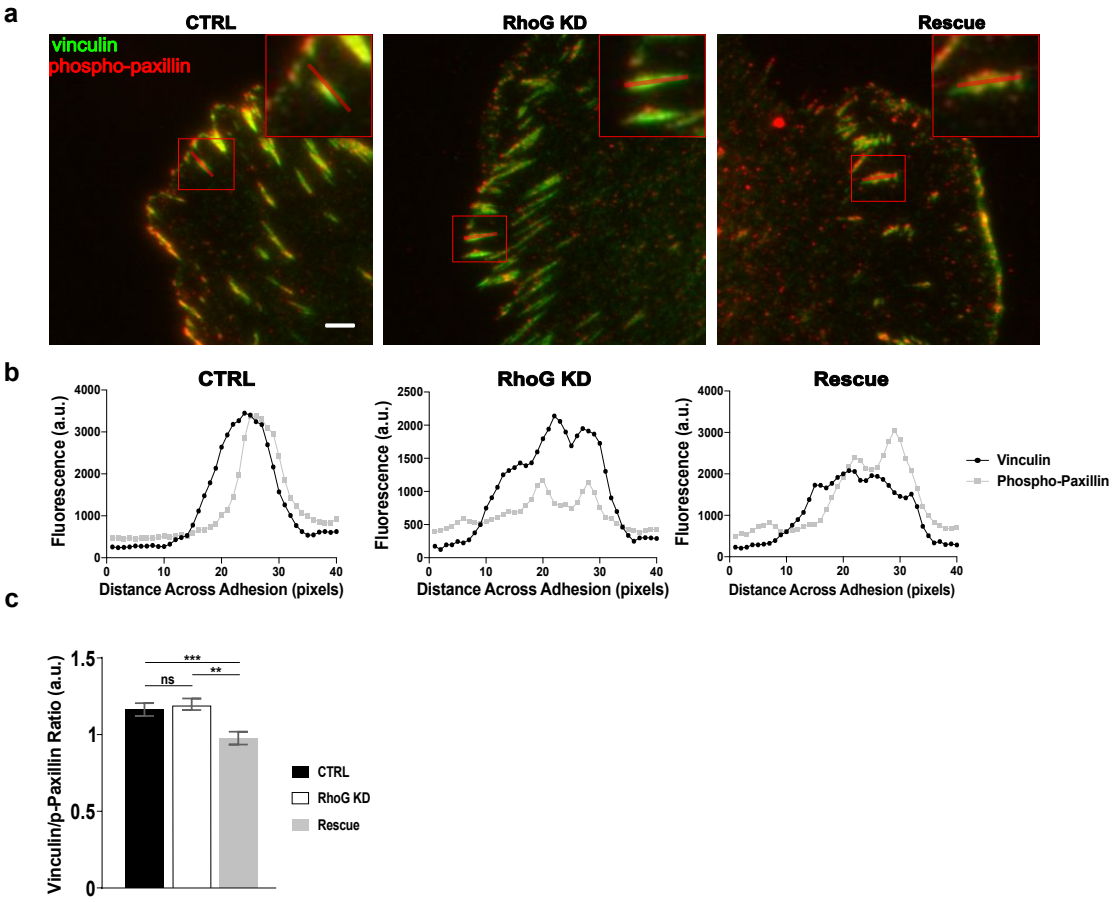

Figure 2-11 RhoG regulates vinculin and phospho-paxillin colocalization. (a) CTRL, RhoG KD, and Rescue cells were stained for vinculin (top panels) and phospho-paxillin (bottom panels). Scale bars represent 3 μm distance. (b) A line of 1-pixel width and 40 pixels length was drawn across an adhesion and intensity values were plotted using ImageJ. Values for one representative adhesion, marked as a ROI in images, are shown for CTRL, RhoG KD, and Rescue cells. All results are shown as mean ± SEM. All data are results of 3 independent experiments where 5 cells and 10 adhesions per cell each were quantified. n = 150. *p<.02.

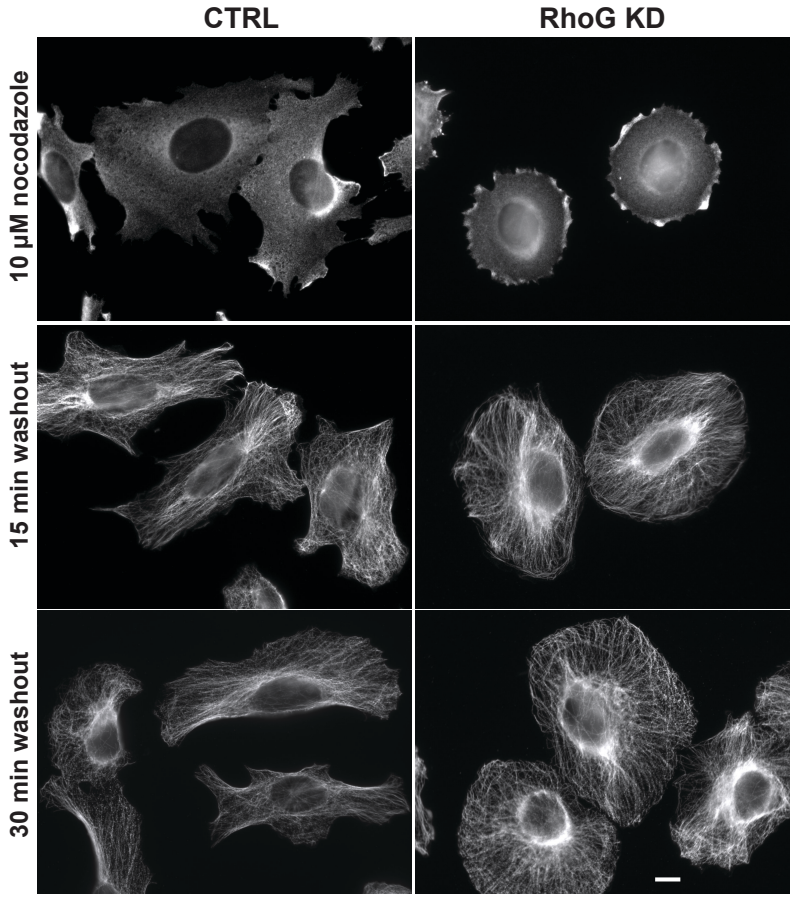

Figure 2-12 RhoG KD does not affect MT-regrowth after nocodazole washout. CTRL and RhoG KD cells were starved overnight and then treated with nocodazole at 10 µM for 1 h. Following treatment cells were washed once with SFM and the incubated with SFM for the indicated times (nocodazole washout). After washout the cells were fixed and stained for tubulin. Scale bar: 10 µm.

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Chapter 3

A Possible Role for RhoG in Force Transmission and Polarization

3.1 Introduction

The phenotypes described in Chapter 2, where RhoG KD cells are smaller, rounded, and lacking protrusive edges, are particularly interesting when considering polarization and force transmission. Additionally, it is known that MT play a critical role in the establishment of cell polarity, resulting in an asymmetrical morphology (Small, J., et al., 2002). Because the morphology of RhoG KD cells is not asymmetrical, and we have identified a novel role for RhoG in a MT-mediated pathway, we hypothesized that RhoG KD cells would also have impaired polarization.

We also noted that RhoG KD cells exhibit a distinct increase in the number, thickness, and alignment of SF (Chapter 2.3.6, Figure 2-6). We have demonstrated that these SF are contractile by measuring pMLC ratio and treating cells with contractility inhibitors (Chapter 2.3.7, Figure 2-7). Initially, we believed the morphology of RhoG KD cells considered with the increase of contractile SF was the result of isometric tension within the cell, which would also explain the mature FA in RhoG KD cells, due to tension-dependent FA maturation. Further contributing to this hypothesis, when MT are disrupted actomyosin contractility is increased (Danowski, B., 1989). The tension held within SF

would be transmitted across FA to the ECM, thus increasing traction force, which prompted us to further explore traction forces exerted by RhoG KD cells.

3.2 Results

3.2.1 Silencing RhoG decreases cell polarization.

To further explore the functionality of phenotypes described in Chapter 2, we performed scratch wound assays to quantify polarization. Cells were stained for the Golgi, a structure known to reorient towards the direction of migration (Figure 3-1a) (Ridley, A., et al., 2003). The angle of polarization was then manually measured as described in Methods and Materials. Our results show that the percent of polarized cells is significantly decreased in RhoG KD cells for all conditions. These results suggest that RhoG's role in MT-mediated FA disassembly further affects the cell, causing disruptions in polarization as well.

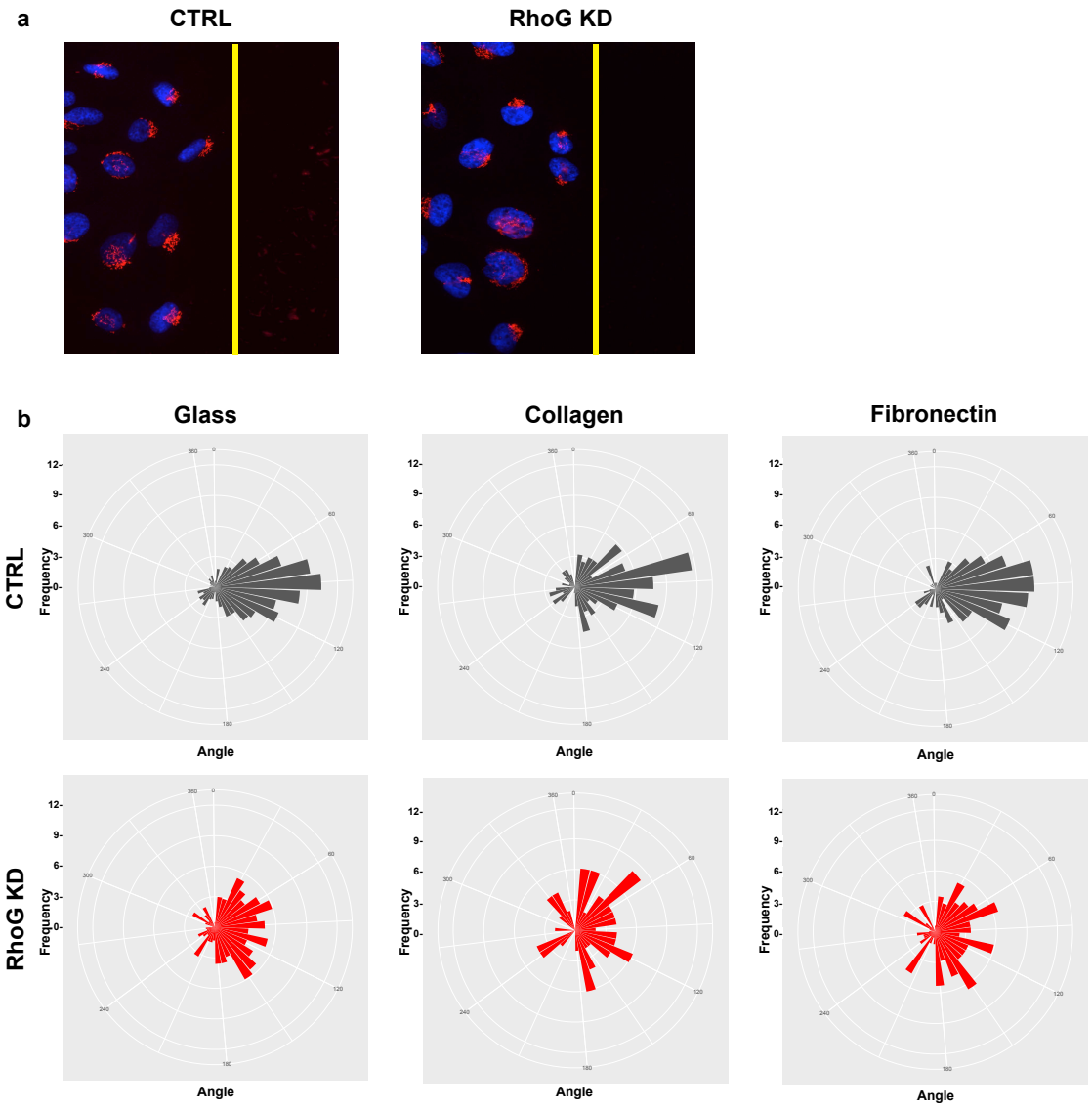

Figure 3-1 Polarization is decreased in RhoG KD cells.(a) Cells were plated to full confluency on either non-coated glass coverslips (pictured) or coverslips coated with collagen or fibronectin for 24 hours. A wound was then manually made on each coverslip. Cells were fixed 4 hours after the wound was made and stained for the Golgi using anti-GM130 antibodies. Yellow lines represent the edge of the wound. (b) The angle of polarization was manually measured. Rose plots were constructed using frequency

distribution of angles in R statistical suite. All data are results of 2 independent experiments where ≥100 cells per experiment were analyzed (n≥200).

3.2.2 RhoG KD decreases force transmission.

To further explore the ability of RhoG KD cells have increased force transmission due to isometric tension, we first performed 3D contractility assays in CTRL and RhoG KD cells embedded in collagen gels. Contrary to our expectations, we found that contractility was significantly decreased when RhoG was silenced (Fig. 3-2a-b). Since cells are embedded in a 3D matrix for collagen assays, where the cells may behave differently than in 2D, we also performed 2D traction force microscopy in CTRL and RhoG KD cells (Fig. 3-2c-d). Again, we found that loss of RhoG significantly reduced traction forces in a 2D environment (Fig. 3-2c-d). These results provide a stark contrast to the FA and SF phenotype in RhoG KD cells. Overall, it seems that SF in RhoG KD cells are able to contract, resulting in FA formation, maturation, and alignment. However, our data suggests that the contractile SF are not able to transmit force across the FA to the ECM.

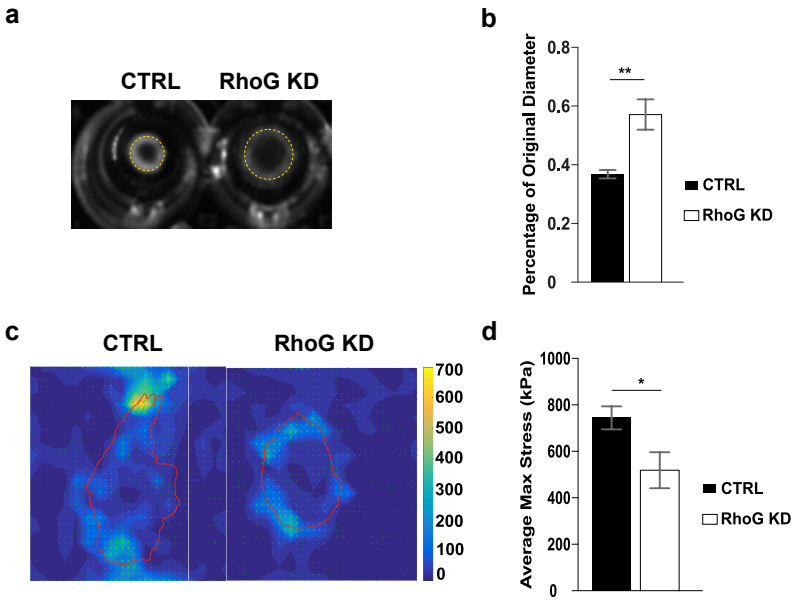

Figure 3-2 RhoG regulates force transmission.(a) CTRL and RhoG KD cells were plated in semi-solid collagen gels for 2 days in a 48-well plate. The gels were released and incubated for an additional 24 hours before being imaged on an Azure C500 system. (b) Gel diameter was manually measured using ImageJ software. Data are the results of three independent experiments in triplicate, n=9. (c) Contractility of CTRL and RhoG KD cells was measured by traction force microscopy. Heat maps were produced using a MatLab based algorithm (Tseng, Q., et al., 2011). (d) All results are shown as mean ± SEM. n=6 *p<.05, **p<.01.

3.3 Materials and Methods

3.3.1 Scratch Wound Assay

Cells were plated to confluency on glass coverslips uncoated or coated with collagen or fibronectin (described in Chapter 2.5.5) and allowed to incubate for 24 hours. A scratch was then manually made with a pipette tip. Cells were incubated for 4 hours and were then fixed and stained for the Golgi, using anti-GM130 mouse antibodies, and

nucleus, using DAPI. The angle of the Golgi relative to the wound was manually measured using ImageJ software. Angles were put into a frequency distribution and rose plots were constructed using R statistical suite.

3.3.2 Contractility Assay

1.5 x 10^5 cells were embedded in 1 mg/mL collagen gels as previously described (Su, S. & Chen, J, 2015). Gels were allowed to stress for 48 hours at 37°C, they were then released manually using a pipette tip and allowed to contract for 24 hours at 37°C. Images of gels were obtained on an Azure C500 imaging station. Gel diameter was then measured using ImageJ.

3.3.3 Traction Force Microscopy

Silicone gels (CY 52-276 A:B=1:1, Dow Corning) were prepared as previously described at room temperature with a Young's modulus of approximately 3 kPa and ν=0.5. Gels were then conjugated to rhodamine carboxylate-modified microbeads (Thermo Fisher Scientific) at room temperature, with 1:25,000 dilution, as previously described (Liu, K., et al., 2013). Bead solution was the prepared using 100 µg/mL EDC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride, Thermo Fisher Scientific). Substrates were coated with 3% APTS ((3-Aminopropyl) triethoxysilane, Sigma-Aldrich) diluted in PBS for 5 minutes at room temperature and then bead solution was added to the surface for approximately 1.5 hours at room temperature to achieve uniform bead density. The entire surface was the immersed in PBS for approximately 1 hour at room temperature to prevent cytotoxicity. Before seeding cells, the substrates were coated with 50 ng/mL fibronectin for 1 hour at 37°C. Cells were plated at a density of 1x10^4 and incubated for 12 hours prior to imaging. Initial images were obtained prior to manually trypsinizing cells.

Images were then obtained every 1 minute until the cell was completely detached from the surface, approximately 20 minutes. Data analysis was performed using the Traction Force Microscopy plugin developed by Qingzong Tseng for ImageJ (Tseng, Q., et al., 2011).

3.4 Discussion

In addition to RhoG's affect on FA through a MT-mediated pathway, we have also identified that these cells exhibit impaired ability to polarize. We expected these results due to the morphology described in Figure 2-1, where RhoG KD cells are markedly round and small. Additionally, in Chapter 2 we have described RhoG's involvement in a MT-dependent pathway, and more specifically the uncontrolled outgrowth of MT in RhoG KD cells. MT are important regulators of cell polarity due to their involvement in vesicular trafficking of proteins to various regions of the cell (Small, J., et al., 2002). Our results suggest that RhoG's effects due to regulation of MT-mediated FA disassembly are further reaching, also impairing the ability of the cell to polarize. This may be due to a decrease in recycling of FA components, thus disabling the cell's ability to respond to cues at the leading edge. However, it is possible that RhoG plays a larger role in MT outgrowth rather than FA disassembly, and therefore FA disassembly and polarity are secondary effects.

FA are thought to regulate force transmission by coupling F-actin movement to the underlying ECM. Prior to FA formation, actin retrograde flow at the lamella is fast and exerts a small amount of force at the cell edge (Alexandrova, A., et al., 2008; Zhang, X., et al., 2008). When FA assemble, actin retrograde flow slows down, and force transmission to the ECM increases (Alexandrova, A., et al., 2008; Gardel, M., et al., 2008). We explored the coupling of contractility and force transmission using 2D and 3D contractility assays. Surprisingly, we found that there was a decrease in force transmission across FA in RhoG

KD cells, despite their contractile SF. Our results suggest that silencing RhoG uncouples contractility of the SF and force transmission across FA. FA have been proposed to function as a "molecular clutch" between the dynamic F-actin and the immobile ECM (Mitchison, T. & Kirschner, M., 1988). Using this analogy, if the clutch "slips", by inefficiently anchoring F-actin to the substrate, then forces cannot be efficiently transmitted (Elosegui-Artola, A., et al., 2018; Case, L. & Waterman, C., 2015), as we observed in RhoG KD cells. Further experiments are needed to characterize the role of RhoG in modulating the clutch.

It is worth noting that others have shown the number of FA may not affect the ability of the cell to exert force upon the ECM, but rather the spread area of the cell does (Oakes, P., et al., 2014). It is possible that, instead of a clutch defect, the reduced area of RhoG KD cells may be the cause of the observed decrease in force transmission. Further experiments will systematically examine the contribution of RhoG-dependent regulation of cell size during force transmission.

Chapter 4

Conclusion and Future Directions

Relatively little is known about the function of the Rho GTPase RhoG. It has been shown to play a role in cell migration and was thought to also be regulated by the MT network (Hiramoto-Yamaki, N., et al., 2010; Meller, J., et al., 2008; Hiramoto, K., et al., 2006; Katoh, H., et al., 2006; Gauthier-Rouviere, C., et al., 1998). Using a systematic approach and detailed analysis of both fixed and live imaging, we have demonstrated that RhoG regulates FA phenotype and cell morphology through increased FA lifetime, not assembly and disassembly rates of FA. The increase in FA lifetime and maturity in turn alters the cell's ability to polarize and protrude, therefore stunting its ability to migrate. Furthermore, we found that increased FA lifetime in RhoG KD cells was the result of disrupted MT outgrowth, an indication that MT capturing at FA is not occurring as normal (Amano, M., et al., 1996; Kimura, K., et al., 1996). Our data provides a link between previous studies concerning RhoG in migration and mechanisms of FA disassembly, showing a novel role for RhoG in the regulation of FA dynamics through a MT-mediated pathway.

It was shown that RhoG localization is affected by the structure of the MT network, but this study did not examine RhoG activity in context (Gauthier-Rouviere, C., et al.,

1998). As mentioned in Chapter 2.4, RhoG localization is disperse throughout the cell; however, localization is not as important as localized activity since Rho GTPases are maintained in either an active or inactive state. Using newly developed methods to track the activity of RhoG in processes, such as fluorescence resonance energy transfer (FRET) biosensors, will be important in understanding its role. Additionally, because Rho GTPases are promiscuous, but carry out specific functions when activated by a specific GEF or deactivated by specific GAP, it will also be important to identify the GEFs and GAPs of this pathway. However, very little is known about the upstream regulators of RhoG. In particular, there are no known GAPs. Further studies examining the effect of specific GEFs and GAPs on RhoG within the context of this process will be needed to understand exactly where RhoG lies within the MT-mediated FA disassembly pathway, if feedback loops exist, and who the regulators of RhoG activity are.

The MT network, contractility within the cell, and force transmission to the ECM are highly connected processes within the cell. MT mediate contractility throughout the cell by transporting proteins to sites involved in mechanosensing and regulation of force (Small, et al., 2002). Therefore, it is important to note the significance of our findings that RhoG KD cells also have impaired force transmission, despite contractile SF. MT are not only involved in the regulation of FA disassembly, but also assembly through regulation of contractility (Small, J., et al., 2002). It is believed that MT mediate localized contractility through the transport or sequestering of regulators, including myosin II and Rho, respectively (Small, J., et al., 2002). Here, we have shown that RhoG KD cells are less sensitive to MT repolymerization using nocodozale washout experiments, meaning that FA in RhoG KD cells are not being effectively targeted by MT for disassembly in the absence

of RhoG. Furthermore, we found that SF in RhoG KD cells are contractile, being responsive to contractility inhibitors and display increased pMLC ratio. Taking our data in consideration with previous findings, it would be easy to speculate that RhoG regulates MT dynamics, resulting in impaired FA disassembly, which should be accompanied with increased force transmission. This would explain the small and rounded cell morphology of RhoG KD cells as well; however, we saw a decrease in force transmission to the ECM. This raises the question of whether the observed decrease in force transmission is an additional effect of RhoG's regulation on MT, or is it a complete separate function, like an altered actin clutch proposed in Chapter 2.4? Future work examining the integrity of the actin clutch in our system will be needed to delineate these observations.

Data shown here also provides new insights concerning our previous findings that RhoG regulates invadopodia formation. As mentioned, it is known that MT capturing at FA lead to their disassembly, but MT are also involved in the regulation of invadopodia elongation (Schoumacher, M., et al., 2010). Additionally, proteins found in the CMSC, responsible for MT targeting to FA, such as liprin, ELKS, and LL5**β**, are also found in a complex involved in the regulation of invadopodia formation (Chiaretti, S. & de Curtis, I., 2016). Through BioID analysis of RhoG-interactions (data not shown), we found several proteins with a high probability of RhoG interaction that are also located in the CMSC (Figure 4-1). It is possible that if RhoG specifically regulates any of the CMSC-associated proteins, RhoG likely plays a role in their regulation in invadopodia formation as well. Therefore, RhoG may be a key component in the balance between non-invasive and invasive cell migration. It will be important to identify the downstream effectors of RhoG

in these pathways through a series of experiments that identify RhoG interacting proteins within the process, and their effect or localization in the absence of RhoG.

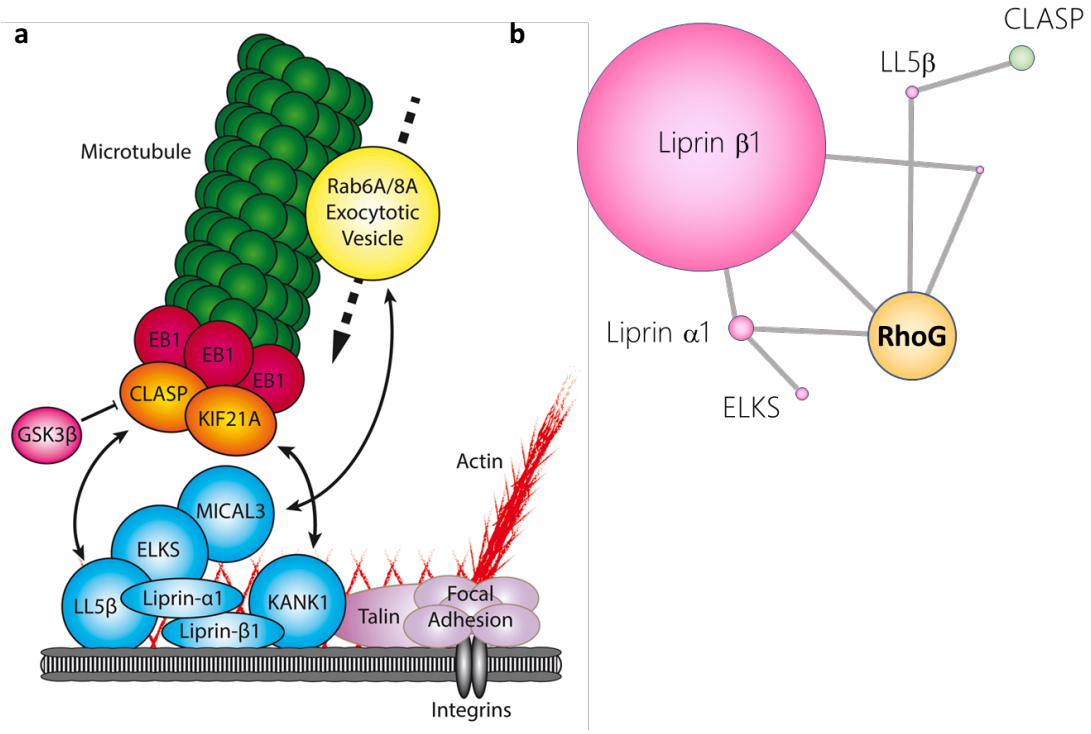

Figure 4-1. RhoG interacting proteins are found in the CMSC**.** Through BioID analysis, we found several RhoG-interacting proteins (b) that were also identified in the CMSC (a) (Noordstra, I. & Akhmanova, A., 2017). b) Grey lines represent known RhoG interactions, the larger the circle, the higher the probability of the interaction based on proteomics quantitative analysis. (Modified with permission from Noordstra, I. & Akhmanova, A., 2017 through CC-BY ISSN: 2050-084X.)

In addition to CMSC-associated proteins, FAK and Src are essential proteins for the disassembly of FA as well (Webb, D., et al., 2004). Of particular relevance to the current study is FAK. Cells from FAK-null mice displayed increased FA formation and impaired migration (Ilic, D., et al., 1995). The increase in FA was later attributed to the role of dynamic FAK phosphorylation in FA disassembly (Hamadi, A., et al., 2005). FAK

has also been implicated in the regulation of invadopodia formation through the shuttling of phosphotyrosine proteins from FA to invadopodia (Chan, K., et al., 2009). We have previously shown that p397-FAK is increased in RhoG KD cells; however, we found that RhoG's role in invadopodia formation was Src-dependent, FAK-independent (Goicoechea, S., et al., 2017). Data presented here, considered with previous studies, leads us to believe that Src and FAK may be important upstream regulators of RhoG's involvement in cell migration.

FAK is also a binding partner and activator of the FA-associated protein paxillin (Tachibana, K., et al., 1995; Bellis, S., et al., 1995; Hayashi, I., et al., 2002). Phospho-paxillin is recruited to adhesions in a contractility-dependent manner (Pasapera, A., et al., 2010). Furthermore, paxillin acts as a scaffold for the capture of MT at FA (Efimov, A., et al., 2008). We have previously shown that p397-FAK is increased in RhoG KD cells (Goicoechea, S., et al., 2017). In the present study, we observed differences in paxillin to tensin ratio in FA, a decrease in contractility, and defects in MT-mediated disassembly when RhoG was silenced. This data, considered with previous studies, leads us to believe that paxillin may be also be an important component in the pathway of RhoG's regulation of FA dynamics. Further experiments examining the functionality, binding, and localization of paxillin will be needed to determine if this is true.

In conclusion, we have identified two novel roles for RhoG in cell migration: regulating FA dynamics through a MT-mediated pathway, and regulating force transmission through FA (Figure 4-2). Taken with our previous findings, it seems that the lesser studied RhoG may play a larger role in cell migration dynamics than previously thought. We believe that delineating the upstream regulators and downstream effectors of

RhoG in these two roles will be critical to further establishing RhoG as an important Rho GTPase in biological functions. Regardless, we have offered new insights to the understanding of the Rho GTPases and their incredibly complex regulation of cell migration. Additionally, we have opened the door to a wealth of new studies that may further the knowledge base, or clarify previous findings, for therapeutic targets based in the Rho GTPase field.

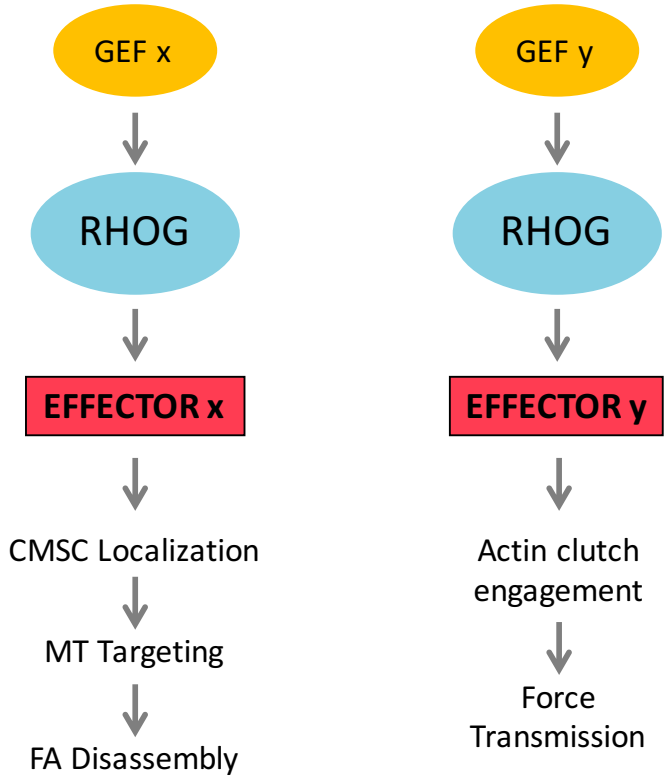

Figure 4-2. Working model. Two proposed pathways, where "x" and "y" represent specific GEFs and effectors involved in RhoG's regulation of MT-mediated FA disassembly (left) and force transmission (right).

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